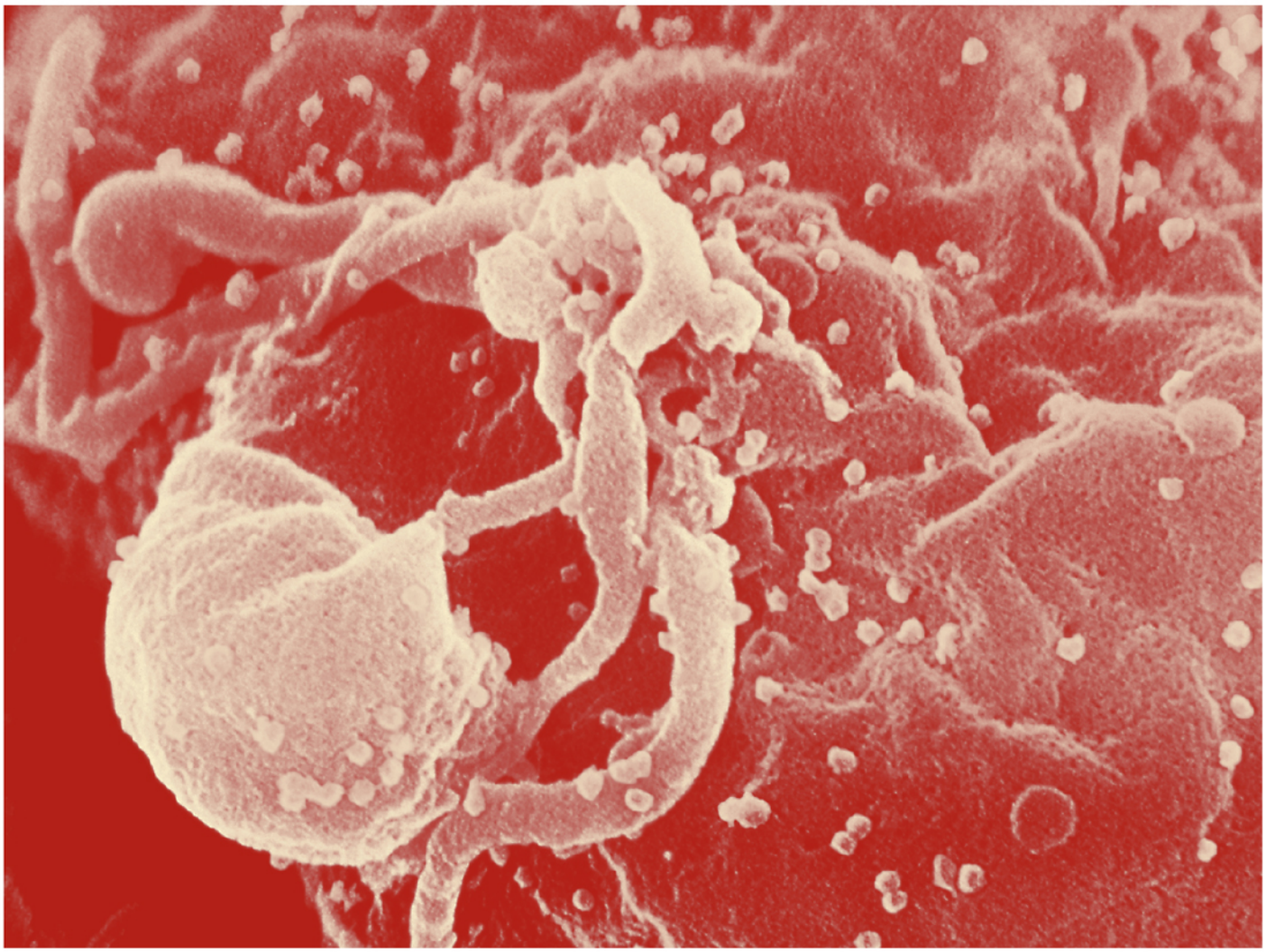
Public Health Laboratories

Analysis, Operations, *and* Management



Wiley D. Jenkins

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Analysis, Operations, and Management

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Production Credits
Publisher: Michael Brown
Editorial Assistant: Catie Heverling
Editorial Assistant: Teresa Reilly
Production Manager: Tracey Chapman
Senior Marketing Manager: Sophie Fleck
Manufacturing and Inventory Control Supervisor: Amy Bacus
Composition: DSCS, LLC/Absolute Service, Inc.
Cover Design: Scott Moden
Cover Image: Courtesy of C. Goldsmith, P. Feorino, E. L. Palmer, W. R. McManus/CDC.
Printing and Binding: Courier Stoughton
Cover Printing: Courier Stoughton

Library of Congress Cataloging-in-Publication Data
Jenkins, Wiley.
Public health laboratories : analysis, operations, and management / Wiley Jenkins.
p. ; cm.
Includes bibliographical references and index.
ISBN-13: 978-0-7637-7102-7 (pbk.)
ISBN-10: 0-7637-7102-3 (pbk.)
1. Public health laboratories. I. Title.
[DNLM: 1. Laboratories—organization & administration—United States. 2. Public Health Administration—United States. 3. Laboratory Techniques and Procedures—United States. 4. Public Health Practice—United States. WA 23 J52p 2011]
RA428.J46 2011
362.1072'1—dc22

2009044577

6048
Printed in the United States of America
14 13 12 11 10 10 9 8 7 6 5 4 3 2 1

Dedication

To my wife, Heather, whose enthusiastic support helped make this possible;

and to my young sons, David and Michael, whose endless curiosity is an inspiration for me.

About the Author

Wiley D. Jenkins, PhD, MPH, is currently a Research Assistant Professor at the Southern Illinois University School of Medicine in Springfield, Illinois. His primary role is Director of Research and Program Development for the Department of Family and Community Medicine, and he is also a faculty member of the Public Health Laboratory Science program in the Department of Medical Microbiology, Immunology, and Cell Biology. He joined the School of Medicine in August of 2007 after 13 years with the Illinois Department of Public Health, Division of Laboratories, where he held positions as laboratory chemist, section supervisor, division bioterrorism coordinator, and laboratory operations manager. In 1999, he was awarded a Centers for Disease Control and Prevention Graduate Certificate Program Scholarship and attended Tulane University in New Orleans, graduating with an MPH in Epidemiology in 2003. He then attended the University of Illinois at Chicago, graduating with his PhD in Health Policy and Administration in 2007. Current research interests include the development and implementation of novel chlamydia and gonorrhea interventions involving primary care physicians.

Acknowledgments

This work would not have been possible without the contribution of time and expertise by many individuals. This book seeks to discuss in detail a wide range of topics, and I was fortunate to be able to collaborate with some of the foremost experts in public health laboratory testing and operations.

First and foremost, I wish to acknowledge and thank the staff at the Illinois Department of Public Health and its Division of Laboratories. Director Damon Arnold, MD, MPH, graciously provided the foreword for this book and encouraged this collaboration between the author and the Division of Laboratories. Tom Johnson, MS, Chief, Division of Laboratories, contributed Chapter 10, "Operations and Management"; provided a technical review of other chapters; and coordinated laboratory staff input and participation. Mike Petros, DrPH(c), and David Jinks, PhD, contributed the section concerning newborn screening in Chapter 5.

Many others provided expert reviews, critiques, comments, and suggestions for portions within their areas of expertise. I would like to thank the following for providing specific technical reviews and offering substantial detail and guidance:

- Pete Dombroski for the Water Testing and Food Testing chapters
- Brian Nicholson for the Laboratory Data Uses and Communication chapter
- Ahmad Abuarqoub for the Tuberculosis section
- Juan Garcia for the Rabies and Enterics sections
- Hope Johnson for the Influenza section
- Mary Konczyk for the Parasitic Protozoa section
- Pete Sutton for the Blood Lead section

I also thank Scott Becker and Eva Perlman at the Association of Public Health Laboratories of their contribution of Chapter 1, "Overview of Public Health Laboratories." The Association is the preeminent organization for the recognition, development, and coordination of public health laboratories in the United States. In the past 20+ years, they have been an instrumental partner in transforming public health laboratories from a collection of individual facilities with varying capabilities and fragmented coordination into a much more robust and united network able to provide a cohesive response to terrorism and infectious disease outbreaks.

I would also like to thank my department Chair, Jerry Kruse, MD, MSPH, for his support of the completion of this work and Judy Walsh for her proofreading of the entire text.

Foreword

The field of laboratory science is continuously expanding, not only from the perspective of truly remarkable benchwork accomplishments, but also in the creation of supportive technological innovations. It has also steadfastly proven its critical role in the arenas of the public and private health sectors, law enforcement, and national defense and security. This book brilliantly encapsulates this swiftly evolving and complex field of modern laboratory sciences. First, the fundamental concepts relating to microbial analysis and quality control are explored to provide background. The book then moves on to delineate concepts pertaining to the fields of chemical and radiological analysis. Clinical testing is also covered in detail, as it relates to biological and chemical agents and genetic abnormalities. Chapters are also devoted to the increasingly important domains of environmental safety concerns related to water, food, and air testing. The book then turns to a laboratory perspective on biological and chemical terrorism preparedness and response issues. It must be stated that, in this arena, laboratories are at the very forefront of our national defense strategies. Laboratory operations and management issues are described in detail and are essential for regulatory compliance, as well as for strategic and financial planning. These topics are vital to the maintenance, and very existence, of our national infrastructure of laboratory systems that are indispensable in our efforts to prevent and treat morbidity and mortality throughout our nation. The critical issues of laboratory data uses and communications are explored in great detail. This has particularly important implications for a nation poised to construct and implement a national health information technology exchange system. Finally, a chapter is devoted to the links between laboratory work and other public health disciplines. Never before has collaboration between the laboratory partners, in the areas of epidemiologic and biostatistical analysis, community health, and health policy and administration, been as important to the overall success of the laboratory system as a whole as now. The laboratory system is essential for guiding clinical diagnosis and treatment, as well as response efforts during manmade and natural disasters on a continuous basis. It is clear that such a book is not only timely, but serves as an invaluable resource and reference for those engaged directly in, or tangentially associated with, the discipline of laboratory sciences. Finally, I want to commend you for the dedication and commitment to the discipline of laboratory sciences which is so vital to our very existence. Truly through your long hours of dedicated and compassionate efforts and services, miracles occur in all of our lives daily, throughout the nation and world we live in.

COL Damon T. Arnold, MD, MPH

Introduction

To our knowledge, this book is the first of its kind. We know of no other text that seeks to describe the wide range of activities in which public health laboratories (PHLs) are engaged. Popular media often imply that a simple injection into an instrument produces a legally defensible result by the next commercial break. This is simply not the case and many laboratory analyses take days for completion. Some, such as the analysis of samples for tuberculosis, may even take 3 to 4 weeks. Many people also do not recognize the wide range of samples and analytes with which PHLs deal. One laboratory may concurrently analyze bats for the presence of rabies, genital swabs for the presence of chlamydia and gonorrhea, stool samples for the presence of enteropathogenic bacteria such as *E. coli* O157:H7, and blood for the presence of lead. PHLs cross many traditional lines of expertise which often separate clinical analyses from those based on environmental, food, and water samples.

PHLs are also different from most private and commercial laboratories by their mission to ensure the health and safety of the public through laboratory analyses. As government entities, they are not-for-profit and provide analyses that promote the public's health; analyses which may be prohibitively expensive, or outright unavailable, elsewhere. It is the combination of mission and lack of a need for profitability that allows PHLs to offer such a wide array of services under one roof. It is also this mission that requires them to be active in other, non-analysis areas as well. Considerable time and effort is spent by laboratory personnel in such areas as emergency preparedness and response, operations and management, and different aspects of data communication.

The skills used and required for successful laboratory analyses and management are as diverse as the tests performed. We often find analysts with Bachelor's degrees in chemistry, biology and microbiology, virology, molecular and cell biology, and associated majors. Many become certified through medical technologist programs and some pursue graduate education. The high levels of education contribute to the research into test methodology and design performed in many PHLs and presented at national/international conferences and in journals. Individuals with a doctoral level education (MD/PhD) provide critical oversight and supervision of many of the high complexity tests performed and the results released. Non-analysts are also a critical component of a PHL, and section chiefs, managers, and accountants must be able to negotiate federal and state rules and regulations, mixtures of revenue streams which may have different rules and reporting, data and workflow oversight to provide adequate day-to-day coverage and still prepare for emergency response, and personnel issues as they relate to employee retention, union rules, and assignments.

In such circumstances, we had a fundamental decision to make about detail. We did not want to provide a text of such detail that one could use it as a guide for actual sample analysis. We determined this level of detail was inappropriate because of the introductory nature of the book, the wide variety of options for many analyses, and the rapid pace with which commercial instrument vendors are developing new and increasingly automated testing methods and techniques. Any detailed methods presented here could be either readily found in a laboratory performing the analyses, or might be subject to obsolescence within a few years. We have been purposeful in attempting to provide sources of information where the reader may go to find detailed method information such that a particular method may be performed.

On the other hand, a simple listing of a disease and its applicable analyses would be unsatisfactory. The reader may well think "But what of it?" if simply told that tuberculosis is analyzed by culture, staining, and an automated system. This brief description does not educate the reader concerning *Mycobacterium tuberculosis* complex, the length of time and safety level required for analyses, and the importance of drug susceptibility testing. For many diseases or conditions, the reader may also be left wondering "Why?" If malaria is not considered endemic in the United States, why do PHLs analyze samples for this parasite? Why are so many blood samples submitted for analysis for the presence of lead?

For each of the four chapters discussing major testing sections, and many of the individual sections in Chapter 5, we decided to include enough information such that the reader could answer the following questions: What is it? Why do we care? How are analyses performed? The section discussing syphilis therefore contains information on the disease itself (progression and treatment), the extent of the disease in the United States, and more detailed information pertinent to laboratory work. This would include descriptions of the organism itself, the types and manner of sample collection, and a description of the tests performed. We find that there are often many different tests that could be performed, and that laboratories create testing algorithms that determine the course of sample testing such that accurate results are reported with a minimum of time and resources. In this manner, the reader will gain an appreciation for why the laboratory performs the analyses, why some results take longer to report than others, and an understanding of how the results were derived.

That said, this book does not provide the actual steps in a given analyses. As described previously, many of these are readily and publicly available, others undergo relatively frequent revision, and still others are subject to obsolescence by newer tests and/or technological advances such as immunoassays and automation. The book also does not present an exhaustive list of every test performed by every laboratory. Such a list would be even more fluid than a listing of test steps because PHLs adjust their offered services constantly based on local and national need. As an example, the Illinois Department of Public Health laboratories changed their influenza testing algorithm several times in the first half of 2009 in response to the emergence of novel influenza A H1N1. Other tests may be offered only by a select few PHLs, such as tests for the toxins responsible for neurotoxic shellfish poisoning.

The book is designed around the idea of imparting an understanding of PHL activities to the reader, who will quickly notice the diverse nature of chapter topics, even in their tone and level of detail. This was purposeful and is in part a reflection of different aspects of PHL work. Chapter 1 presents an overarching view of PHLs in the United States and globally. It provides a brief description of their history, mission, and core functions; distribution and staffing; coordinated activities; and future directions. From this, the reader will be able to understand the context of the PHL and its importance to local, national, and even global health. This is followed by Chapters 2, 3, and 4, where we provide a general discussion of microbiological, chemical, and radiological analysis techniques. These chapters were written so that the reader would have a better understanding of specific laboratory activities. He or she will know the difference between selective and differential media, between PCR and immunoassay, and between HPLC/UV and GC/MS. Many of these concepts are not taught at the undergraduate level, and especially so across disciplines. So while an individual with a degree in chemistry may be able to describe a mass spectrometer and how it is utilized for chemical analyses, they may have no knowledge of the steps and utility of Gram staining. A basic understanding of these analytical activities will assist the reader in understanding the diverse nature of many tests and testing algorithms described in later chapters. These three chapters in particular were written to be merely general descriptions.

The chapters that follow, Chapters 5 to 8, discuss actual tests. For this edition of the book, they are divided according to sample type: clinical, water, food, and air. These are readily recognizable differentiations and often have quite different rules and regulations. They are also the highest volume types of sample submitted, with other sample types such as animal tissue and soil being extremely variable across PHLs and almost universally in low numbers. It is in these chapters that we will discuss the individual contaminants, their impact, and the associated analyses. The reader will also notice substantial variation in the delivery, types of information, and level of detail. Chapter 5, for example, discusses the analysis of clinical samples. These analyses are most often done to diagnose a disease or condition. There are frequently a large number of options when it comes to these analyses, with the US Food and Drug Administration (FDA) regulating some of the test kits and instruments used for disease diagnosis. However, many of the microbiological identification techniques are based on historically documented organism characteristics (e.g., tuberculosis identification via staining and growth in selective media). The laboratory must therefore simply show itself competent in these procedures in accordance with clinical laboratory regulatory requirements. Testing done for water samples in Chapter 6, on the other hand, has an entirely different basis. These are often done to see if the submitter is in compliance

with federal regulations such as the Clean Water Act and are regulated by the US Environmental Protection Agency (EPA). Because these tests are by nature regulatory, and facilities are potentially subject to action if noncompliant, it is incumbent to the EPA to promulgate suitable methods for analysis that have known limitations. We therefore briefly describe these rules, and also mention the mandated analysis methods.

To highlight the differences, let us consider the analysis of two samples: a clinical sample for chlamydia and a water sample for organochlorine pesticides. In Chapter 5 we find that the clinical sample may be analyzed by a commercially available system using DNA analysis (FDA-approved), by various tests using antibody/antigen reactions (enzyme-linked and direct fluorescence), and by culture. The choice of test(s) is largely up to the laboratory based on costs, need, and required expertise. In Chapter 6 we find that if the water sample analysis is to meet regulatory requirements, the PHL must strictly follow one of the following EPA Methods: 505, 507, 508, 508.1, 525.2, or 551.1. Other methods may be as accurate, but they would not meet the requirements. The analysis of water samples for nonregulated parameters is much more flexible, though methodology often varies case-by-case and laboratory-by-laboratory. Food analyses described in Chapter 7 are a mix of laboratory methods based on organism characteristics, commercial test kits and instruments, and analytical methods promulgated by the FDA. We also find that sample testing procedures may be different for samples submitted as part of regulations versus an outbreak response. Finally, we find in Chapter 8 that air samples submitted for the analysis of regulated pollutants must be analyzed by the allowed EPA method. However, unlike the methods described for water in Chapter 6, these are almost always titled after, and associated with, a specific analytical instrument (e.g., *Andersen Model RAAS10-100 PM10 Single Channel PM10 Sampler* for the analysis of particulate matter 10.0).

From discussion of the different testing sections, we move to other PHL activities. These are critical to laboratory continuing operations and integration with other partners, but seldom noticed or appreciated. Chapter 9 in particular discusses national terrorism and emergency preparedness and the role of the state PHL. This chapter provides a somewhat more lengthy description of individual terrorism agents because of considerable public interest. PHL personnel are considered by many in the public to be experts in such matters and we judged a more thorough review was warranted. Chapter 10 goes on to discuss laboratory operations and management and is complemented by a discussion of laboratory data uses in Chapter 11. Finally, in Chapter 12, we discuss how PHLs and their data impact the research and practice of the five traditional fields of public health: biostatistics, community health, environmental health, epidemiology, and policy and administration. PHLs do not operate in a vacuum for their own benefit, and the data they produce has profound impacts in many areas of public health practice.

Acronyms

AFB	Acid-fast bacilli
APHL	Association of Public Health Laboratories
BL	Blood lead
BT	Bioterrorism
CDC	Centers for Disease Control and Prevention
CLIA	Clinical Laboratory Improvement Amendments (Act)
CT	Chemical terrorism; chlamydia
DFA	Direct fluorescent antibody
ECD	Electron capture detector
EHR	Electronic health record
EIA	Enzyme-linked immunoassay
EPA	US Environmental Protection Agency
FDA	US Food and Drug Administration
FERN	Food Emergency Response Network
GC	Gas chromatography; gonorrhea
GIS	Geographic information system
HIPAA	Health Insurance Portability and Accountability Act
HPLC	High performance (pressure) liquid chromatography
IFA	Indirect fluorescent antibody
IDPH	Illinois Department of Public Health
LRN	Laboratory Response Network
MCL	Maximum contaminant level
MCLG	Maximum contaminant level goal
MS	Mass spectroscopy
NBS	Newborn screening
NIH	National Institutes of Health
PCR	Polymerization chain reaction
PHIN	Public Health Information Network
PHL	Public health laboratory
qRT-PCR	(quantitative) Real time polymerization chain reaction
RT-PCR	Reverse transcriptase polymerization chain reaction
rRT-PCR	Real time polymerization chain reaction
STI	Sexually transmitted infection
TB	Tuberculosis
USAMRIID	US Army Medical Research Institute for Infectious Diseases
USAMRICD	US Army Medical Research Institute for Chemical Defense
UV/VIS	Ultraviolet/visible
WHO	World Health Organization
WNV	West Nile virus

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An Introduction to Public Health Laboratories

Scott Becker and Eva J. Perlman

From tracking a meningitis outbreak to testing drinking water to training laboratory professionals on how to identify anthrax, public health laboratories (PHLs) operate where scientific inquiry meets on-the-ground community impact. PHLs provide diagnostic testing, disease surveillance, applied research, laboratory training, and more. Without the critical information generated by these scientists, many of the more familiar activities that characterize the practice of public health could not take place. Laboratory science and practice is a common denominator for fulfillment of fundamental public health services, including monitoring health status to identify and solve community health problems and diagnosing and evaluating diseases and environmental hazards that threaten the health of populations.[1–8] For example, laboratory services have been integral to public health efforts to prevent and control vector-borne illnesses such as West Nile virus, diarrheal diseases initiated by food- and water-borne organisms like Salmonella, conditions caused by exposure to environmental toxicants, highly infectious illnesses such as severe acute respiratory syndrome, and illness that is the result of biological terrorism.[2] When health risks emerge or re-emerge, laboratories in public health analyze the threat, provide the answers needed to mount an effective response, and act to protect the public.[3]

> "We're emergency responders from the lab perspective."
>
> Peter Shult, Director, Communicable Disease Division and Emergency Laboratory Response, Wisconsin State Laboratory of Hygiene[4]

Thus, PHLs must concern themselves not only with high-quality performance of complex laboratory procedures, but also their roles as part of a larger regional, national, and even global public safety network. Achieving uniformity of data and prompt, integrated reporting is both more possible and more difficult than ever before. Today's laboratories on all levels must have multidisciplinary capabilities in addition to good, up-to-date science: Knowledge of informatics, the skills to negotiate budgets and policy, understanding of communications among laboratories and with the public, the ability to train others and to anticipate workforce and resource needs, practical experience in emergency procedures, grounding in standards and performance measurement—the list can go on.

This chapter will look at the growth of PHLs, their mission, and what makes them unique, including their core functions and how they interact with the greater network of public health organization and agencies to carry out these functions. Finally, it will project future directions and challenges and offer some insight on how PHLs might meet them.

A Brief History of US Public Health Laboratories

More than a century ago, as the United States shifted from a mostly rural society to a modern, urban-centered one, PHLs emerged in concert with public health departments.[5] Both were working toward a common goal: to identify the causes behind—and stem the spread of—the many diseases ravaging a growing nation. In the late 19th century, urbanization and population growth were

causing health problems unlike any the United States had seen before. Cities dumping untreated sewage into rivers and streams caused typhoid fever to run rampant. It was not uncommon for young children to die from diseases we today consider obscure, nonexistent, or treatable, such as measles, mumps, diphtheria, whooping cough, and scarlet fever. At the turn of the century, tuberculosis alone killed 194 of every 100,000 residents.[6]

To tackle their dire hygiene and sanitation issues, regional and municipal governments adopted formal health boards and implemented public health policies to protect their residents. The budding area of laboratory-based investigation played an important role in their solutions. Building upon the work of Louis Pasteur and Robert Koch, researchers applied the germ theory of disease and discovered causative agents for leprosy, typhoid fever, tuberculosis, cholera, and diphtheria—all common diseases for the time. In the world of science, applied research now focused on addressing pressing real-world problems.[5] State and federal government created facilities to conduct this research. One of the first, the Marine Hospital on Staten Island in New York, moved to the Washington, DC, area in 1888 to become the precursor to the National Institutes of Health (NIH). PHLs soon opened their doors at the state level, starting in Rhode Island, Kansas, and Michigan, and growing across the nation.[6]

Food safety and the elimination of water-borne disease were the first targets of the early PHLs. Over the next years, technological advances made tackling infectious diseases more possible and more critical. World Wars I and II presented the public health threat of sexually transmitted diseases, also leading laboratories to make advances in handling and planning for crisis and surge-condition situations. In the 1960s and 1970s, as awareness grew of the long-term health effects of environmental hazards, PHLs became a first line of defense and developed important networking and communications skills.[6]

As the century drew to a close, threats became more diverse and more global. Localized outbreaks of food-borne illnesses and episodes of conditions such as Legionnaire's disease emerged as potentially national threats, as travel habits, technology, and economies changed rapidly.[6] Two crises brought home the importance of PHLs, their advanced science, their emergency response capabilities, and their importance as a hub in the public health network: the prospect of bioterrorism and the reality of human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS).[6] These pushed PHLs to develop technology, communication, and policy links and skills that will serve PHLs well against new health threats, such as the H1N1 pandemic, which is being fought worldwide as this is being written.

Today, state PHLs can be found in every state supporting America's national PHL network as well as individual state public health systems. At the local level, about 250 local PHLs are now in operation across the nation. The Association of Public Health Laboratories (APHL) describes a PHL as, "a local, state, or federal governmental entity. . .[that] provides testing for public health programs in assessing health status and preventing disease [and] fulfills core public health functions in partnership with private clinical, hospital, and commercial laboratories, healthcare organizations, and other institutions."[2]

Interestingly, the need for PHLs has often been debated during their history. For instance, in the 1970s and 1980s, an argument surfaced that, because antibiotics had reduced many of the major public health threats, the United States could discontinue tracking, detecting, or communicating data on diseases.[6] That was before the reality of HIV/AIDS or antibiotic-resistant infection set in. During the writing of this text, each day's news bears out the importance of PHLs: novel influenza viruses and new food-borne outbreaks are only two of the areas over which nations must be constantly vigilant to protect lives, peace, and economic stability.

Public Health Laboratories: A Unique Mission and Role

To shape a strong, secure, productive society, leaders must ask certain questions about health: How can we make sure environmental pollutants and disease does not take an economic and human toll? How can we help mothers and children thrive—and thus ensure our future? How do we keep a health emergency from compromising national security? This is where the mission of PHLs becomes clear. Environmental health, disease control and prevention, maternal and child health, epidemiology, and emergency response—the mission of a PHL is to support these kinds of programs on the federal, state, and local levels.[5]

PHLs do so in many ways. They perform analytic testing. They consult with lawmakers and regulators on the formation and implementation of public health policy. They help train laboratory professionals across the public and private sectors. In times of crisis, PHLs play a key role in emergency preparedness and

response. In regions serving at-risk populations, PHLs can help community clinics provide primary laboratory services.[7]

This is only the tip of the iceberg. As needs and opportunities expand, so do the many roles of the PHL. To advance knowledge in the health and sciences, PHLs often are called upon to conduct epidemiologic studies and other applied research. As new technologies emerge, PHLs often are enlisted to evaluate and try out these innovations.

What Makes a Public Health Laboratory Different?

There are nearly 200,000 private-sector, clinical laboratories in the United States, as well as a few thousand public health, veterinary, food safety, and environmental testing laboratories. While PHLs often provide support or "surge capacity" for one another during crises, their main focus is to serve a local jurisdiction, a state, or, in the case of federal PHLs, the nation. Unlike laboratories in commercial settings, PHLs are integrated into the broader public health system. State health laboratorians, for example, work closely with public health agency epidemiologists to identify trends and "sentinel events" that may signal emerging health problems.[5]

Laboratories range in capacity from basic water testing services to more complicated testing involving human clinical samples or, in some cases, characterization of potential bioterrorism agents. State laboratories are available to local health agencies in the jurisdictions that lack resources to fund a local laboratory.[2] PHLs interact with federal laboratories and private laboratories run by hospitals, physicians' offices, and other independent parties. However, core distinctions exist.

PHLs must meet the requirements of the federal Clinical Laboratory Improvement Amendments of 1988 (CLIA) as well as the mandates of external agencies, which often include the US Environmental Protection Agency (EPA) or the US Food and Drug Administration (FDA). PHLs also conduct more training and regional and community interaction than federal laboratories. PHLs also differ from private-sector laboratories, even though their internal operations are similar. Private laboratories focus on individual patient care, which makes them vulnerable to cost containment pressures. Unlike private medical laboratories that perform tests to diagnose illnesses and conditions afflicting individual patients, PHLs safeguard entire communities.[5] In one way or another, the work of these laboratories affects the life of every American.

Protecting the Nation: The National Laboratory System

Despite differences in capacities, function, and mission, all of these parties, and PHLs across the nation, work together to protect and preserve the health of our communities. But because a specimen that indicates the first sign of an outbreak could show up at any type of laboratory, it is important to link the efforts of all types of laboratories across the nation. A national laboratory system—one overarching network of state laboratories coordinating efforts among the states and federal agencies such as the Centers for Disease Control and Prevention (CDC)—has existed in practice for many years.[2] However, only recently have organizations more formally articulated their roles and responsibilities. Linking these laboratories to create seamless systems within each state, for public health surveillance and laboratory support and improvement, is the mission of the National Laboratory System (NLS) initiative. While the NLS is being built state by state (as reflected in the vignettes that follow), the eventual goal is to connect 50 individual state laboratory systems into a national system that promises even greater value to America.[8]

Since 2000, the CDC and APHL have sponsored many projects to explore creative ways public and private stakeholders can communicate and coordinate. Some of these have been put to the test in crisis situations. For instance, in the aftermath of Hurricane Katrina, hospital laboratories along the Gulf Coast of Mississippi were able to continue operations, thanks in part to an influx of reagents and state-owned vehicles for specimen transport arranged by the Mississippi Department of Health laboratory.[8]

Fast and First: The Laboratory Response Network

In the late 1990s, biohazards and biological and chemical terrorism became a looming global threat, and PHLs throughout the United States began to prepare to meet any possible incident. At that time, the vast majority of PHLs in the United States were not able to test for some of the most virulent biological agents. Those that could were using testing methods that were inordinately time-consuming. New procedures had yet to be developed or validated.[2]

In 1999, to address this situation, the CDC and APHL established the Laboratory Response Network (LRN). The network is an integrated, multitiered system of state and local PHLs as well as national laboratories at the CDC, FDA, Federal Bureau of Investigation (FBI),

the Department of Defense (DOD), and other federal agencies. The LRN also includes select private clinical, veterinary, and agricultural laboratories that are seen as the front lines of detecting microbial agents of bioterrorism. The goal of the LRN is to bring the most accurate and rapid testing methods closer to the patient, as well as assure laboratory capabilities and capacity adequate for rapidly responding to biological terrorism and other public health emergencies. The role of the LRN is not confined to responding to terrorism events; however, it is also equipped to respond to emerging infectious disease, natural disasters, and other public health threats.[2]

The LRN is just one more example underscoring the importance of networking, communication, and enhanced scope in today's PHL. State and local PHLs support the network with advanced diagnostics and disease monitoring; hospital and clinical laboratories refer suspicious specimens to LRN reference laboratories.[9] Through practice, a structure evolved to deal with events:

- Thousands of clinical and hospital laboratories serve as sentinel laboratories, monitoring for possible agents in clinical specimens or environmental samples.
- All state PHLs serve as reference or confirmatory laboratories, with the ability to isolate and definitively identify threats.
- Federal LRN laboratories at the CDC and the DOD conduct investigations and provide oversight, training, and new technology.

Within the LRN, state PHLs are recognized as first-responder laboratories. In the event of a confirmed biological or chemical attack, they are the first point of contact for public safety officials to arrange testing. The structure of the LRN and the role of PHLs are described in more detail in Chapter 9.

Sidebar 1-1 Public Health Laboratories Networking Put to the Test[2]

The LRN was tested—and rose to the challenge—during the anthrax events of September 2001, when an employee in a Florida tabloid publishing company was infected with anthrax. Between October and December 2001, the LRN conducted nearly 122,000 work-ups based on environmental samples, the results of which guided hundreds of decisions to evacuate or reoccupy buildings, as well as to determine what areas to deem "affected." This was accomplished even though the LRN was originally structured to test clinical, not environmental, samples. Thanks to the collaboration of CDC and state laboratorians working around the clock, an environmental test protocol for anthrax was quickly developed and disseminated in time for the crisis.

Training, relationships, and communication were key to a timely and effective response. First, the LRN, though still in its infancy, was up and running before the crisis hit. Second, a protocol to identify anthrax in human specimens had already been developed, validated, and broadly disseminated to state laboratorians. Third, the state PHL in Jacksonville, Florida, had a good working relationship with the clinical laboratories in the state. When a laboratory worker in a Boca Raton hospital received the specimen from a physician who suspected that his patient had anthrax, the hospital laboratorian knew the name and phone number of the appropriate contact in the Florida Bureau of Laboratories who was able to perform confirmatory testing for anthrax in a clinical specimen.

In any emergency situation demanding quick response, triage is essential, and this event was no different. LRN circumvented bottlenecks to a certain degree through structuring a "pyramid" of laboratories, each level designed to respond to a certain category of threat. Initial screening was conducted by thousands of sentinel laboratories across the United States. Samples for which analysts could not rule out the presence of a potential bioterrorism agent were then sent to a confirmatory laboratory. At the top of the pyramid, two federal LRN laboratories had the capacity to conduct highly sophisticated forensic and epidemiological investigations. Lessons learned from the 2001 anthrax response provided laboratorians with real experience upon which to improve their ability to triage potentially contaminated items.[2]

What Public Health Laboratories Do: The Core Functions[10]

In 2002, through an article in the *Morbidity and Mortality Weekly Report*, the core functions of PHLs were formalized.[1] This listing (see Sidebar 1-2) continues to work as a practical and complete framework for evaluating the specific functions and services provided by

a laboratory.[1,7] The list does not necessarily mean the laboratory will provide all these functions, but that the laboratory is responsible for assuring these functions are available. PHLs do not exist in a vacuum, and as these core functions are refined over the years, it will be apparent that other types of laboratories sharing the PHL goals may share in providing these services.

Sidebar 1-2 Eleven Core Functions of State Public Health Laboratories[1]

1. *Disease prevention, control, and surveillance* by providing diagnostic and analytical services to assess and monitor infectious, communicable, genetic, and chronic diseases and exposure to environmental toxicants.
2. *Integrated data management* to capture, maintain, and communicate data essential for public health analysis and decision-making.
3. *Reference and specialized testing* to identify unusual pathogens, confirm atypical laboratory results, verify results of other laboratory tests, and perform tests that are not typically performed by private sector laboratories.
4. *Environmental health and protection*, including analysis of environmental samples and biological specimens to identify and monitor potential threats and ensure regulatory compliance.
5. *Food safety assurance* by testing specimens from people, food, and beverages implicated in food-borne illnesses and monitoring radioactive contamination of foods and water.
6. *Laboratory improvement and regulation*, including training and quality assurance.
7. *Policy development*, including development of standards and providing leadership.
8. *Emergency response* via provision of rapid, high-volume laboratory support as part of state and national disaster preparedness programs.
9. *Public health-related research* to improve practice of laboratory science.
10. *Training and education* for laboratory staff in the private and public sectors in the United States and abroad.
11. *Partnerships and communication* with public health colleagues at all levels and with managed care organizations, academia, private industry, legislators, public safety officials, and others to participate in state policy planning and to support the core functions outlined here.

Core Functions in Action

These core functions include several that were previously viewed as outside the realm of PHL responsibilities, but which have, in recent years, been recognized as important and have been fulfilled by PHLs. Specifically, these functions involve surveillance, furnishing finished "data products" to all parts of the health system, creating meta-data, and developing policy.

After clinical specimens and environmental samples come into the laboratory, PHLs convert them into useable information, which is then confirmed, organized, analyzed, stored, and communicated to those with a need to know—ideally, in real time. In a meningitis outbreak in Minnesota, PHL analysis played a crucial role in stopping an outbreak. Information gained then provided a valuable foundation for pinpointing the source of a second outbreak, enabling the state to target vaccinations only to those at risk.[11] In South Salt Lake City, Utah, environmental chemists at the state's PHL tested and analyzed material from a tanker crash to reveal the cause to be simple negligence, rather than chemical terrorism as first responders initially feared.[7,11]

PHL research can contribute to the meta-data that shapes key findings and, consequently, health practices. For example, the director of California's Genetic Disease Laboratory heard of a new technology, tandem mass spectrometry (MS/MS), that identified dozens of treatable genetic diseases that previously had gone undetected in newborns. Over a period of 18 months, the PHL performed MS/MS testing on the blood samples of roughly 375,000 babies, 51 of whom tested positive for one of the new conditions. As a result, the state enacted a law mandating the addition of the new genetic conditions to the standard panel of tests for all infants born in California. With informed parental consent, babies would be tested free of charge at the state PHLs.[11]

PHLs increasingly play a pivotal role in how governmental agencies and other authorities shape policy, as well as set, interpret, and revise regulations. In 2001 and 2004, because of concerns about mercury levels, both the EPA and the FDA issued nationwide advisories that women of childbearing age restrict consumption of fish. However, fish is a key nutritional staple for many rural Alaskans. Targeted testing by Alaskan PHL scientists and state epidemiologists over the course of 2 years reported mercury levels well below the "no observed effect level" set by the World Health Organization. Based on these findings, the Alaska Division of Public Health recommended unrestricted consumption of fish caught in Alaskan waters.[11]

Funding and Oversight for Public Health Laboratories: General Structures and Practices

All this laboratory activity costs money and needs to be regularly evaluated for quality performance. Where do PHLs get their funding? What are the policies and practices that govern their operations? Because so many different types of PHLs exist, these essential activities will be carried out differently. What remains constant are the changes in politics and local and national needs to which laboratories will need to respond, and the lifesaving missions that continue to require top-quality resources and performance.

In the beginning, it was not uncommon for a PHL to receive a significant amount of its funding from one source, whether a university, a federal agency, or a state department of health. Today, PHLs tap numerous funding streams, including:

- Appropriated state or local revenue
- Direct federal funding
- Indirect federal funding (through other departmental grant recipients)
- Fees or other earned income
- Third-party reimbursement, such as Medicaid

In the 1970s and 1980s, debate began on privatizing PHLs and has since been a perennial topic of discussion.[5] Outsourcing regulatory oversight and data integration are two of the most obvious sticking points to privatization. Furthermore, proposals have been unable to model cost savings from privatization. This being said, PHLs have needed to become adept at making their case, and advocating for, their public role (for more information concerning the role of PHLs visible to the public, see Chapters 10 and 12).

New funding streams have opened up in response to the expanded roles of PHLs. A recent and significant example is homeland security. For instance, in the event of a confirmed biological attack, state PHLs are recognized as first-responder laboratories. This has significantly impacted funding trends for the decades around the turn of the 21st century and will likely continue to do so into the future. A focused effort to build basic public health infrastructure as a matter of national security began in the early 1990s with the CDC's strategy for preventing emerging infectious diseases. It led to the creation of two federal programs: the Epidemiology and Laboratory Capacity Program and the Emerging Infections Programs, through which states can compete for funding. In 1998, the CDC strategy was revised to include an explicit reference to bioterrorism.

At the same time, the US Congress authorized specific bioterrorism preparedness activities at the CDC, including an extramural program through which all states were funded to build public health capacity to respond to a deliberate release of infectious organisms. State PHLs used these new resources for various purposes, such as purchasing advanced instrumentation, upgrading safety facilities, hiring coordinators to oversee bioterrorism preparedness activities, improving information management and communications systems, and helping to upgrade other state laboratories that agree to provide surge capacity in a serious bioterrorism attack. But after this surge, economic constraints led to funding cutbacks in many areas affecting PHLs. A look at future challenges that might affect funding and what laboratories might do to weather those challenges is at the end of this chapter.

Just as PHLs get funding from multiple sources, they also receive oversight from multiple organizations, agencies, and regulatory groups. Most PHLs must meet quality standards established by the CLIA, which were established in 1988 and finalized in 1992. The aim of these standards is to ensure accurate, reliable, and timely diagnostic test results for clinical specimens, no matter where testing is performed. Sites that conduct testing of clinical specimens for diagnosis, treatment, or prevention of disease must obtain a CLIA certificate corresponding to the complexity of the tests performed.[11] PHLs may also need to comply with relevant laws and regulations such as the Clean Water Act, as well as oversight by other agencies.[5] In addition, today's PHLs are often seeking ways to improve quality and service, and are using tools such as performance management to achieve this.

Distribution and Staffing

Public Health Laboratories at the National, State, and Local Levels

The organizational structure of a PHL can vary based on where it fits within its governmental department or university system, when and how it serves agencies and customers outside of its host institution, how much testing is conducted in-house, and several other factors. PHL funding and organizational structures generally follow three models, each with advantages and challenges. Most commonly, PHLs are housed within a state health department. This structure enables them to access

facilities a local agency might not have and increases opportunities for communication and collaboration, often with other organizations of the health agency and local health departments.[5]

However, an increasing number of PHLs (such as those in Massachusetts, Nevada, and Nebraska) are affiliating themselves with a state university system. This not only provides access to an extensive library and skilled workforce, but it also frees a PHL from the political influence and funding fluctuations that can be experienced at a state agency. A university-affiliated PHL also has the freedom to create separate funding streams.[5]

Still other PHLs are "consolidated laboratories," which means they are housed in a state agency that is not a health agency. A successful example can be found in the state of Virginia, where the Virginia Department of Government Services supports the Virginia Division of Consolidated Laboratory Services to serve a number of state agency "clients," from Agriculture and Consumer Services to Criminal Justice Services. Shared infrastructure and economies of scale across a comparatively large "customer base" often means that these kinds of PHLs can adopt new technologies more quickly.[5]

Federal agencies operating laboratories related to public health include:

- The CDC
- The FDA
- The DOD
- The FBI
- The US Department of Agriculture (USDA)
- The NIH
- The EPA[5]

Staffing Trends: Past, Present, and Future

Workforce needs will vary according to type of PHL. However, key staff members often include the following.

- A laboratory director
- A leadership team of section directors and managers
- Supportive administrative staff
- Highly trained employees such as epidemiologists and laboratory technicians to perform core laboratory functions
- Specialists in areas such as environmental health, global disease, and bioterrorism
- A quality assurance officer
- Communications professionals who can relate the PHL's work to media, policymakers, clients, leading scientists and public health practitioners, and other key audiences.[7]

PHLs have found they need to augment their staff during critical emergencies. As these emergencies have compounded around the turn of the 21st century, many PHLs have been faced with a serious workforce shortage. Options they use to cope include cross-training laboratory personnel, establishing pools of volunteer microbiologists, or making arrangements to "borrow" staff from private laboratories.[2]

Public Health Laboratories Outside of the United States

In the latter half of the 20th century, it became clear to public health workers worldwide that all capacities would need to be better connected and aligned. Several factors lent urgency to this global view:

- Emergence of HIV/AIDS and drug-resistant tuberculosis
- Economic interdependence and global trade making health and stability of all nations a priority
- Loss of life and productivity in developing nations because of malaria and other infectious diseases
- The pandemic potential of influenzas and acute respiratory disease
- Increased immigration and travel among all countries

It is worthwhile to note that training and education of laboratorians abroad was made part of the formal listing of PHL core functions. Around the time of this writing, the World Health Organization adopted standards for all member states to develop surveillance capacity to detect, report, and respond to public health risks and emergencies. To these ends, US PHLs participate in several initiatives to strengthen laboratory systems and practices and workforce development, as well as connect and mutually improve PHL services worldwide. Here's a closer look at two such efforts:

World Health Organization Twinning Program

This program matches laboratories in developing countries with more established institutions to improve the quality of their laboratory practice and their surveillance of and response to international infectious diseases. Accordingly, US PHLs set up matching programs with national laboratories in developing countries; for instance, the California State PHL is matched up with the Ethiopia Health and Nutrition Research Institute and the Michigan State Laboratory and the Los Angeles County Laboratory are matched up with the NIH Immunology Laboratory in Mozambique. The US PHLs provide expertise, technical assistance and training to improve capacity and quality. For example, Guyana has a new national public health reference library for which the North Carolina PHL is providing mentorship in quality assurance and biosafety activities.[12]

George Washington University-Association of Public Health Laboratories International Institute for Public Health Laboratory Management

The George Washington University School of Public Health and Health Services in Washington, DC, and the APHL developed this international educational resource. Advanced seminars are provided for laboratory professionals who manage laboratory systems and hold responsibility for the planning, managing, and direction of national PHL systems in developing countries. Again, a major focus is surveillance, as well as quality assurance, policy development, and public health program planning. The senior health professionals participating are provided with practical knowledge conferring competency and leadership in quality PHL practice.[13]

Interstate Coordination and Training

Because threats and pandemics do not respect state boundaries, PHLs continually need to learn how to work seamlessly across them. Concurrently, PHLs also must uphold their responsibility to provide training across the nation to ensure the quality of all medical and environmental laboratories. Here are a few examples of how PHLs and the organizations with which they work are paving the way toward a more integrated, more skilled public health infrastructure.

The Centers for Disease Control and Prevention

PHLs have collaborated with the CDC from the time the agency was created in 1946. Throughout the 1950s and 1960s, the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD), the predecessor organization of APHL, worked hand in hand with CDC staff and scientists. The CDC worked on the federal level and ASTPHLD on the state and local levels, enabling both to stay current on the latest developments in the laboratories and on the ground.[6] By the 1980s, despite the influx of federal funding for HIV/AIDS testing, the relationship between PHLs and the CDC was less close, particularly in the area of training, which was strongly affected by the CDC reorganization of the early 1980s.[6] This left a workforce gap that local and state PHLs had difficulty filling. The result was an agreement that led to the formation of the National Laboratory Training Network (NLTN; see later in the chapter).[6]

PHLs and the CDC collaborate on dozens of essential programs in areas from infectious diseases to newborn screening. The May 2009 novel H1N1 influenza crisis provides a good case in point as to how PHLs work with the CDC: The CDC developed and deployed novel H1N1 diagnostic kits, delivering them to over 60 state and local PHLs in only 10 days. Confirmatory testing, which previously had been handled only by the CDC, then could be conducted by these laboratories. Such a step meant that results could be produced in less time and disease control measures put into place faster.[14] These improvements could not have happened without the CDC and PHLs working together.

Public Health Laboratories and Federal Departments and Agencies

PHLs find themselves interacting on several levels with multiple federal departments and agencies. To give a picture of the complexity of these collaborations, here are just a few examples.

- PHLs work with the FBI in bioterrorism and chemical terrorism event planning and response.
- The US Department of Health and Human Services (DHHS) has launched programs such as Healthy People 2010, which has objectives that necessitate laboratory involvement.
- The President's Emergency Plan for AIDS Relief has tapped PHL knowledge to help with PHL systems building and training abroad.
- The EPA needs laboratory information to implement pollution-control programs under acts such as the Clean Water Act and the Clean Air Act.

- PulseNet, the nation's food-borne disease surveillance laboratory network, has become an essential partner to epidemiologists concerned with food-borne outbreaks. PulseNet also has a growing international component.

Challenges—and opportunities—also exist in the area of technology, as the data systems used by non-traditional public health partners, such as the FBI and other law enforcement agencies, are not likely to be revamped to comply with Public Health Information Network (PHIN) standards.[2]

Association of Public Health Laboratories

Since 1951, when its predecessor organization ASTPHLD was founded,[6] the APHL has served as both a resource for its member PHLs and as a liaison among these members, federal officials, and other partners. Its mission is to promote the role of PHLs in support of national and global objectives and to promote policies and programs that assure continuous improvement in the quality of laboratory practice. The APHL provides guidance on federal protocols and directives as well as advises federal agencies on the development and implementation of national initiatives that involve PHLs.[6] Reading about the diversity of skills and responsibilities in the PHL, it is understandable that current or prospective laboratorians and administrators would ask how one could cover all these bases and keep a laboratory running. APHL is the primary place for the answer to this question—as well as a link to the resources needed to fulfill these responsibilities.[3]

Other Associated Organizations

After the 2001 anthrax crisis, it became clear that a broader umbrella of individuals, organizations, and responders must work collaboratively to plan and practice response activities. This wider network includes the National Guard, emergency management personnel (hazardous material teams, fire departments, and other safety workers), and law enforcement personnel. It is now commonplace to have public health and clinical laboratory leaders involved in all aspects of bioterrorism response planning at the state and local levels.[15]

In addition, outside of the emergency response area, PHLs often work with other organizations nationally and globally. Here are a few examples.

- The World Health Organization has looked to US PHLs in training programs and for data collection.
- In response to infectious diseases such as influenza easily crossing borders, US PHLs have collaborated with their counterparts through the Pan American Health Organization and the Canadian Public Health Laboratory Network.
- Organizations such as the World AIDS Foundation, the World Bank, and more have been sources of funding and expertise for PHLs, and PHL laboratorians have worked with these organizations to teach, learn, and improve quality and alignment of PHLs worldwide.

The National Laboratory Training Network

The NLTN, a partnership between CDC and APHL, operates toward one ambitious overarching goal: to be the best possible laboratory training vehicle in the United States. In operation since 1989, the NLTN provides training on a regional level to laboratorians performing testing of public health significance, on subjects ranging from molecular diagnostic techniques to food-borne disease investigations. In keeping with the overall PHL mission to collaborate and connect, and bridging regional differences, the training programs are available in diverse formats and re-evaluated with greatest access in mind. NLTN provides its consistently highly rated, reasonably priced, laboratory-specific, credit-earning continuing education via traditional "wet" workshops, seminars, and distance learning programs, including teleconferences, webinars, and computer-assisted resources, and it is flexible and receptive to new formats and content.[16]

The Emerging Infectious Diseases Laboratory Fellowship Program

To build PHLs' capacity to respond to new health threats, as well as provide unique opportunities to explore careers in PHL science, APHL and the CDC developed the Emerging Infectious Diseases (EID) Laboratory Fellowship Program. It trains and prepares selected scientists for careers in PHLs and supports public health initiatives related to infectious disease research. Areas of training and research include development and evaluation of diagnostic techniques, antimicrobial sensitivity and resistance, principles and practices of vector or animal control, emerging pathogens, and laboratory–epidemiology interaction. Fellows participate in either a 1-year program designed for bachelor's or master's level scientists, with emphasis on the practical application of technologies, methodologies, and practices related to EID, or a

2-year program in which doctoral level (PhD, MD, or DVM) scientists conduct high-priority research in infectious diseases. PHLs have the opportunity to host an EID fellow.

Credentialing

The PHL community has grappled with the issue of licensure since the middle of the 20th century. Initiatives began on the state level. In the 1940s and 1950s, the state of California created a model adapted by many others. The 1965 passage of Medicare legislation, however, brought with it sweeping changes with the CLIA. This evolved into the 1988 CLIA, and CLIA certification is now required by all clinical laboratories that receive Medicare or Medicaid payments. The CLIA regulations of 1992 further codified certification,[5] most notably stipulating stringency of requirements based on the complexity of an individual test. The Centers for Medicare and Medicaid Services provides oversight and enforcement for CLIA compliance, so PHLs need to be aware of their relationships with this agency. The CDC and the FDA also play roles in support and test categorization.

Yet, as the role of the PHL evolves, work still remains to make sure laboratory credentialing keeps pace. Today, a minority of states have licensure or credentialing requirements for medical and PHL scientists. Most states have no such requirements and rely only on local institutional policies and practices or job descriptions to specify the minimum knowledge, skills, and abilities required of laboratorians.[2] However, CLIA certification or credentialing through the College of American Pathologists can ensure laboratorian competency.

To address this, APHL has collaborated with the American Board of Bioanalysis to offer board certification in public health microbiology. The certification will afford doctoral level scientists in PHLs a new means to qualify for certification under CLIA, as well as establish the qualifications for nonphysician laboratory directors in medical and PHLs that conduct high-complexity testing on human specimens. The certification will be the first to specifically examine the training and experience required to direct a state or large municipal PHL. This represents one influential step into a changing landscape of certification that will affect PHLs into the future.[17]

Future Directions[15]

PHLs are part of a rapidly evolving era in public health—one in which global, flexible, and immediate response is essential to saving lives. The future challenges for these laboratories include:

- Standardized, multidirectional electronic communication of data and information
- Molecular biological assays
- Rapid, nonculture point-of-care infectious disease assays
- Ultrasensitive chemical analysis instrumentation
- Emergency response preparedness
- All-hazard surveillance
- Population biomonitoring
- Expanded newborn screening
- Genetic testing
- Emerging chemical contaminants
- Potential terrorism
- Emerging pathogenic microorganisms

PHLs also face a changing laboratory culture: a culture of connectivity and high expectation. The need for communication, collaboration, and cooperation with a multitude of essential partners, both within and outside governmental agencies, demands new goals and skills. Cultivating this new culture will strengthen PHLs and public health overall. Following is a look at a few of the areas that will drive these changes.

Emergency Response

America's increased focus on emergency response has deep repercussions for PHLs. While only one core function of a PHL is explicitly developed to emergency response, it is important to note that the entire laboratory infrastructure—skilled staff, instrumentation, specimen containment facilities, information management systems, linkages with private sector laboratories, and more—must be in place and functioning well in advance of a crisis in order to maintain the vigilance necessary to detect the unannounced release of an infectious organism or emergence of a new disease and the readiness to mount a swift and appropriate crisis response.

Achieving success will demand a continued commitment to partnerships and collaboration:

- Infectious disease and environmental epidemiologists

- Sentinel clinical laboratories
- Local and state first responders
- Federal agencies including CDC, FBI, FDA, USDA, the US Department of Homeland Security, DOD, and the DHHS
- State and local health officials

These broader working relationships will subject PHLs to new responsibilities. For instance, when working with first responders in field tests, PHLs will need to communicate the possible drawbacks and the importance of proper collection.

Biomonitoring investigations will undoubtedly be part of the new frontier. Biomonitoring is the direct measurement of people's exposure to environmental contaminants by measuring substances or their metabolites in blood, urine, or other specimens. With the early 21st-century influx of emergency preparedness funding through the CDC, many PHLs now have the technical expertise and instrumentation to support biomonitoring. To design biomonitoring studies, laboratories need to work in close partnership with environmental and chronic disease epidemiologists, as well as others in the environmental health community.[15]

Technological Advancements

For PHLs to provide optimum value to infectious disease surveillance and investigation, they will need to operate in as close to real time as possible. In surveillance, delay exposes more people to a possible pathogen, decreasing the efficacy of prevention and control measures.[2]

Technology will continue to play a big role in speeding up the flow of information. To this end, the CDC and partner organizations are continuing to build the PHIN. The purpose of the PHIN is to enable the secure transmission of population-based healthcare data across a patchwork of public health-related data streams for the purposes of surveillance and detection of emerging national health threats. These streams—which include FoodNet, PulseNet, and eLEXNET—currently function in isolation. Awareness of the vital importance of healthcare-related information flow has been increasing in all levels of government. The DHHS established the National Health Information Network (NHIN) in 2004 to improve the quality and efficiency of transmission of all healthcare data—both personal and population-based. It is a goal of the NHIN to promote the adoption of electronic medical record technology across the nation so ultimately every American can have unfettered access to their healthcare information. The PHIN works in collaboration with the NHIN to ensure that responders to the nation's population-based health care have access to and are providing appropriate data to protect the public's health.

A Workforce Crisis

In the early part of the 21st century, as infectious diseases multiply, environmental contaminants turn up in human tissues, and biological terrorism looms as a credible threat, it is scary to contemplate a scarce supply of scientists skilled in laboratory testing. Yet, the United States is now in the midst of a severe shortage of PHL scientists that threatens the nation's emergency response capability.[18] This highlights the concurrent needs for training and credentialing. For instance, the analysts who conduct bioterrorism testing must be trained in the standard methods and must be able to demonstrate competency before they are called on to run tests for actual events. The use of laws and regulations to assure the competency of laboratory staff in clinical and PHLs differs widely among states. Given the importance of public health testing, it is imperative that the analysts who perform these services receive standardized training with an established means to assess their understanding and abilities in method performance.

A 2007 survey showed that the United States had 50,000 fewer public health workers than it had in 1987.[19] Senior qualified PHL staff were retiring in large numbers, with no one to replace them. At this writing, organizations such as APHL are taking steps, from promoting PHL careers to advocating for national policy changes, in order to address this problem. Yet the confluence of increasingly demanding technical training and increasingly urgent emergency needs against decreasing numbers of scientists entering the workforce and decreasing amounts of money to pay them point to a "perfect storm" condition in the PHL workforce. Everyone in the larger health sector, from emergency response to private practice, should be aware of this trend in PHLs and how it could affect their fields in the next several decades.

Politics, Policy, and Funding

PHL funding, and even to some extent practice, has always been affected by politics and policy. This is as it should be, because the laboratories serve the needs of the country and community. However, it also sets up a situation in which funding and resources are released

in response to a crisis—sometimes only after significant efforts in political advocacy—and then laboratories are forgotten in the interim. This makes sustaining an adequate workforce and essential equipment difficult and compromises public safety. No one can predict when the next crisis will hit, and PHLs are at constant risk of being caught unprepared when they are underfunded.

The response to the novel H1N1 virus in 2009 is a good case in point. As the crisis emerged, PHLs were themselves in crisis. A global economic downturn caused federal and state funding shortages; laboratory workers had been laid off or placed on leave. Although the United States developed an economic stimulus package, PHLs did not benefit from any increased funding. After weeks of operating under surge conditions with staff shortages, laboratories received help in the form of emergency funding. However, in some cases it was "too little, too late" for bringing back the resources that had been lost in the downturn.

This typical "funding roller coaster" demands that today's PHL workers become adept at politics and advocacy. This may necessitate additional skills; once again, collaboration and communication are keys to success. PHL leaders are better learning how to state their case and make their needs known and how to communicate the value of laboratories in order to strengthen the chances for obtaining consistent, reliable funding. A perennial issue in the United States is the reform of the healthcare system. PHLs are positioned to play an important role in increasing efficiencies and quality of care; however, they must be proactive in both looking for opportunities to do so and to communicate their capabilities.

Cultivating a "Culture of Quality"

Given the scope of people and decisions that depend on their data, quality is a top priority for PHLs. Their operations are regulated by several national agencies, including the Centers for Medicare and Medicaid Services (for diagnostic testing performed on specimens of human origin), the EPA (through the Safe Drinking Water Act), and the FDA (for testing milk that will be transported across state lines). The standards are multifaceted and rigorous. For instance, to ensure operations are up to standard, an incoming PHL director will often review the following.[10]

- The current CLIA license
- The state license (if applicable)
- Select agent registration
- Any EPA certificates and related correspondence
- Other relevant laws and regulations (e.g., Clean Water Act, Interstate Milk Shippers Act)
- The activities the PHL can legally engage in

In keeping with the spirit of partnerships and collaboration in today's public health world, PHLs often work with regulatory agencies to support their efforts. This can involve developing a jurisdictional laboratory response network, facilitating coordination among the many private and public sector players, and conducting training and outreach programs. Does the state have a laboratory improvement and regulation initiative? If not—or if the current one needs to be improved—PHLs can provide a vital role.

But in addition to quality assurance—meeting important regulatory compliance and internal standards—PHLs are also striving toward quality improvement. This is part of an evolving role that will position them for greater flexibility and stronger performance. For instance, APHL and the CDC partnered to create the Laboratory Systems Improvement Program, which has a mission to establish a system that measures the performance of state public health systems and supports their continuous improvement. It provides an assessment tool, technical assistance, and a wealth of accessible resources in quality improvement.[20]

Too often over the years, the PHL has been seen as a "black box"—separate unto itself, sometimes slow-moving, and reluctant to reach out. As a consequence, the PHL's lifesaving mission and its importance to national and global health have been overlooked. Significant leaps forward in PHL functioning have always come in response to crises, and the future certainly will not lack for comparable opportunities. Yet as this overview of the history and challenges in PHLs shows, it is the decisions and changes made between the crises that set the foundation for these advances.

Discussion Questions

1. Should funding be allocated for creating more and better equipped PHLs at the local level? Why or why not?
2. PHLs are expensive to create, maintain, and staff. What major activities do they perform that are worth this expense?
3. PHLs at the state level are critical components of the national response to terrorism and other emergencies

(e.g., influenza). Should PHLs be funded by the federal government as part of national security? Why or why not?

4. PHL work is increasingly technical, yet there are serious shortages in the available workforce. What might be done, and what types of training required, to alleviate the shortfall in qualified laboratory workers?
5. What types of analyses or research should PHLs become involved with in the coming years?
6. Given that PHLs are expensive to maintain and often provide services at reduced or no charge, why should a PHL offer a test which is also available commercially (e.g., for sexually transmitted diseases)?
7. Many countries are just now developing their first, comprehensive PHL at the state/regional level. Given often limited resources for start up and maintenance, what types of analyses should they first focus on, and why?
8. Describe at least two manners in which PHLs differ from private laboratories.
9. Of the 11 core functions of PHLs, of which two are the general public least likely to be aware? Why might that be, and how can it be changed?
10. Describe how state PHLs and the CDC complement each other in their response to infectious disease of bioterrorism events.
11. Funding for PHLs is frequently inconsistent and subject to "roller coaster" effects. Federal funds may provide for analytical instruments and personnel for several years, but are likely to taper off. What should state PHLs consider before accepting the funding for instruments and personnel given that it may be short lived?

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2

The Basics of Microbial Analyses

Introduction

The purpose of this chapter is to impart to the reader a better understanding of what occurs in a laboratory when a sample is analyzed for the presence of microorganisms. There are aspects of this process that may be easier or more difficult than sample analysis for the presence of chemical or radiological elements. On the one hand, the simple growth of a colony on a specific type of agar, or how it reacts to a specific stain, may be characteristic of a specific organism. This process is likely much cheaper to perform than a chemical analysis requiring instrumentation. On the other hand, it may be quite difficult and time consuming to differentiate between two closely related species (e.g., *Bacillus cereus* and *B. anthracis*; the difference is quite important!) whereas the identification of two chemical cousins may be much easier. Also similar to chemical analysis is the differentiation of microorganisms into different groups with different properties and methods of analysis. We will thus discuss how selected methods apply to the analysis of bacterium, viruses, and parasitic protozoa.

The purpose of this chapter is to provide an overview of the methodologies used for the analysis of different sample types for microbiologic organisms. It will cover different types of samples that may be analyzed, some of the methods employed for sample preparation, and, finally, an overview of different analysis methods. It will not discuss techniques and methods used for the analysis of fungi and mycobacteria (except as it relates to *Mycobacterium tuberculosis*). While these are all important and have varying degrees of public health importance, their inclusion is beyond the scope of this book. Indeed, later chapters discussing sample analyses and contaminants will focus on bacteria, viruses, and select protozoa. A section in Chapter 5 discussing tuberculosis is the single slight exception.

The reader is advised that the topics discussed in this chapter were chosen to represent the more common techniques and methods employed in modern public health laboratories (PHLs). It is by no means exhaustive of all that PHLs do, and certainly not exhaustive of all microbiology. There are many texts that deal with aspects of this chapter and we do not seek to duplicate that level of detail. Instead, the chapter was purposefully written to be a general and brief overview of different techniques. The desire is for the reader to come away with an understanding of what actually occurs in the laboratory so they, in turn, will have a greater appreciation for the expertise involved and the potential limitations of the test methods. Sources of further reference will be provided at the end of this chapter for those seeking greater detail on specific methodologies. Later chapters of this book, dealing with typical analysis sections in laboratories (e.g., food analysis for microbial contaminants), will reference this chapter when describing the specific techniques involved and provide more detail into the specific order of activities.

Sample Types and Collection

When looking for microorganisms, the types of sample amenable for analysis are almost infinite. That is, an individual can bring in almost any type of substance and a subsequent analysis for the presence of almost any type

of microorganism could be attempted. However, this is an excellent example of how simply because something is theoretically possible does not also mean it is practical. A level of logic must be employed to ensure the sample has a higher likelihood of containing the pathogen of interest. It is for this reason that PHLs often specify which sample types are allowable for which methods (e.g., stool samples for parasites, sputum samples for tuberculosis).

To clarify the nomenclature, clinical samples (those obtained from humans) are often referred to as specimens. In fact, much of the regulatory language concerning the analysis of such samples and the operations of the laboratory use this term. However, there are also many occasions for overlap with other terms, such as sample and substance. A clinician collects a blood or stool sample, but it may then be called a specimen in a laboratory. When shipping such items, they are classed as either diagnostic specimens or infectious substances. On the other hand, samples such as water, soil, and food collected from the environment are near universally referred to as samples. To maintain consistency throughout the text, we will therefore use the term "sample" to refer to that which is collected for laboratory analysis no matter the source.

Clinical

Blood samples are collected when the pathogen of interest may be found in circulation, or if the test will look for the presence of pathogen-targeted antibodies. A common type of blood sample seen in PHLs is that obtained from a vein and collected into an evacuated tube with a volume of 2–10 ml. The tube may or may not contain reagents such as heparin or EDTA. Care must be taken to prepare the site of venipuncture so that the collected sample is not contaminated with dermal flora. Some tests do not require this much volume. The analysis for the presence of genetic disorders in newborns requires a simple drop of blood. This blood spot is collected by penetrating the skin on the heel of the newborn with a fine needle and blotting a drop of blood onto a prepared paper card. Blood samples are routinely collected for the analysis of some parasites (e.g., *Plasmodium*) and lead levels.

Sputum samples are collected when the pathogen is expected to reside within the lungs or nasopharyngeal area. An example is the collection of such a sample for *M. tuberculosis* where the individual coughs their sputum directly into the sample container. A variation of this is the nasopharyngeal swab where a clinician physically rubs this area with a swab to collect a sample. Such swabs may then be analyzed for organisms causing respiratory illness.

Swab samples are generally useful when the pathogen is likely to be found on the surface of an accessible part of the body. In addition to the nasopharyngeal swab just described, other sites include the vagina and cervix (for sexually transmitted infections) and the inside of the mouth (for human immunodeficiency virus [HIV]). Swab samples, especially those collected for viral analysis, usually require the use of a transport medium. Transport media compositions vary in formula and differ in composition for bacteria and viruses. Many test kits provide specific media and containers compatible with their test.

Stool samples are collected primarily in response to a suspected case of water- or food-borne illness. The individual is ill and the symptoms lead the clinician to suspect an enteropathogenic organism (i.e., one which may cause disease in the gastrointestinal tract) as the culprit. Samples are collected in clean, wide-mouthed containers. Unlike many other clinical sample types, these are often chemically preserved if the analysis is for the presence of parasitic protozoa (discussed in more detail in Chapter 5). Like most clinical samples, these would ideally be delivered to the laboratory within 2 hours of collection, but if this is not possible they are preserved either thermally (4°C) or through the addition of various reagents.

Slant/stab samples are usually sent to the laboratory from a hospital or clinic that has already performed the initial culture. They may need assistance with colony identification, tests for drug susceptibility, or may be simply complying with state/federal regulations requiring the referral of certain sample types for further characterization. A common example is the culturing and analysis of a clinical sample for *Bacillus* species (spp.). The hospital may have identified the isolated colony as belonging to *Bacillus* but they lack the reagents to rule out *B. anthracis*. The state health department maintains stocks and expertise to perform this type of "rule out," or confirmatory, testing in support of local clinicians.

Other sample types are certainly possible, but their appearance in PHLs is less frequent and may vary considerably by geography and season. Cerebrospinal fluid may be collected for the analysis of arbovirus infection (viral encephalitis). Hair samples may be collected for the analysis of metals, indicating exposure.

Environmental

Water samples are collected for a variety of reasons. These include routine testing of drinking and recreation waters for contaminants regulated by the US Environmental Protection Agency, well contamination from field runoff, and requirements for home sales. The great majority of

analyses performed on a routine basis look for regulated parameters, such as fecal coliform in samples from private and semiprivate wells and recreational waters such as beaches. Water-borne pathogens and testing methods and requirements will be discussed in Chapter 6. The collection of such samples, often done to meet regulatory requirements, has fairly stringent requirements for details like sample size, container material, and shipping conditions. These are discussed in more detail in Chapter 3.

Food samples are often submitted as part of a food-borne outbreak or illness investigation. These samples include almost any conceivable type of food that is suspected to be associated with the adverse health event. Examples include the intentional poisoning of food to target an individual or group and the accidental contamination of food during processing or transportation. There is no set standard for sample type and collection for this broad category. In general, those investigating the occurrence will contact the laboratory and determine (1) what the laboratory is capable of in terms of analysis; (2) what type of sample and what amount should be collected; (3) how the sample should be collected, packaged, and shipped; and (4) the time frame for collection and delivery to the laboratory. Many dairy samples (e.g., milk, ice cream) are submitted on a regular basis for the producer to remain compliant with the Grade "A" Pasteurized Milk Ordinance.

Other sample types include gauze wipes to ascertain the presence of surface contamination. There were many of these collected during the anthrax attacks in 2001 when investigators were attempting to determine which parts of mail distribution facilities were contaminated, and if decontamination efforts were successful. These were associated with the submission of "white powders" for suspected anthrax analysis. These were actually almost any type of substance someone thought suspicious enough to test for bioterrorism agents. PHLs gained much experience with the screening and analysis of such environmental samples in the year after the anthrax attacks of 2001. Since then, the submission of such samples has decreased more than 90%, but there are still the occasional samples for which an analysis must be attempted. Many of these more esoteric sample types are decided on a case-by-case and lab-by-lab basis.

Sample Preparation

The great majority of samples received in the laboratory may be forwarded on to analysis without any prior preparation. Examples include swabs submitted for analysis of chlamydia and gonorrhea and stool samples submitted for parasitic analysis. There are some sample types and analysis techniques that do require some level of sample preparation before the analytical technique may be employed.

Lysing involves taking the sample and mixing it with a solution that will break apart the cellular components (cell lysis). This is done so that the genetic material of any target organisms is freed from the cell/viral matrix and available for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) analysis. Intact cellular/viral walls prevent such analysis because the DNA/RNA is not accessible to the nucleic acid (NA) probes, but the lysing agent disrupts these walls and releases the contents. This solution is termed the lysate. Mechanisms to cause cell lysis include the use of viral phages that invade cells, the use of enzymes that cause the walls to weaken, and the use of various solutions that create osmotic pressure causing the walls to rupture.

Other preparation activities include the simple plating of the sample onto solid media and the inoculation of the sample into growth media such as broth. Plating the sample onto nonselective media allows for the formation of colonies after incubation. Isolated colonies may then be selected for further examination. Inoculation into nonselective broth allows for the rapid growth of organisms. Having a much larger amount of sample organisms may be useful for conducting trace-level analyses for biologic toxins and for creating a larger sample for use in various analyses such as sequencing and pulse-field gel electrophoresis (PFGE).

Sample Analysis

Although there are nearly innumerable types of tests and assays that can be performed to identify a microorganism, the actual tests selected to be done are often chosen based on previously determined information. That is, there is often a series of tests that must be performed, and the results of an initial step might well determine which test will be done subsequently. For example, the examination of a stool sample by light microscopy may reveal the presence of the protozoa *Cryptosporidium*. There might then be no point in then culturing the sample for an enteropathogenic bacterium such as *Salmonella*. In Chapters 3 and 4, where we discuss chemical and radiological analyses, we will be walking the reader through the steps of sample collection, preparation, extraction and cleanup, and analysis—essentially including the most common procedures for each because they are all fairly common for most analyses. While chemical and radiological analyses follow a fairly linear path, microbiologic analyses often follow more circuitous paths. Microscopy may be followed by culture, and more microscopy and further

culture, with sample portions subjected to other analyses such as NA and immunologic assays at any time. Given that microbiologic analyses are so varied, we will take a different tack here. Instead of following a sequence of events, we will discuss some of the more common techniques and methods used in PHLs. The order of their presentation is not strictly related to their order of use.

Microscopic Examination

Microscopy is a method of viewing extremely small objects. While many microscopes may be quite complex, light microscopes all contain at least four basic components: an illumination source, a sample platform, an objective, and an eyepiece. The illumination source may be as simple as a light bulb, and may emit in the ultraviolet (UV) range as well. This light is usually focused with one or more lens to pass through the sample, which is mounted on its platform. As the light passes through the sample, some is scattered away and the rest enters the objective as an image. The objective is actually a series of refractive lenses where the magnification occurs. Finally, the image reaches the eyepiece where it is viewed. The eyepiece may actually be electronic—such as a charge-coupled device (CCD) camera, which digitally records the image. The level of magnification achievable is limited by the wavelength of the light used, and a 1000× magnification is the highest commonly used. Electron microscopes (EM) use a beam of electrons as their illumination, and different types operate under different principles. As electrons have a much smaller wavelength than light photons, the level of magnification possible is much greater, with 20,000× commonly achieved by scanning EMs. Transmission EMs may resolve individual atoms with specialized techniques and conditions. EMs are somewhat expensive to maintain, require a relatively high level of expertise to use, and are not terribly useful for much diagnostic analyses. They are therefore infrequently used in PHLs and few maintain them.

Light microscopy makes use of white light to illuminate the sample, which is then directed through the objective and observed through the eyepiece. This is the simplest of microscopic techniques, but also suffers from several disadvantages. First, out-of-focus elements within the view may blur or obscure the point of interest. Secondly, many bacteria, parasites, and other organisms potentially observable in this manner do not refract enough light to become sufficiently visible. That is, they are not dark enough to resolve and remain indistinct from either the background or each other. This weakness may be offset by alterations in technique. An example of simple light microscopy is seen in Figure 2-1.

Figure 2-1 Unstained wet mount of a *Giardia lamblia* trophozoite visualized (poorly) by light microscopy. (Courtesy of Centers for Disease Control and Prevention/Dr. Mae Melvin.)

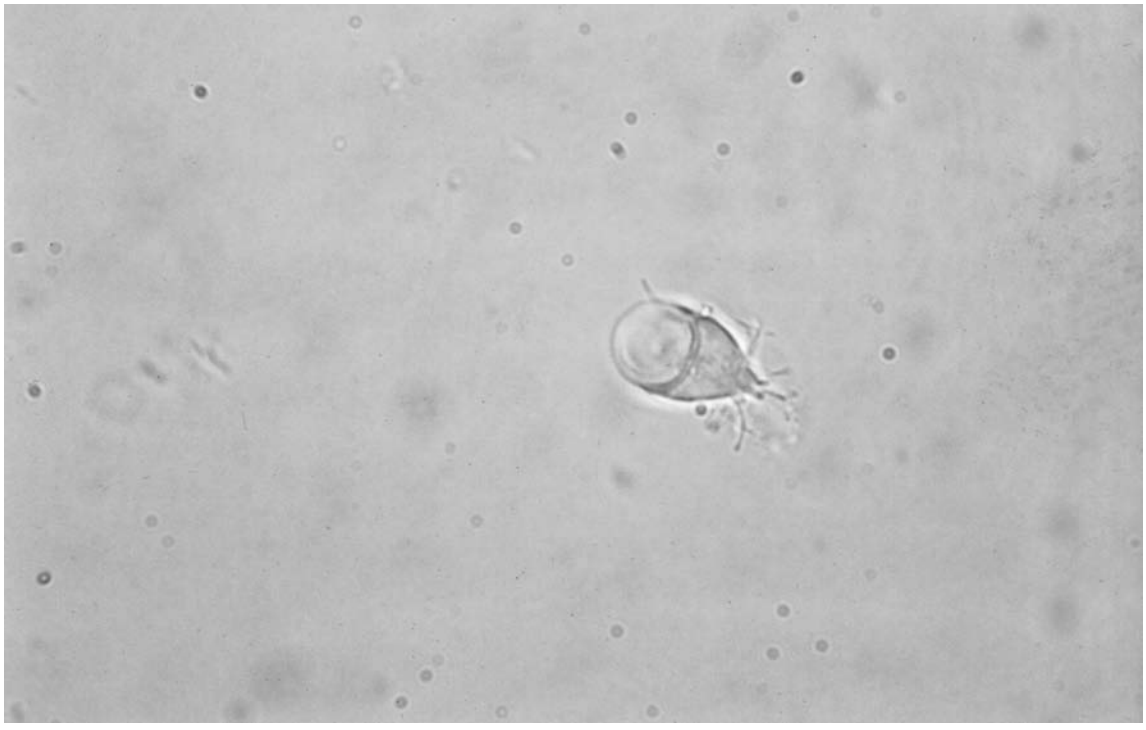
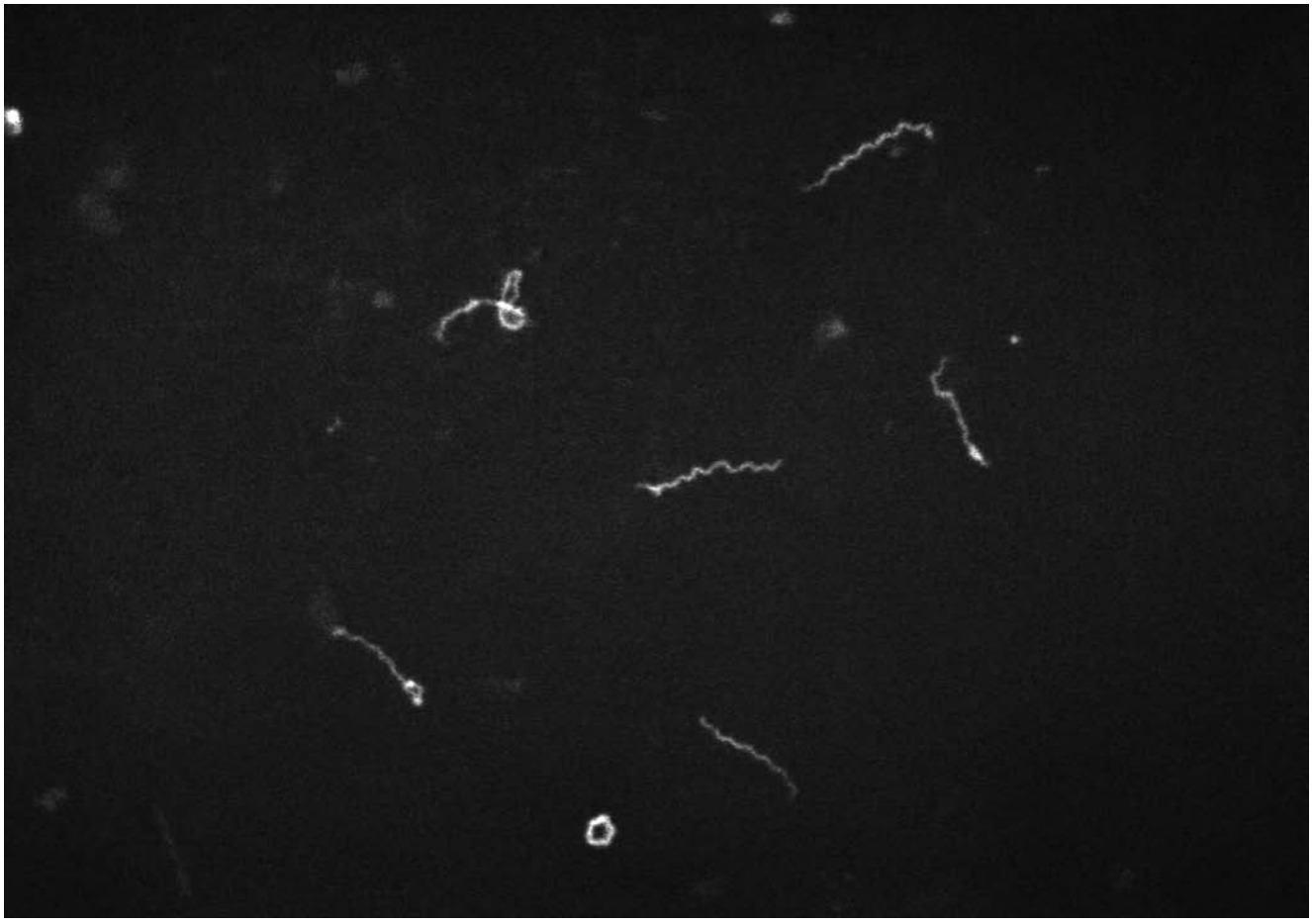


Figure 2-2 ***Treponema pallidum*** **(syphilis) bacteria visualized by darkfield microscopy.**



Darkfield microscopy is a technique whereby the source illumination is actually directed away from the objective lens. The view therefore appears dark (hence the name). Bacteria have a different refractive index than the medium they are in, and the light is redirected into the objective by passage through them. The technique is useful for the detection of organisms that are thin, such as *T. pallidum* spirochetes. An example is seen in Figure 2-2.

Phase-contrast microscopy utilizes destructive interference to resolve near transparent microorganisms. A ring annulus placed between the source illumination and the sample produces a hollow cylinder of light that passes around the specimen and to the objective. Light that passes through the specimen is slowed slightly and arrives at the objective ~1/2 wavelength out of phase with the cylindrical light. This produces destructive interference and specimen details (which slowed the light) appear dark in comparison to the surrounding medium. An example is seen in Figure 2-3.

Fluorescent microscopy takes advantage of the properties of some compounds (fluorochromes) to absorb UV light and re-emit light in the visible spectrum. The sample is illuminated with a UV source and any fluorescent components in the sample that absorb the emitted UV spectrum emit their visible own light. Filters are used to separate the illuminated and background lights from the emitted light and the slide appears dark with bright areas where the fluorochromes are observed. Many staining techniques use this principle and some of them will be discussed later in this chapter. An example of a naturally fluorescing microorganism (*Cyclospora*) is seen in Figure 2-4.

Electron microscopy is a technique which is not directly observable. That is, a beam of electrons is aimed through the sample. Resolution is determined by how the beam is refracted, which is based on sample density. Thus, cell walls and solid objects are "darker" than less dense areas, such as the cytoplasm. There are many variations in techniques and technology (including scanning

Figure 2-3 ***Trichomonas vaginalis*** **protozoa visualized by phase contrast microscopy. (Courtesy of Centers for Disease Control and Prevention.)**

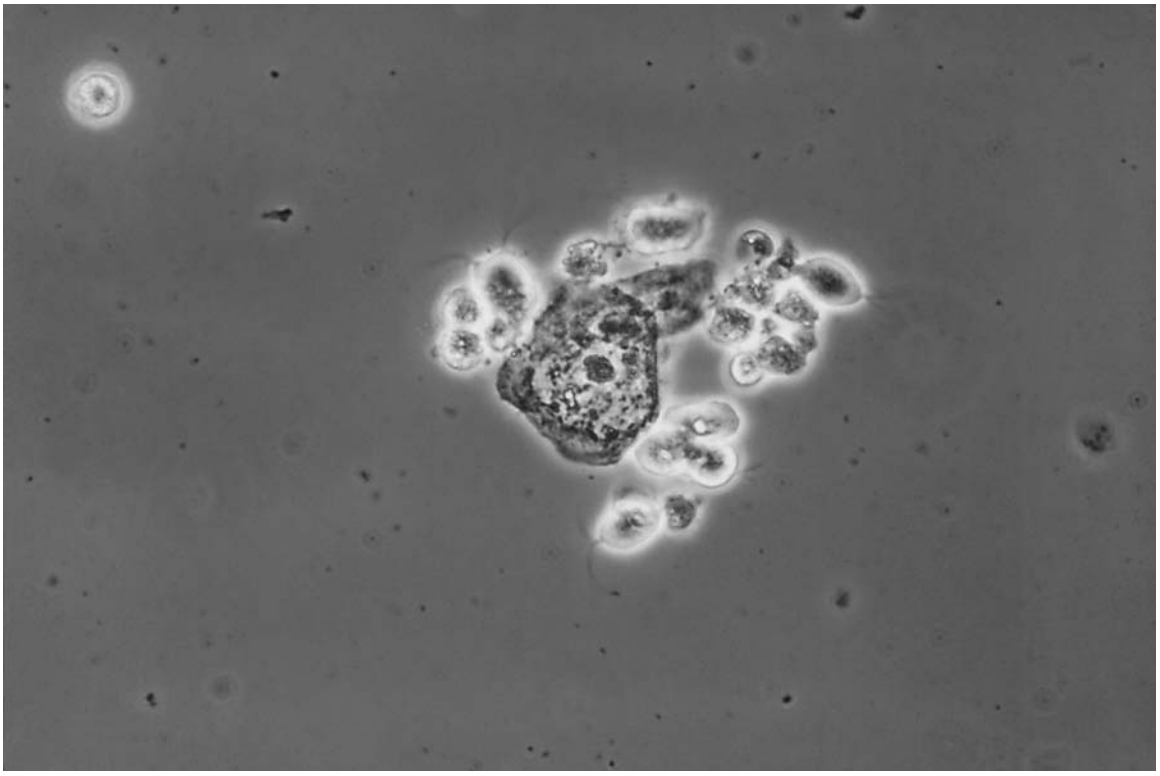


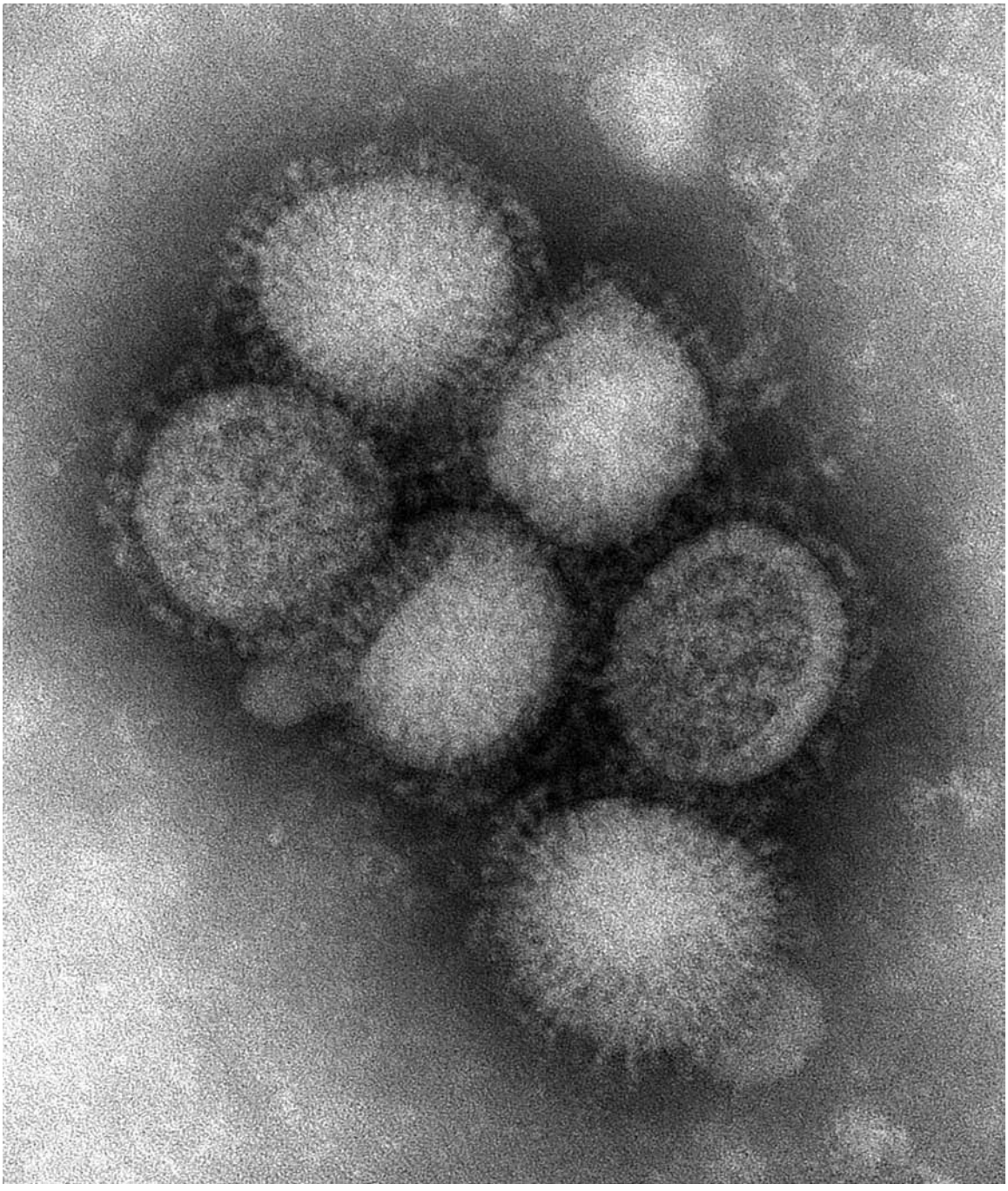
Figure 2-4 ***Cyclospora cayetanensis*** **oocyst visualized by fluorescence under UV microscopy. An image bank of full-color photos is available online at http://www.jbpub.com/catalog/9780763771027/. (Courtesy of Centers for Disease Control and Prevention/DPDx.)**

and transmission), but these are highly technical, very expensive to maintain, and not often used outside of national level and research laboratories. An example is seen in Figure 2-5.

As is evident by the previous discussion, many of the organisms one might wish to observe are not readily visible by simple microscopy. They may be almost entirely transparent, or at least transparent enough, so that few characteristic details may be seen. In addition, we find that many microorganisms that may be observed look alike. How might one be able to tell the difference between two different rod-shaped bacterium of approximately equal size? To overcome these problems, researchers have developed a number of staining techniques to provide color contrast and aid identification.

One may say that the overarching goal of staining it to cause target structures to become more visible by the creation of contrast. Figure 2-1 is an example of a wet mount, where the sample is observed without any fixation or staining. While few structural details are visible, this technique is useful for observing an organism's motility (movement), which may often be characteristic. Observation may be improved by staining the sample. In this process, chemical compounds are added to the sample on the slide to provide a color contrast.

Figure 2-5 Swine flu virions (A/CA/4/09) visualized by negative-stained transmission electron microscopy. (Courtesy of Centers for Disease Control and Prevention/C. A. Goldsmith and A. Balish; photographer: C. A. Goldsmith and A. Balish.)



These compounds may be nonspecific and stain all cells equally, or specific and only stain specific target compounds. The process of staining follows a basic series of steps.

- *Fixing* the sample is done to preserve cell structure and/or make the cells more ready to chemically react with staining compounds. This is done through the use of chemicals such as methanol, or heat, and may not be necessary for all methods.
- *Primary stain* is a chemical compound that reacts with different cellular components; being retained if the components are present. Single-stain methods may be used on fixed or unfixed samples and provide contrast by using a color reagent that binds nonspecifically to structures.

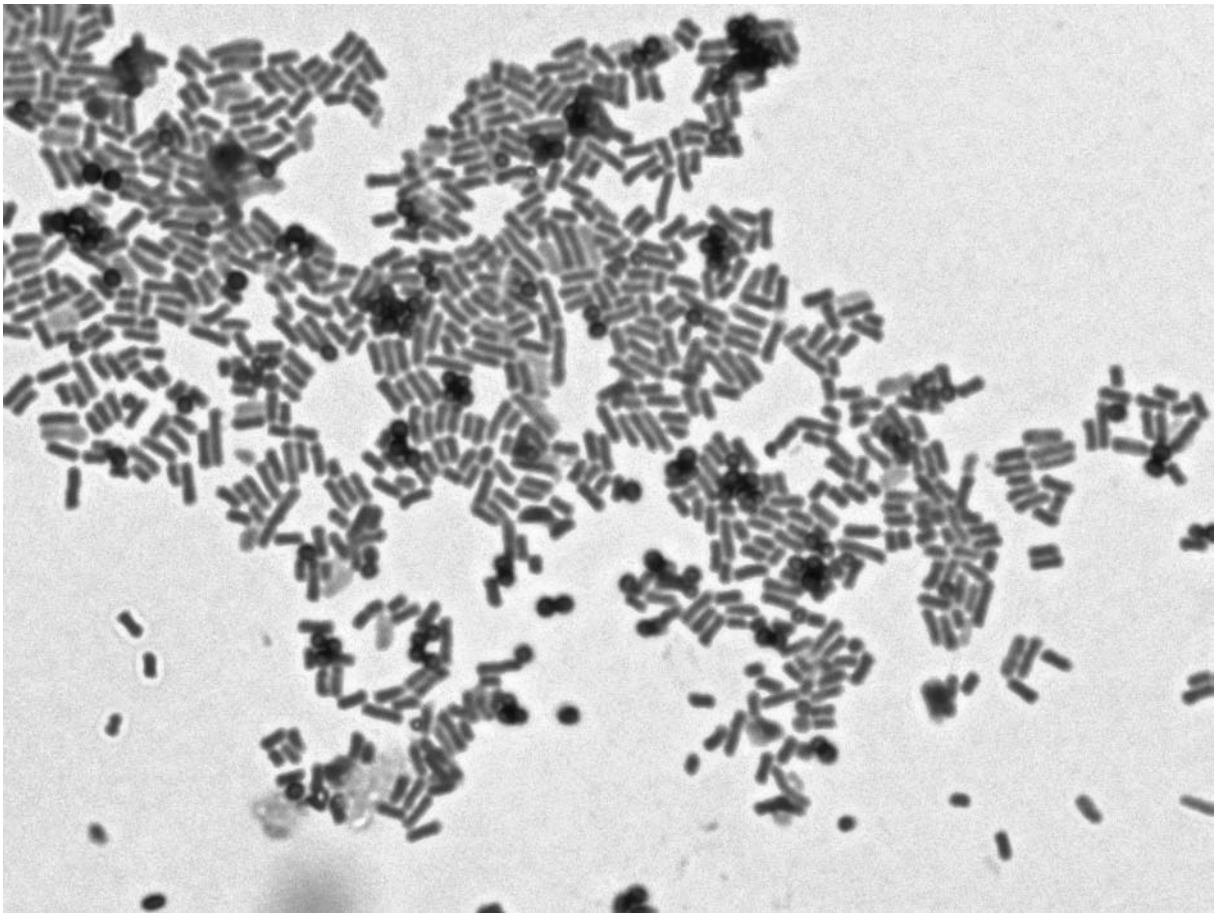
- *Mordants*, such as iodine solution, may then be added to precipitate the retained stains in the cellular structures so they are not removed during decolorization.
- *Decolorization* is the next step where the slide is washed and all nonbound stain removed. These solutions vary, but typical components include acids, alcohol, and organic solvents. At this point, only the cells that react with the primary stain are colored. However, it is frequently useful to view others present as a contrast.
- *Counterstaining* is now often done to ensure that all microorganisms are now stained and visible. However, those organisms that reacted to the primary stain will be a different color than those reacting now.

Bacterial Staining

Gram stain is perhaps the most widely used staining technique, and one that is used to differentiate bacteria into two groups. Bacteria are often referred to as either Gram-positive or Gram-negative (though some may be Gram-variable). Gram-positive organisms have few lipids in their cell membrane, allowing for binding of the crystal violet primary stain, and appear purple in color. Gram-negative organisms have a higher lipid content in an outer membrane that prevents this binding, but are subject to the safranin or fuchsin counterstain, and appear pink or red (depending on the counterstain). Variations of this technique (e.g., Wayson) are used to observe difficult to visualize organisms which are weakly Gram-negative. An example is seen in Figure 2-6.

Ziehl–Neelsen (Z–N) stain is useful for organisms with long chain fatty acids (mycolics) in their cell walls that make them impervious to most basic dyes. The application of heat allows the penetration of the carbol fuchsin primary dye into the organism where it forms a stable complex. This is not washed away by the decolorization step and the organism is termed *acid-fast*. Acid-fast bacilli (AFB) are thus colored red, while

Figure 2-6 Example of Gram staining showing Gram-negative *E. coli* and Gram-positive *Staphylococcus*. (Courtesy of Illinois Department of Public Health, Division of Laboratories.)



non–acid-fast organisms react with the methylene blue counterstain to become blue colored. This technique is particularly useful for *Mycobacterium* species. *Kinyoun* is nearly identical to Z–N stain with the exception that the heating step is replaced with a higher phenol concentration in the primary stain solution.

Acridine orange is a compound that is permeable to cell walls and becomes incorporated into cellular NA. This is a single-stain method (no counterstain) and, under UV illumination, it fluoresces orange.

Auramine-rhodamine are both fluorochromes that bind to mycolic acids (long fatty acids incorporated into the cell walls of some organisms such as *Mycobacterium* spp.). They resist decolorization and are used as equivalents to acid-fast techniques in some laboratories because the method is easier and faster to perform. AFB fluoresce orange-yellow, but if the potassium permanganate counterstain is not used, the color changes to yellow-green.

Fluorescent antibody (FA) is a staining method using the high degree of specificity offered by immunohistochemistry. It will be discussed in more detail later in this chapter.

There are other stains also in use, including McFadyean and India ink capsule stains, to show the presence of bacilli capsules. Iodine stain is used to provide nonspecific contrast.

Protozoa Staining

Modified Z–N and *modified Kinyoun* are acid-fast stain variations utilizing a less harsh acid solution during decolorization (Z–N) or a lower concentration of acid during decolorization and no heat requirement (Kinyoun). Acid-fast protozoa are colored red, while non–acid-fast organisms react with the methylene blue counterstain to become blue colored.

Giemsa and *Wright's* stains are used on blood films and differ only in that Giemsa stain contains no fixative and the blood film must be fixed *a priori*. Methylene blue and eosin are combined into a primary stain and no further counterstain is required. These stains characteristically color erythrocytes, leukocyte nuclei, eosinophilic, and neutrophilic granules.

Trichrome stain (Wheatley) utilizes chromotrope 2R, fast green FCF, and light green SF stains on fixed fecal smears that have been obtained from preserved samples. The stains are combined into a single solution and no further counterstain is required. The combination of stains allows various structures within cysts and trophozoites to become colored differently.

Modified safranin stain is useful for *Cyclospora* and provides more consistent staining than the modified acid-fast stains. Safranin stains *Cyclospora* red and the methylene blue counterstain provides a blue background.

Other important stains include hematoxylin, iron-hematoxylin, iodine, acid-fast trichrome, modified Field's stain, and Lugol's iodine stain.

Viral Staining

The direct staining of either selected samples or infected cells for viruses is not often specific enough for a diagnosis. Some cell morphologies, such as characteristic cellular inclusions, may be observed, but require further tests for confirmatory identification. Immunofluorescent staining most commonly uses either fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TMIT) as markers that fluoresce apple green and reddish-orange, respectively. An example of viral immunofluorescent staining is the rabies slide in Figure 5-16.

By this point one may have learned a lot. Parasites may have been observed and identified via morphology, size, and immunoassay reaction and there may be no need to go further. The analyst may have gathered information about some pathogenic bacteria based on size, shape, staining, and immunoassay reaction. This would then tell them what next steps to take as far as culturing, biochemical reactions, or molecular analyses. The analyst might find no protozoa or bacteria (of interest), but have an immunoassay positive for virus. This would lead them to confirmatory analyses by molecular and/or other immunoassay methods.

Culturing

The ability to grow microorganisms is one of the most basic, yet powerful, techniques in microbiology. This allows the analyst to preserve the sample for extended time periods, transport the sample to other laboratories, obtain greater amounts of isolated organisms for selected analytical purposes, and identify the organisms through observation of how they react in different conditions. There are literally hundreds of identified media, and we will not attempt to discuss them all. While many have very specific functions, all include a nutrient source, a specific pH, a solidifying agent (for solid media such as plates, agar from red seaweed is almost exclusively used), and selected specific additives (e.g., antibiotics). We will be discussing solid media (plates) for the most part in this section. Broths will not be discussed separately because they have many of the same variations, with the excep-

tion of a solidifying agent. See Atlas and Snyder (2006) for more information on media composition conditions.

Most of the organisms encountered by PHLs grow best in temperatures between 25° and 40°C (though there are exceptions such as those described in Chapter 6). Most organisms also grow well under ambient atmosphere (aerobic), though some are facultative anaerobes. *Staphylococcus*, for example, is able to grow with or without oxygen and *Campylobacter* is an example of a microaerophilic organism requiring decreased levels of oxygen and increased levels of carbon dioxide.

Transport media are used to sustain the sample from collection to arrival in the laboratory. These are usually tubes with a small amount of nonnutritive agar or broth into which the sample is placed. Additives help maintain pH, reduce desiccation, and indicate oxidation. Media for viral and *Chlamydia* samples differ in that the main requirements are more for an isotonic environment with a consistent pH and antibiotics.

General purpose media are used to nonselectively grow organisms from the sample. They are useful for both aerobic and facultatively anaerobic organisms and isolates obtained are subjected to further analysis for identification. Examples of general purpose media are 5% sheep blood agar, known as a blood agar plate (BAP), and chocolate agar. There is information that can be drawn from some of these types of media alone. Some organisms produce enzymes (hemolysins) that can lyse the red blood cells in BAP and degrade the contained hemoglobin. This is referred to as β-hemolysis and there is a clear area in the media around the colony. A partial breakdown of the red blood cells results in a greenish area in the media and is termed α-hemolysis. The absence of hemolysins and lack of any breakdown is termed γ-hemolysis. This in itself aids in the identification of the organism.

Enriched media is used to grow organisms that do not propagate well on general media. Termed *fastidious*, these organisms require special nutrients in order to grow. An example is the addition of the amino acid cystine in chocolate agar to grow *Francisella* spp.

Selective media are used to aid organism identification by setting growth conditions that are favorable for only certain organisms. This is usually done by the addition of dyes and/or antibiotics that inhibit the growth of organisms that cannot tolerate them well. A media may contain penicillin, for example, and organisms resistant to penicillin will grow while those that are susceptible will not do well. However, this selection is not always perfect and small colonies of partially inhibited organisms may be present. An example of selective media is MacConkey agar. The addition of crystal violet dye (discussed previously along with Gram staining) inhibits the growth of Gram-positive organisms. The systematic testing of an isolate's ability to grow in the presence of different antibiotics is the basis of drug susceptibility testing done for such organisms as *M. tuberculosis*.

Differential media are used to aid organism identification by setting conditions that alter their appearance on the media. This is done by the addition of additives that may/may not alter colony appearance depending on genus or even species. An example is the use of Hektoen agar to differentiate between *Salmonella* and *Shigella* in stool sample analysis. While both grow on Hektoen agar, their appearance is distinctly different. This is discussed in more detail in Chapter 5.

These media, with their general, selective, and/or differential purposes, allow for the growth of most bacteria and are of paramount importance for the isolation of bacterial organisms. The strategy is to enhance the growth of target organisms while inhibiting competing normal flora for the purpose of identifying the infectious pathogen. While we have described several types of media already, the following are also used to varying extents in PHLs.

Bacterial Cultures

Media may be both selective and differential. Sorbitol-MacConkey (SMAC) agar is selective for *Escherichia coli* spp. and differential between O157 and non-O157 strains (O157 does not ferment sorbitol while most non-O157 strains do). O157 colonies will be pale/colorless in color while non-O157 colonies will appear pink. The color differences vary with media, and the use of commercially available CHROMagar for the same purpose will result in different color variations. This is discussed in more detail in Chapter 5.

- *Bismuth sulfite* agar is selective for *Salmonella enterica* Typhi and other enteric bacilli. Hydrogen sulfide gas produced by *Salmonella* reacts with iron sulfate to produce green/black metallic colonies and a black/brown precipitate. *Brilliant green* agar is selective for *S. enterica* non-Typhi.
- *Columbia* agar is a general purpose agar for the growth of many organisms, including fastidious ones. The addition of 5% sheep blood (blood agar) allows for the observation of hemolytic reactions.

- *Cystine tellurite blood* agar is a selective and differential media for the detection of *Corynebacterium diphtheria*. Potassium tellurite inhibits most upper respiratory tract flora (except *C. diphtheria*) and Gram-negative organisms.
- *Middlebrook 7H11* agar is nonselective for the growth of *Mycobacterium* spp. It contains casein hydrolysate (versus 7H10 which does not) to promote the growth of drug resistant strains. This media has largely replaced *Lowenstein-Jensen* medium, which is egg-based and requires more time for colony growth.
- *Modified Thayer-Martin* agar is selective for the growth and identification of *Neisseria* spp. from clinical samples. The modifications from Thayer-Martin include the inclusion of less agar, more dextrose, and the addition of trimethoprim. These promote the growth of *Neisseria* while inhibiting *Proteus* spp.
- *Regan-Lowe* agar is a selective medium for the identification of *Bordetella pertussis*. Starch and charcoal are added to neutralize compounds toxic to *Bordetella*. Regan-Lowe is more effective at isolating *Bordetella* than *Borden Gengou* media. Both contain blood for the detection of hemolysis.

Protozoa Cultures

There are only a few parasitic protozoa that are able to be cultured (e.g., *Trichomonas vaginalis*) and few laboratories perform this procedure. They rely instead on microscopic and immunologic techniques for identification.

Viral Cultures

Viruses (and *Chlamydia trachomatis*) are obligate intracellular parasites and cannot grow in culture alone. They must infect other cells which may then be propagated. While cell culture lines may be maintained by a laboratory, most are available commercially. The infection of a cell line by a virus may be deduced by subsequent cytopathic effects (CPE). These are visible and characteristic effects of viral replication within the cell monolayer. Genetically altered cell lines exist whereby the presence of the specific virus induces a biochemical reaction that may be observed (e.g., via a color change or physical effect in the surrounding media). These lines are collectively termed enzyme-linked virus-inducible system (ELVIS) and contain additional genetic code from viral, bacterial, or cellular sources. Some of the more widely used and generally useful cell lines are as follows.

- C6/36 and AP61 are cell lines both derived from mosquitoes and are useful for the isolation of arboviruses.
- HEK is a cell line derived from human embryonic kidneys and is becoming less commonly available. It is useful for the isolation of adenoviruses, enteroviruses, herpes simplex virus, and others.
- McCoy cells are derived from a mouse fibroblast line and used for the isolation of *C. trachomatis*.
- Madin-Darby canine kidney (MDCK) cells are used for the isolation of the A, B, and C influenzas.

Biochemical Reactions

Biochemical identification becomes an essential part of the microbiology work when attempting to identify bacteria that have distinctive biochemical profiles or when multiple subspecies share closely related biochemical reactions. Just as one may use media to differentiate or select organisms, one may observe the reaction of colony isolates to specific compounds. While a confirmed organism identification is unlikely to be made based solely on one biochemical reaction, the results of several may be indicative of a specific genus or species. Biochemical reactions are not usually useful for the identification of protozoa or viruses, but one used for limited viral identification will be described. The following reactions are some of the more common tests used in PHLs.

- *Catalase* is an enzyme produced by some bacteria and will covert hydrogen peroxide to water and oxygen (releasing O_2 gas). A H_2O_2 solution is added to the test colony and the presence of subsequent foaming or bubbling indicates the presence of catalase. This test is useful for the identification of *Neisseria* spp.
- *Coagulase* is an enzyme capable of clotting plasma and some *Staphylococcal* spp. produce two types. Free coagulase is an extracellular enzyme produced when the organism is cultured in broth, and bound coagulase (clumping factor) remains attached to the cell wall of the organism. Commercially available rabbit plasma reagent is mixed with sample. The slide method is observed for immediate clumping. The tube method is incubated for 4 hours and observed to a clot. Clumping and clots indicate the presence of free or bound coagulase.

- *Hippurate test* relies on the production of the enzyme hippuricase (hippurate hydrolase) by some bacteria. A solution of sodium hippurate is inoculated with sample and incubated for 2 hours. A ninhydrin reagent is added that reacts with glycine, one of the breakdown products of hippurate hydrolysis. The reaction of ninhydrin and glycine results in a deep blue-purple color. The test is useful to identify *Listeria* spp. and *Campylobacter jejuni*.
- *Nitrite reduction test* is based on the ability of some organisms to reduce nitrate to either nitrite or nitrogen gas. An overnight broth culture is used to inoculate the reagents. The development of a pink color in 1–2 minutes is positive for the reduction of nitrate to nitrite. A negative reaction (no color change) is confirmed by the addition of ~20 mg of zinc powder with subsequent color change. However, no color change indicates the reduction of nitrate to nitrogen gas and is a positive reaction. The ability of an organism to reduce nitrites is especially useful for the speciation of members of the *Neisseria* and *Moraxella* groups.
- *Oxidase test* detects the presence of a cytochrome oxidase system in the test organism. The test reagent (Kovacs, Gordon and McLeod's, Gaby and Hadley) is made up and several drops placed on filter paper. A loopful of test organisms is placed on the paper. The production of a blue color within 15 seconds (Kovacs) to 30 minutes (Gaby and Hadley) indicates a positive test.
- *Urease test* is used to determine the presence of the enzyme urease by the splitting of urea. The test reagents are mixed with sample and allowed to incubate for up to 4 hours. As urea is enzymatically split, hydrogen is taken from solution to form ammonia. This increases the solution's pH and the phenol red indicator turns red as a result. The development of a red color is considered a positive test.
- *Hemadsorption test* is used for viruses. Some viruses, such as influenza and mumps, may not produce any CPE and need to be detected by other tests. These (and some other) viruses produce hemagglutinin that is expressed on the surface of infected cells. Infected cells will cause erythrocytes to clump when mixed. Guinea pig red blood cells are used in buffer suspension. Other common tests include indole production (for which we use sulfur reduction, indole reduction, and motility [SIM] medium), the methyl red test, the Voges-Proskauer test, and citrate production.
- *Motility test* is an additional nonbiochemical test that is routinely performed in the microbiology laboratory. Bacteria with flagella (or other motive force) can be differentiated from nonmotile species/strains based on their ability to exhibit movement in motility medium (observed microscopically).

Immunoassays

Immunoassays are at once almost amazingly simple yet equally powerful. They are based on the great levels of sensitivity and specificity associated with the mammalian immune response to foreign invaders. These invaders are recognized by the body by the presence of novel compounds (antigens; usually proteins) that are found on the invader's surface. Because the immune systems does not recognize them, they are identified as foreign and the body seeks to eliminate them. To do this, the immune system creates a chemical complement (antibody) to the invading antigen. When an antibody meets its complementary antigen, they bind and the resultant antigen–antibody complex is thus marked for destruction by white blood cells. Surface antigens recognized by the body are often highly specific to that genus, and may be unique to that species. The power of this system arises from the great specificity of the created antibody. They seldom cross-react with any other antigen, even one with a closely related form. Unlike the previous sections, where the methods were often specific to bacteria, protozoa, or viruses, many immunoassay techniques are equally useful, and similar in design and use, between these three types of organisms. We will therefore not differentiate methods between the three.

Any organism or compound that can elicit an immune response may be a target for immunoassay analysis. The test reagent analyte may be the resultant antibody obtained from an exposed animal host or the antigen itself, which may have been artificially produced through genetically modified cell lines (recombinant). Once obtained, the reagent may be labeled so that it may be observed for analysis. The first labels used were radioactive isotopes, but these have been largely abandoned. Reagents are now labeled with enzymes that react with a substrate to produce a color or chemical compounds that fluoresce under UV illumination (e.g., fluorescein, FITC, TMIT).

Agglutination assays rely on the binding of antibodies to their target antigens when placed together in suspension. They bind together and form clumps that are visible to the naked eye (agglutinate) and there is therefore no labeling or fluorescent display. To aid this process, the antigen may be bound to an inert substance, such as latex or erythrocytes, that makes the clumps more readily visible. Both the Venereal Disease Research Laboratory (VDRL) and rapid plasma reagin tests for syphilis use this technique.

Immunofluorescent antibody (IFA) staining utilizes a fluorescent compound. In the direct fluorescent antibody technique (sometimes referred to as DFA), the fluorochrome is bound directly to a specific antibody. This is then mixed with the sample and binds to any target antigen (if present). The observation of subsequent fluorescence indicates the presence of the target microorganism. The immunofluorescent detection of the rabies virus in brain tissue is an example. The indirect fluorescent antibody technique, by contrast, binds the fluorochrome to anti-immunoglobulin. The sample is mixed with target antigen (usually recombinant) and complementary antibodies in the sample bind to them to form a complex. After the reagents are washed away, the labeled anti-immunoglobulin is applied and binds to the antigen–antibody complex. The presence of fluorescence indicates the presence of the target antibody in the sample, indicating the host's immune response to infection by the target organism. The indirect detection of arbovirus infection is an example.

Competitive enzyme immunoassays are usually used to test for the presence of target antigen in a sample. Target organism-specific antibodies are first coated onto a solid surface (such as a microtiter plate). The sample is mixed with reagents, including enzyme-linked antigens and added to the test. The sample antigens and enzyme-linked antigens compete for the antibodies. After the incubation time, unbound antigens are washed away. Next, an enzyme substrate is added that the linked enzymes convert to produce color. The amount of color produced is directly proportional to the amount of sites bound by the enzyme-linked antigen, and inversely proportional to the amount of antigen in the sample. Thus, the absence of color indicates the binding of antibody by sample antigen, and intense color indicates little/no target antigen present in the sample.

A variation of this coats the solid surface of the microtiter plate with target antigen, rather than antibody. The test sample is placed in a small tube and mixed with enzyme-linked antibody. Any target antigen present in the sample will bind to the antibody. This mix is then added to the microtiter plate. If there was target antigen present in the sample, it will have bound to the enzyme-linked antibody and there will be little left to bind to the plate and the result will be zero/little color. If there were no target antigens in the sample, the enzyme-linked antibodies will be free to bind to the microtiter plate in much greater numbers and the result will be a much brighter color.

Noncompetitive enzyme immunoassays are similar to competitive assays in that antibodies or antigen are bound to a solid surface. Nitrocellulose is commonly used, and this type of test is the basis for many analyses (including home pregnancy tests). If antigen is bound to the substrate, target antibodies are captured from the sample and detected with a second enzyme-linked anti-immunoglobulin antibody. If antibodies are bound to the substrate, target antigen are captured from the sample and detected with an enzyme-linked antibody directed toward a different site (epitope) on the antigen. This is often referred to as a "capture" assay where the antigen is sandwiched between two antibodies. As in competitive assays, the result is a color change. Unlike competitive assays, the color change is solely qualitative (i.e., present/absent; pregnant/not pregnant).

Binding the test antibody or antigen to a solid substrate is useful for the mechanics of this type of test. These may be precoated by the manufacturer. There is a degree of quality control (QC) and, perhaps most importantly, they are fixed in place and may be washed or rinsed of reagents as the testing procedure unfolds. However, there are limitations when the reaction surface is essentially two dimensional. There is a fairly small surface area of the microtiter plate well (or other solid substrate) in comparison to the volume of sample and the opportunity for antibody–antigen binding via contact is limited. This may be resolved in part by mixing and increasing incubation times, but these are imperfect fixes. One solution is to fix the test antibody/antigen to small latex (or other material) beads with diameters < 1 mm. These beads are able to mix with the sample in solution, providing greater opportunity for antibody–antigen interaction, and also have a much greater surface area. For the washing and rinsing steps, the beads may be forced into a somewhat solid pellet at the bottom of the test chamber via centrifugation and resuspended with a vortexer. A variation of this idea coats the test antibody/antigen to small metal particles. These are then captured to the side of the test chamber with magnets.

It is worth mentioning a specific assay to provide an indication of one of the ways in which technology is rapidly advancing in this field. The Luminex xMAP® system uses very small microbeads (5.6 μm in diameter) that contain two different dyes. By varying the intensities of the two dyes, they have created a panel of up to 100 "different" beads that can be individually recognized by laser absorbance. These 100 beads could each be individually coated with one of 100 different antibodies (e.g., bead #1 coated with influenza A H5N1 antibody, bead #2 coated with *Yersinia pestis* antibody, and so on). The advantage of individually recognizable beads is that they may all be contained in one sample for analysis. Thus, a few drops of sample may be mixed in a single well with all 100 beads for the examination of 100 different antibodies. After incubation, washing, and the addition of fluorescent markers, the sample mix is run through a flow cytometer where the beads are strung into a line and examined individually by multiple lasers. Two of the lasers are used to identify the particular bead (e.g., bead #3 = *Y. pestis*) and another is used to detect the fluorescence that indicates the binding of the target (e.g., fluorescence present = the presence of *Y. pestis*).

Nucleic Acid Assays

The analysis of NA sequences, whether DNA or RNA, offers the potential for analyses even more powerful and sensitive than immunoassays. For each organism, there are regions of the DNA/RNA that are conserved (i.e., they do not change regularly), and many have unique sequences that are known. By designing probes that seek out these unique sequences, analysts are afforded a very high degree of specificity. While antigens from different species may have very similarly shaped epitopes and allow some degree of cross-reactivity (infrequent at best), it is unlikely for two species to have the same 50 base pair (bp) sequence (if chosen carefully). And, unlike antibody–antigen bonding where a very close match may be enough for a cross reaction, DNA probes require an exact match with few opportunities for error. Like the previous section on immunoassays, NA analysis techniques are equally useful, and similar in design and use, between these three types of organisms. We will therefore not differentiate methods between the three.

There are two main types of NA analyses. *Nonamplified* probes look for matching NA sequences within the sample. An example of a liquid phase hybridization protection assay for *C. trachomatis* and *Neisseria gonorrhoeae* is the PACE 2C CT/GC by Gen-Probe, Inc. The sample is lysed and mixed with a DNA probe labeled with an acridinium ester. If sample DNA complementary to the probe is present within the sample, it binds to the probe. After incubation, the sample is hydrolysed and peroxides added. The binding of the probe to complementary sample DNA protects the acridinium ester from hydrolysis, and it now emits light in reaction with the peroxides. This is detected by a luminometer. This technique is useful for samples and cultures that have large amounts of sample NA.

For most other analyses, there exists a need to *amplify* the NA present into greater numbers. That is, take a vanishingly small number of target NA strands (nearly undetectable) and create many more copies. The amplification process identifies target NA by the use of oligo primers. These are short strands of NA (~15 bp long) that are complementary to a sequence in the target organism's genome. Once the primers anneal to their target sequence, polymerization enzymes extend them to make a copy of that sequence (an amplicon). They are then denatured to separate the strands. After this first cycle we now have two copies of the target sequence, and both may be copied again. After the second cycle we have four copies, and so on for multiple cycles. This process allows for incredible sensitivity in that the test can, in theory, detect a single copy of the target sequence in a sample. The ability to detect very low levels of a target sequence may have significant impact on disease identification and treatment where the NA present in the sample may be quite low and the disease otherwise undetectable by other means. An example is HIV, where the initial immune response is undetectable for the first week or so of infection (this may be changing with advances in immunoassays) but the individual may be infectious. The ability to detect low levels of HIV RNA in the patient's serum would allow the opportunity for counseling and the potential prevention of the spread of disease to others.

Polymerase chain reaction (PCR) is perhaps the most widely known and used variant of NA amplification. An example is the AMPLICOR *M. tuberculosis* test by Roche Diagnostics (Indianapolis, IN). Target DNA is amplified in this process through the systematic denaturing, annealing, and extension steps. Once the sample has been lysed, the double-stranded DNA is heat-denatured into single strands. Oligo primers specific to regions on the target organism's genome then anneal to their complementary sequences in the sample (if present). A polymerization enzyme such as Taq polymerase then extends the oligos on their 3′ side to create replicant strands of the target DNA sequence. The mix is then heat-denatured again and the original and newly created strands are available

for additional copying. This cycle is usually repeated 20 to 40 times, theoretically amplifying a single strand into 1,073,741,824 amplicons after 30 cycles.

Reverse transcriptase-polymerase chain reaction (RT-PCR) is a variation of PCR where the target NA is cell/virus RNA, not DNA. An example is the COBAS AMPLICOR HIV-1 Monitor for HIV-1 by Roche Diagnostics. The primers anneal to their target RNA, transcribe it into the complementary DNA (cDNA) strand, and then amplify that newly created strand by the process described previously for PCR. This acronym can be misleading at times as it may be used to refer to real-time PCR.

Real-time polymerase chain reaction (rRT-PCR) is a PCR technique where a detection agent is included in the polymerization mix. The simplest such agents are fluorescent dyes (e.g., SYBR green I) that preferentially bind to double-stranded DNA or fluorescently labeled oligo probes that fluoresce when bound to their target DNA strand. As the number of DNA copies increase, the level of fluorescence also increases and is detected. The fluorescence can be detected after every cycle and the analysts can "see" whether there is target NA being amplified. The procedure may also be used to not only identify the presence of the target organism, but also quantify the amount of target NA (qRT-PCR). The specificity of this type of probe may be also further characterized by a melting curve analysis, based on the different temperature at which DNA strands denature and the subsequent loss of signal. Other techniques utilize fluorescent resonance energy transfer (FRET) probes, dual hybridization probes, molecular beacons, and other variants and combinations of these techniques.

Nucleic acid sequence-based amplification and *transcription mediated amplification* are similar methods that actually amplify target RNA. An example is the APTIMA combo 2 assay for *C. trachomatis* and *N. gonorrhoeae* by Gen-Probe, Inc (San Diego, CA). A primer anneals to its target RNA sequence (if present) and a cDNA strand created. This RNA–cDNA duplex is then denatured, and a second primer annealed to the cDNA to generate double-stranded DNA containing an RNA polymerase promoter. This strand is then used to make multiple single-strand antisense RNA copies. These can also instigate the creation of more cDNA and propagate the cycle.

Several of these techniques do not provide a means of NA detection either during or postamplification. That is, the analyst has generated millions of copies of the target NA but now needs a method to detect their presence. One of the earlier methods used agarose gel for this purpose. Here, the sample is mixed with a stain (e.g., ethidium bromide) and injected into one end of an agarose gel slab. When an electric current is applied, the NA pieces migrate through the gel at a speed inversely proportionate to their size, with NA fragments of different sizes traveling at different rates (separating). After a suitable time, the distance traveled by sample components is compared to a standard containing NA pieces of known sizes to determine if the amplified product is a size match compared to the standard. The DNA may then be transferred to a nitrocellulose membrane and hybridized for other specific detection techniques and to create a more permanent record (agarose gels degrade fairly quickly). A variation of this technique, restriction fragment length polymorphism (RFLP) utilizes restriction enzymes to cut the NA into smaller fragments. Restriction enzymes cleave NA at very specific NA sequence patterns. SmaI, for example, cleaves DNA in the middle of the sequence . . . CCCGGG . . . The pattern of subsequent fragments may be specific to individual amplification products.

The amplification product may also be detected colorimetrically in microtiter plates. In this technique, amplicon probes are coated onto the microtiter well surface and bind to the amplicons when then the test mix is added. This is followed by washing and the addition of both an enzyme conjugate and a colorizing substrate (very similar to their use in immunoassays discussed previously). The binding of the conjugate to the probe–target complex results in an observable color change. There are multiple variations of this technique.

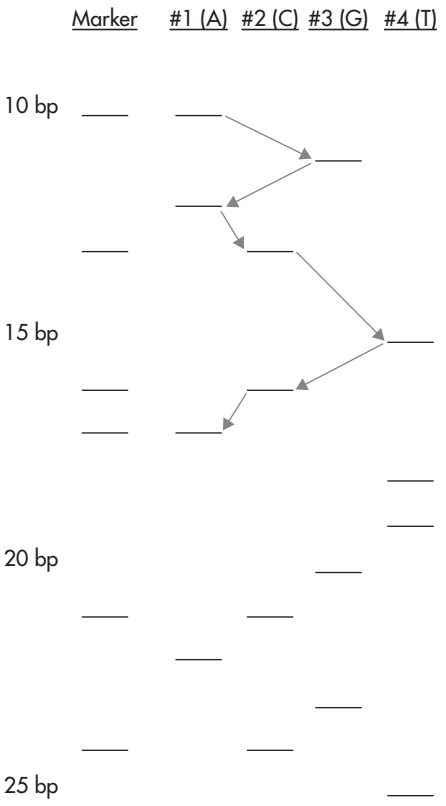
PFGE is important in the PHL to specify a known organism's strain or subtype rather than to provide organism identification. While different *S. enterica* serotypes may have similar immunologic and biochemical reactions, they have slight variations in their genetic code. There may be alterations and deletions such that the number of base pairs between restriction enzyme cleavage points differs. Where the NA for the particular strain is cleaved by a restriction enzyme is very characteristic of that strain and is often called its "fingerprint." A downside to using RFLP with cellular DNA, versus PCR amplicons, is the size of the fragments. Whereas amplicons are typically in the 10s to 100s of bp long, genomic DNA fragments may exceed 600,000 bp. This is far too large for separation by standard gel electrophoresis because the fragments become entangled in the agarose and cannot progress in the straight manner dictated by the electric current. However, if the direction of the current is altered (pulsed) such that it is directed in

alternating directions, the fragments may make forward progress. This is much like wriggling a stuck kite through the branches of a tree. While PFGE analysis may not affect diagnosis or treatment, it has immense value in epidemiologic investigations of disease, especially when cases may be widely dispersed geographically.

Sequencing is the last NA technique we will describe. The basic process involves the addition of a primer oligo, a polymerization enzyme, a mix of NAs to be used as building materials, and chain termination reagents to a tube containing target NA. This setup is duplicated in four tubes, each containing a different termination reagent (one each for the four nucleotides adenine, cytosine, guanine, and thymine). The oligo finds its complement on the target NA and the polymerization enzyme starts extending the chain. When it selects a terminator reagent rather than another nucleotide, the extension stops (say at 12 bp). For the next copy, the extension might proceed to 33 bp before termination. In each tube, the termination reagent corresponds to a different nucleotide, resulting in every chain in that tube stopping at a place where that specific nucleotide would go. Thus, tube #1 has chains of a variety of lengths that all stop where an adenine would go. Tube #2 has chains that all end in cytosine, and so on. The contents of these tubes are then separated in parallel (i.e., lanes 1 to 4) via electrophoresis and form a series of "ladder" patterns corresponding to chain length. Comparing these patterns to a standard mix, we can deduce the sequence of the target NA. For example, lane #1 has a band at 12 bp, and the other lanes are blank at that position. We know that the 12th nucleotide in the sequence is therefore adenine. Following the lanes down the gel, we find the next band in lane #3 (corresponding to guanine). The next nucleotide in the sequence is thus guanine. This process of finding terminal nucleotides in successively longer chains by moving down the gel and from lane-to-lane allows the analyst to construct the sequence of the NA to almost any length (limits are more associated with gel composition and size). This technique is quite useful for differentiating between closely related species or strains where the only readily identifiable differences are point mutations in a target gene.

Figure 2-7 is an example of a hypothetical sequencing result. As the tube contents are driven down the gel, they separate by size (smaller fragments traveling more quickly). We find the smallest fragment corresponds to the 10 bp marker and is in the first lane (indicating it ends with A). The next larger fragment (the next one down the gel) is in the third lane, corresponding to a terminal G.

Figure 2-7 Example of a hypothetical sequencing result.



By continuing down the gel in this fashion we find that our visible sequence is . . . GACTCATTGCAGCT . . . A significant point mutation might be the change of the C for a G in the fourth nucleotide.

The Role of Technology and Automation in Time Saving

It would be appropriate at this point to briefly mention the growing importance of the role of robotics and automation in microbial analysis. In a perhaps perfect world, the laboratory analyst would have plenty of time to devote to individual samples, performing tests in succession to determine what, if any, microorganism is present in a sample. Unfortunately, the real world presents the analysts with multiple samples of different types that must be analyzed near simultaneously. Analysts are also prone to the occasional error, which may compromise a single biochemical test or contaminate an entire PCR workstation (where the presence of just a few stray target DNA strands may cause a sample to become falsely positive). Lastly, the number of organisms for which a

sample may be screened seems to be only increasing. The simple manual screening of a sample for the presence of 12 different organisms may take considerable time, not including the subsequent identification of any potential positives.

In the section discussing immunoassay procedures, we described a technology and instrument that has the potential to screen a sample for up to 100 organisms at one time within a couple of hours. This feat could not be duplicated manually by one person in less than several days. There is some small automation in the plate washing and rinsing, but the real time saving is in the technology. The combination of tests is also observed in bioMerieux's API-20 E® (Durham, NC). This test kit for the identification of enteropathogenic bacteria consists of a plastic strip of 20 wells containing reagents for different biochemical tests. The wells are inoculated with sample, incubated, and observed for reaction (as evidenced by color change). The pattern of positive and negative responses is indicative of genus and species. While the test may take up to 24 hours to complete, more than one strip can be done at once and is a time saver for the analyst with multiple samples submitted as part of an outbreak investigation. It is also useful as a screening tool, and presumptive positives must be confirmed by traditional means. The development of a new analysis method may also lead to automation, as exemplified by the Becton-Dickinson ProbeTec ET™, (Franklin Lakes, NJ) system for the analysis of chlamydia and gonorrhea in clinical samples. The company developed a proprietary NA amplification system (strand displacement amplification) and incorporated it into the ProbeTec instrument. Further automation and robotics are evident in the companion Viper™, which automates many of the sample preparation steps required before the analysis steps taken by the ProbeTec.

Every year brings new advances in technology that have the potential to shorten analysis times, increase sample analysis volume, and lead to more accurate results. The use of automated incubation and instrument detection during the testing for tuberculosis provides preliminary results ~1 week sooner than traditionally grown cultures (discussed in Chapter 5). Instruments can detect significant changes before they become apparent to the human eye. The increasing use of robotics and automation for high-volume samples is resulting in faster overall analysis times and reduced opportunities for error and cross-contamination. Further advances in technology will also aid in the subtyping of important organisms and further organism characterization.

Quality Control

Measures to ensure that analyses are correct are both important and extremely variable when it comes to microbial analyses. We will find in the next two chapters that QC requirements during the analytical portion of chemical and radiological analyses are focused on ensuring that the analytes in question are removed from the sample and that the instrument performs properly. These analyses, as well as microbial analyses, also have comprehensive rules for documenting such things as sample receipt and handling, reagent purchase, preparation and storage, and results reporting and sample disposal. These types of QC are quite detailed and beyond the scope of this book to enumerate. What we will discuss, albeit briefly and generally, are some of the measures commonly encountered during the analytical process.

Culturing

- When streaking a sample unto selective and/or differential media, the sample may be streaked onto a general media as well. If there is no growth on the selective media, but there is on the general, then one may conclude that the organism selected for is not present. If there is no growth on either media, then the sample may simply not contain any viable organisms, perhaps due to improper shipping or preservation.
- Purchased media and cell lines are stored according to manufacturer directions, most often refrigerated and in darkness, and checked regularly for growth and general condition, and also checked before use.

Staining

- QC largely revolves around the documented and correct creation of reagents.
- Analysts may document their proficiency in identifying organisms using staining methods, but the quality of each actual stain procedure is not judged or recorded.

Biochemical Tests and Immunoassays

- Additional test tubes/plates used for biochemical analyses may be inoculated with sample-free broth or saline to act as a negative control.

- Positive controls are not as frequently employed as it may be difficult and/or expensive to maintain stocks of target organisms for the purpose. Some may be obtained commercially for this purpose, or organisms that mimic characteristics of the target organism may be used (e.g., produces gas).

Nucleic Acid Testing

- Positive control samples are available for some molecular (NA) tests. These are designed to test the lower limits of the NA method's detection. They show that the test has/has not been performed properly. They may also be used to spike a sample before amplification to test for the presence of inhibitors that might cause a false negative. If the sample is negative, but the sample plus spike is positive, then the sample may be considered truly positive. If both are negative, then there is likely a problem with the method or analyst.
- Negative controls (those without any sample) may also be carried throughout the molecular analysis process. Their negative result indicates that there is no cross-contamination leading to false positives.
- College of American Pathologists is the only Centers for Medicare and Medicaid Services (CMS)-approved provider of molecular proficiency studies for molecular analysis of infectious disease.

Many kit manufacturers include negative and/or positive controls with their tests. That is, of the 96 wells in a microtiter plate, 20 may be reserved for controls. These may be negative and positive controls and sample duplicates and are done to ensure that the test reagents and instrumentation are working properly. These controls vary by kit and manufacturer.

Microbiologic analyses have become exceedingly sensitive in the past 20 years, with advancements in antibody and NA analyses. While culturing has also advanced with the advent of newer and differential and selective growth media, it is not always considered the gold standard by which all other tests are measured. We see how the mastering of a relatively few techniques allows the analyst to analyze a variety of sample types for a wide range of pathogenic microorganisms. Genus and species identification is often a process of elimination as colony isolates appear/do not appear on selective media and exhibit specific biochemical reactions. The combination of these techniques into modern automated systems also allows the analysts to process a volume of samples unimagined 50 years ago.

Discussion Questions

1. Microscopy is a powerful tool when it comes to the identification of many microorganisms but is subject to significant limitations. Describe three potentially serious limitations and how they may be addressed.
2. Describe at least two advantages of microscopy over other analysis methods.
3. Describe the difference between selective and differential media and how they may be used to identify an unknown pathogen.
4. Describe the elements of the staining process.
5. Describe the rationale for biochemical tests and how they may assist in microorganism identification.
6. What is the difference between direct and indirect immunoassay analysis?
7. How does immunofluorescence differ from enzyme immunoassay? (Hint: they both utilize antigens and antibodies.)
8. Why might laboratory contamination with target pathogens be such a concern for PCR analyses?
9. Describe two different methods to identify the products of either PCR or RT-PCR.
10. Go on the Internet and find three good sources for detailed information on some or all the techniques described in this chapter. They cannot be government sponsored (.gov) or listed under Additional Resources. Describe what they provide and why they are good sources of reliable information.

Additional Resources

Atlas, R. M., & Snyder, J. W. (2006). *Handbook of media for clinical microbiology*. Boca Raton, FL: CRC Press.

Difco & BBL Manual. (2004). Retrieved November 9, 2009, from http://www.bd.com/ds/aboutUs/news/News072504.asp.

Forbes, B. A., Sahm, D. F., & Weissfeld, A. S. (2007). *Bailey and Scott's diagnostic microbiology*. St. Louis, MO: Mosby.

Murray, P. R., Baron, E. J., Jorgensen, J. H., Landry, M. L., & Pfaller, M. A. (2007). *Manual of clinical microbiology* (9th ed). Washington, DC: ASM Press.

Specter, S., Hodinka, R. L., Young, S. A., & Wiedbrauk, D. L. (2009). *Clinical virology manual*. Washington, DC: ASM Press.

3

The Basics of Chemical Analyses

Introduction

The purpose of this chapter is to impart to the reader a better understanding of what occurs in a chemistry laboratory when a sample is analyzed. Too often, there is the perception that analyses are swift, easy, and absolute. In the past, in our laboratory in Illinois, we have had individuals bring in samples for fairly complex analyses and sit down to wait; not realizing that what they have requested will take 2 to 3 days. Modern television crime shows often utilize analytical equipment such as gas chromatographs in crime labs, but imply that all it takes is a single analysis over a matter of minutes to produce an ironclad result. The truth is often much more complex and time-consuming (though usually has much better lighting).

The chapter is divided into sections based on the flow of work associated with chemical analyses (see Figure 3-1): sample collection, preparation, and extraction followed by extract cleanup, reduction, and analysis. Techniques, instruments, and reagents are usually exclusive to one of these steps. Each section will cover the general principles behind the techniques and provide examples. The sections will "walk" the reader through a sample analysis to show what is involved. It is not of such depth that an actual analysis could be performed without further references. The reader will also notice that chemical analyses follow a much more linear path than those for microbes. Whereas we had a more general discussion of microbial analysis techniques in Chapter 2, here we can present a more limited set of actions in the order in which they typically occur.

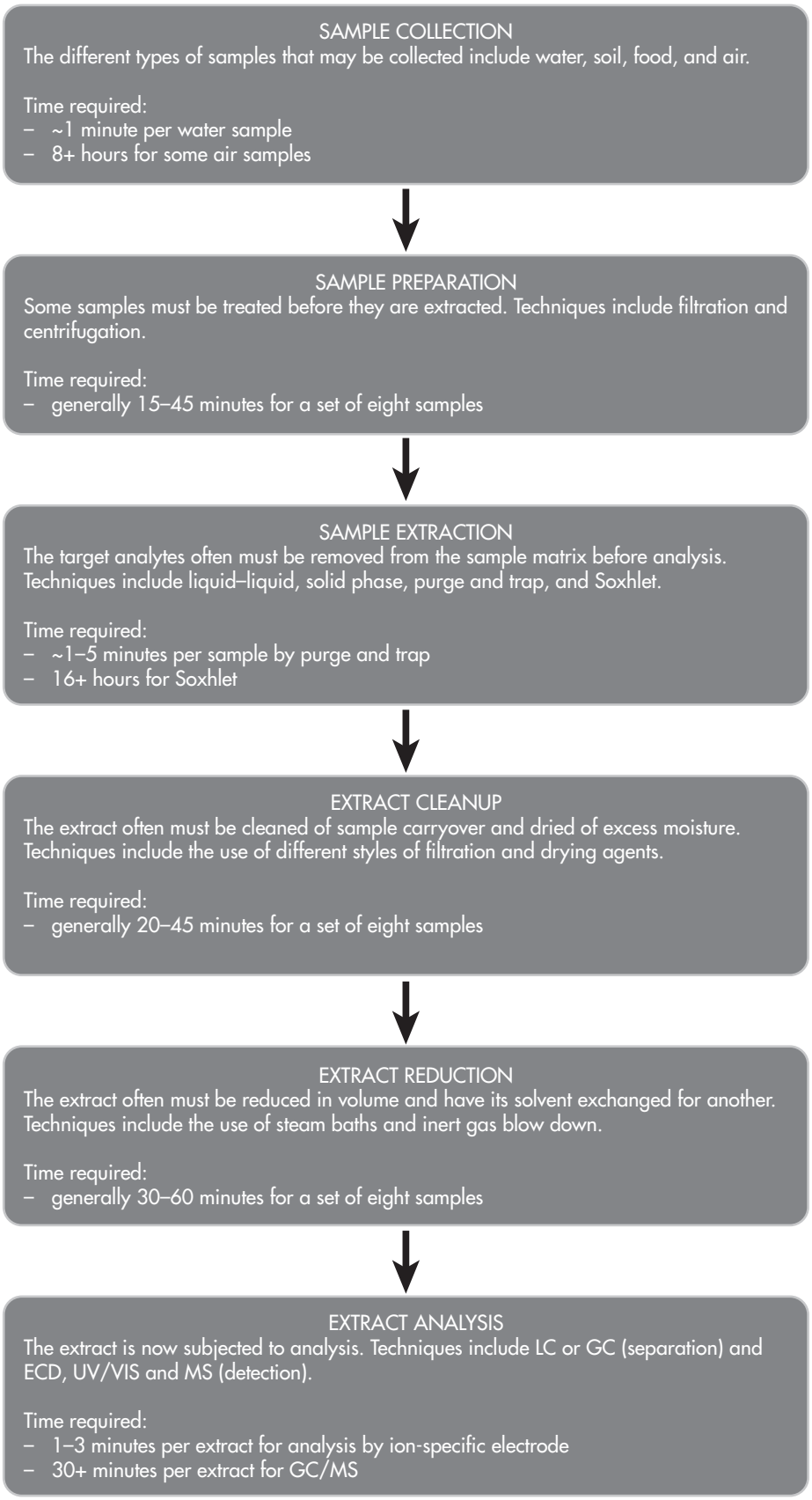
In comparison to analyses for microorganisms (Chapter 2) and radiologicals (Chapter 4), chemical analyses are generally much more subject to biases and generally much more expensive to conduct. They are also quite important as the presence of chemical contaminants may often be quite difficult to detect and/or remove from the sample medium. For example, the presence of ^{241}Am (Americium, an alpha and gamma emitter, commonly used in smoke detectors) may be readily detected in the surface of a hamburger using a handheld Geiger counter, whereas the presence of trace levels of dioxin can only be detected through substantial sample preparation and concentration followed by analysis with very expensive instruments.

The purpose of this chapter is to provide an overview of the methodologies utilized for the analysis of different sample types for chemical compounds. It is purposefully written to be both brief and general. There will be additional resources provided at the end of the chapter for the reader who desires to know more about chemical analysis techniques. The chapter will discuss different types of samples that may be analyzed, some of the methods employed for sample preparation, and, finally, an overview of different instrumentation and analysis methods. Figure 3-1 shows the steps involved in going from sample collection to analysis results.

Sample Types and Collection

When looking for chemical contaminants, the types of samples amenable for analysis are almost infinite. That is, an individual can bring in almost any type of

Figure 3-1 Flowchart showing the steps from sample collection to analysis for chemical compounds.



substance and a subsequent analysis for the presence of almost any type of compound could be attempted. However, similar to microbial analyses, this is another excellent example of how simply because something is possible does not also mean it is practical. Sample type, that is, the matrix from which the target compound is to be detected, can have profound impact on the sensitivity and reliability of the method. It is for this reason that the methods of analysis used in public health laboratories (PHLs) for most compounds stipulate which sample types are allowed, how they should be collected and handled, and their manner of preservation and/or time limits from sample collection to analysis.

Water

Water samples are collected for a variety of reasons. These include routine testing for contaminants regulated by the US Environmental Protection Agency (EPA), well contamination from field runoff, and requirements for home sales. The great majority of analyses performed on a routine basis look for different EPA-regulated compounds such as NO_3/NO_2 (nitrate/nitrite), organochlorine pesticides, and toxic elements. The full listing of drinking water contaminants regulated by the EPA can be found at http://www.epa.gov/safewater/contaminants, and will also be discussed in further detail in Chapter 6. The following examples highlight some of the different requirements.

- NO_3/NO_2 by EPA Method 353.2, *Determination of Nitrate–Nitrite Nitrogen by Automated Colorimetry*: specifies that samples be collected in either plastic or glass bottles of sufficient quantity for analysis (usually 30+ ml), preserved with sulfuric acid, and chilled to 4°C for transport to the lab.[1]
- Chlorinated pesticides by EPA Method 508.1, *Determination of Chlorinated Pesticides, Herbicides, and Organohalides by Liquid–Solid Extraction and Electron Capture Gas Chromatography*: specifies that samples be collected in glass bottles, usually 1+ liter in size.[1]
- Volatile organic compounds by EPA Method 524.2, *Measurement of Purgeable Organic Compounds in Water by Capillary Column Gas Chromatography/Mass Spectroscopy*: specifies that samples be collected in duplicate in glass bottles with polytetrafluoroethylene (PTFE)-lined caps and no residual air bubbles remaining inside.[1]

The methods here highlight some problems associated with following the letter of the method. Water samples are often collected by either local health department sanitarians or homeowners. There are serious concerns on the part of some laboratories about distributing sulfuric acid, a preservative, to those who are not trained in its safe use. There is also the question of availability of chill packs for cooling the samples. These are expensive to purchase and provide on a large scale. Some laboratories that perform this analysis on a large scale (>1000 samples per year), therefore, do not require that samples be chemically and thermally preserved. This may add a caveat to the analytical result as it was not done in complete accordance with all method requirements, but does serve the public interest.

From these three methods (of many promulgated by the EPA and other sources), we see some of the variety of sampling requirements as well as some commonalities. Glass is the container material of choice where trace compounds from plastics may leach into the sample and become a noticeable contaminant or interference. Where this is not an issue, such as nitrate or fluoride analysis, labs often go with the much less expensive option of plastic. We find that chlorine removal is needed for the majority of sample analyses because chlorine may provide significant interferences during analysis. Chemical preservation is also often called for, but at times neglected for the sake of expedience and safety concerns. Thermal preservation is also often needed. This serves several purposes including retarding microbial growth and retarding chemical reactions and evaporation.

Food

Food is an incredibly complex sample when it comes to chemical analysis. While there are many analytical methods in existence, PHLs do not always have responsibility for testing food for chemicals. This often falls on regulatory agencies such as the US or state Departments of Agriculture. While widespread chemical analysis in food is not done, the following examples are performed in some PHLs.

Milk: One matrix analyzed for chemicals on a regular basis is milk. Milk is analyzed for the presence of aflatoxins (B1, B2, M1, and M2). The milk is collected before pasteurization in 30+ ml plastic bottles. They are chilled to 4°C and analyzed within 7 days.

Seafood decomposition and toxins: These samples are often collected in coastal areas, areas with large bodies of water, and fisheries. Fish and other seafood

suspected of containing the toxins associated neurotoxic shellfish poisoning (NSP) are collected either during an outbreak/suspected case or as part of routine monitoring in areas where it is more likely to occur.

Elemental analyses: These samples are most often submitted for the analysis of various metals. Some imported medicine, or even jewelry made of dried beans, may have added or high lead content which may be poisonous if consumed. In many areas, fish are caught and analyzed for the presence of mercury and other toxic metals, which are a result of environmental contamination. High levels of mercury, for example, result in advisories against eating certain types of fish caught in specified areas. Elemental analyses are not typically affected by the sample's storage condition, but for the sake of those transporting and ultimately analyzing these samples, they are usually transported quickly and/or chilled to reduce spoilage.

Illness/complaints: These samples include almost any conceivable type of food that is suspected to be associated with a food-borne outbreak or food-related adverse health event. Examples include the intentional poisoning of food to target an individual or group (a criminal event or bioterrorism event) and the accidental contamination of food during processing or transportation. There is no set standard for sample type and collection for this broad category. In general, those investigating the occurrence will contact the laboratory and determine (1) what the laboratory is capable of in terms of analysis; (2) what type of sample and what amount should be collected; (3) how the sample should be collected, packaged, and shipped; and (4) the time frame for collection and delivery to the lab.

Relatively few PHLs analyze food for chemical compounds on a regular basis. Those that do usually follow established guidelines for specific analyses they perform, and these guidelines may come from analysis kit instructions, regulatory agencies such as the EPA, or from methods published by organizations such as the Association of Analytical Communities (AOAC) or the Food Emergency Response Network (FERN). The collection of samples for nonroutine analyses is often negotiated at the time of collection between the investigators and laboratory personnel.

Air

The methods for air sample collection are variable, but not to the extreme of food. There are a few different mechanisms for air sampling, utilizing one of two basic techniques. The first is a simple collection of air in a container of some sort. These samples are collected by the use of evacuated stainless steel canisters. The traditional SUMMA canisters have electropolished inner surfaces, and newer MiniCan canisters are coated on the inside with fused silica. The canisters are evacuated to low pressure prior to sampling. When ready, a sampling regulator is attached and the canister opened. The low internal pressure causes the surrounding air to enter the canister until the pressures are equalized. Aluminized bags may also be forcibly filled.

The other method involves the concentration of air constituents by passing the air over/through a collection medium such as impregnated activated beaded carbon (IABC). Tubes and cassettes are variations of the theme that air is passed through them with the analytes of interest being captured by the filtering medium. There are a variety of natural and artificial medium constituents and numerous commercially available tubes, each with specific target compounds. All rely on the principle that as air is passed through the medium, the target compounds are retained and the remaining nontarget compounds are not. Some compounds undergo reaction with the sorbent material. Not all compounds are collected and analyzed equally well by the different sample techniques.

Other

Wipes are often employed to search for surface contamination. It is a very simple procedure. Typically, a gauze pad is wiped over a defined area and placed in a vial. Depending on the target compound(s) characteristics, the size of the area wiped may be increased or decreased, and a solvent may be added to the gauze to increase absorption of the target(s) to the pad. Wipes may also be combined with paint chips for the analysis of lead in homes.

Soil samples are also occasionally received, though in many states such samples are directed to state EPA, Department of Natural Resources, or Department of Agriculture labs. These are usually almost as simple as wipes in terms of collection, though there may be some differences based on the target analytes. Soil is placed in to a 25+ ml bottle with a PTFE septa-lined cap. The sample is to be protected from light (or an amber glass bottle used). If the sample is not corrosive, foil may be used instead of PTFE. Finally, the bottle should be filled as much as possible to minimize headspace where target analytes may gather. Depending on target analytes and analysis, sample sizes may be as small as 1 g and sample refrigeration may or may not be required.

Laboratories, depending on their level of instrumentation and skill, may often receive a "miscellaneous" sample that does not fit into any neat category. For example, the Illinois Department of Public Health (IDPH) laboratory at one time received a stack of Chicago Transit Authority bus passes that were associated with potential illness in users. There is no method tailored or designed for this circumstance, so laboratories take the basic sample collection and extraction methods that most closely match the sample medium and analyte and "create" a specific method. Such base methods may be taken from the EPA manual *Test Methods for Evaluating Solid Waste, Physical/Chemical Methods* (commonly referred to as SW-846). Reports of such analyses usually describe the nonstandard manner of sample type, collection, and analysis with its accompanying caveats.

Sample Preparation

There are often occasions when a sample arrives in the laboratory in a less-than-optimal condition. This occurs most often in water samples that may have fine particles of dirt, rust, or other debris suspended within. While not necessarily an analytical concern in itself, the presence of these contaminants may decrease the effectiveness of extraction techniques, or simply make them take much longer. For example, small dirt particles may quickly clog the pores in filters used in solid phase extraction, and turn a 15-minute extraction into 90 minutes. Preparing the sample by removing these interferences is thus important.

One of the simplest (and often quickest) techniques is mechanical filtration. In this method, the sample is passed through a medium that retains some macro component(s) through mechanical action. Separation is based on particle size, density, or other physical property.

Simple filtration: The most basic is the pouring of a liquid sample through a filter paper. The paper has a defined porosity that only allows particles of sufficient size to pass. In this manner, particles down to the micron size (10^{-6} m) are trapped on the paper while the rest passes through unhindered. This is quick and effective method to remove small particles often collected in environmental water samples.

Centrifugation: Separation in this technique is based on the idea that some sample components are more dense than others. When subjected to increased gravitational pull, the denser components will separate from lighter (less dense) components. The desired component(s) can then be obtained. For example, centrifugation can cause solid particles to form a clump at the bottom of the vessel, and the solute may be poured off.

Sample Extraction

Some of the techniques used for the analysis of chemicals are very sensitive to contaminants and very specific about what samples are acceptable. A simple analogy is the use of gasoline for your car. Internal combustion engines are not designed to burn unrefined oil. Instead, this oil must be treated to both remove obstructive components (which could harm the engine) and concentrate the desired fractions (which increases available energy). Many clinical and environmental samples must go through similar preparation procedures so that they are compatible with the instrumentation and/or the chemical(s) of interest are concentrated so they are more easily identified.

Basic Solvent Elution

This technique is used when there is no need for mechanical separation of the sample medium and extracting solvent. An example would be the beads from an air sampling tube, which are removed and placed in a flask, mixed with solvent (e.g., hydrogen peroxide), and allowed to sit for 60 minutes. The solution is then subjected to analysis without further preparation. Other extraction solvents include carbon disulfide and methylene chloride.

Soxhlet Extraction

Liquids boil when the liquid's internal pressure equals its vapor pressure. At that point, bubbles can form without being crushed. In a distillation process, the solvent vapor is passed though a chilled condensing column where it condenses back to a liquid and is collected by gravity. Soxhlet extraction is essentially a circulating distillation device that continuously washes a sample with solvent. The solid sample is placed in a thimble and loaded into the Soxhlet chamber. The extracting solvent is heated to boiling in the still pot (round-bottomed flask), which is below and separate from the chamber. The vapor is then cooled to a liquid in a condensing column (Snyder column) and the warm solvent is delivered to the chamber. The target compound(s) is somewhat soluble to this warm solvent and dissolves into it. When the solvent

volume in the chamber reaches a certain level, it is gravitationally siphoned out of the chamber through a side arm and back into the still pot, carrying any components extracted from the sample. This process is repeated multiple times over hours or days. It is most useful when the solute is marginally soluble in the solvent. This method is particularly useful for the extraction of semivolatile and nonvolatile organic compounds.

Liquid–Liquid Extraction

This is a straightforward method where a water sample is mixed with an immiscible solvent (e.g., methylene chloride [CH_2Cl_2]) in a separatory funnel. As these two liquids are not mutually soluble (similar to oil and water), they form readily identifiable layers. Many organic target compounds have a greater affinity to the solvent, and will dissolve into it when the funnel is shaken. The extent of this transfer from the aqueous (water) phase to the organic (solvent) phase is largely determined by the compound's distribution ratio (partition coefficient). Thus, the water sample is mixed with the solvent in the separatory funnel, shaken to effect transfer of the solute, and the water and solvent allowed to settle and form two layers. The funnel has a spigot at the bottom that is used to draw off the lower layer. This method is particularly useful for the extraction of semivolatile and nonvolatile organic compounds.

Solid Phase Extraction

In solid phase extraction, the mobile phase (liquid sample) is passed through a stationary phase (solid) to separate out different components based on their physical and/or chemical properties. Thus, the components of interest are either retained on the stationary phase and the remaining sample discarded, or impurities are retained and the remaining sample is further processed and analyzed. There are essentially four different kinds of stationary phases, each designed to retain different types of compounds. *Normal* phases adsorb polar molecules such as organochlorine pesticides. *Reversed* phases adsorb weakly polar and nonpolar compounds such as hexane. *Anion exchange* phases adsorb negatively charged ions such as acids, and *cation exchange* phases adsorb positively charged ions such as ammonium.

The most widespread use of solid phase extraction is for the analysis of water samples using either disks or cartridges. The disks are essentially PTFE circles (usually 47 or 90 mm) that are impregnated with C18 (branched carbon compounds containing 18 carbons). Cartridges are small tubes filled with ~20 g of C18. Both methodologies use a vacuum to pull the sample through the capture medium (C18) which will retain the target analytes. Once the sample is passed, the disk/cartridge is dried, and solvent is passed through to elute the captured analytes. This method is particularly useful for the extraction of semivolatile and nonvolatile organic compounds.

Purge and Trap

A sample is loaded into the mechanism and an inert gas (e.g., high purity helium) is bubbled through. Volatile organic compounds (VOCs; e.g., benzene, toluene) have affinity for the gas, and pass to a short column (trap) filled with absorbent material. Once the purge is complete, the trap is rapidly heated to release the concentrated compounds to the analytical instrument. As this cannot be done without immediate analysis, it is often described in conjunction with analysis methods and will be discussed further in the analysis section. This methodology is generally limited to the analysis of VOCs in water.

Sonication

The principle of sonication is that high-energy sound waves (ultrasonic) are able to disrupt liquid surface tension and break apart the sample matrix (e.g., soil or sludge) in some cases. Thus, a solvent may mix much more thoroughly with a solid sample and come into physical contact with much more of it at the microscopic level. This contact is critical if the solvent is to extract the target analyte(s). However, the limited contact time between solvent and sample results in significant extraction inefficiency in comparison to other methods. The sonic waves are provided by a sonicating horn. This is a steel probe of varying diameters (3/4-inch or smaller typically used) that vibrates at high frequency when power is applied. This method is particularly useful for the extraction of semivolatile and nonvolatile organic compounds.

Mechanical Mixing and Blending

There are occasions whereby the sample must be physically agitated or even ground in a blender for the purposes of analysis. This is typical of food samples where the sample is often mixed with a solvent and subsequently agitated by shaking, mixing, or blending. The purpose is to allow the solvent to come in contact with as much of the sample as possible so that any target

analytes have an opportunity to enter the liquid phase. For example, simply placing a whole fish in a beaker of methylene chloride would be quite ineffective. The solvent simply does not have access to the internal fatty tissues where the chemical would reside. By homogenizing the sample and solvent in a blender or grinder, the mechanical shredding of the tissue allows the solvent to interact with the exposed fatty tissue and extract any target compounds present. There are no "general" methods for this type of sample preparation as the methods vary significantly by both the sample and analyte(s) of interest. This method is particularly useful for the extraction of semivolatile and nonvolatile organic compounds.

Supercritical Fluid

Supercritical fluid uses carbon dioxide or other solvents under high pressure/temperature to extract solids. By increasing the pressure and temperature in a defined manner in a closed container, the extraction solvent enters the supercritical phase (as opposed to simple liquid or vapor phase). This phase has properties of both liquids and vapors. For example, the extraction solvent now has no viscosity or surface tension (associated with liquids), which allows for much deeper penetration into the sample. It also has increased solubility of target compounds over liquid, which allows for more efficient extraction. The conditions for super criticality to exist are relatively narrowly defined. This method is particularly useful for the extraction of semivolatile petroleum hydrocarbons, polynuclear aromatic hydrocarbons, polychlorinated biphenyls, and organochlorine pesticides.

Microwave

An extraction can be performed on solid matrices with solvents utilizing microwave as the heating source. Microwave emissions can be very closely controlled, thus closely controlling the system's temperature. By heating a closed system, the boiling point of the extraction liquid is greater than it would be in the open atmosphere. This increased temperature also decreases the viscosity of the solvent, allowing it to penetrate more deeply into the sample. These two conditions combine to cause greatly reduced extraction times. This method is particularly useful for the extraction of semivolatile and nonvolatile organic compounds.

Acid Digestion

This method of extraction is exactly as it sounds. Solid samples are mixed with acids and heated to break organometallic bonds. The analyte(s) of interest (usually metals such as lead [Pb] and arsenic [As]) are thus freed for analysis. The analysis is most often performed on soils, sludges, and paint chips from homes (to test for potential lead exposure to children). This method is particularly useful for the extraction of metals.

The purpose of sample extraction is to both remove the target analytes from their original matrix and allow them to be concentrated. The sample matrix may simply not be amenable to analysis. You cannot place soil or water into a gas chromatograph, the compounds must first be removed and placed in a solvent. There may also be many other compounds and interferences included with the sample matrix (e.g., residual chlorine, salts) that may interfere with analysis. Extraction selects compounds of the target class and allows the others to be removed. This makes the analysis much easier and reduces potential analytical "noise" from unwanted sample constituents. Extraction also allows the volume of liquid containing the target analytes to be significantly reduced, concentrating the analytes and making analyses more sensitive.

Sample Extract Cleanup

From the extraction procedures outlined previously, one may see that the resultant extract is not necessarily "clean." The sonication extraction of dirt may result in extremely fine dirt particles becoming suspended in the solvent and carried out. A water sample subjected to liquid–liquid extraction will have some moisture carried in the solvent as well. Both these contaminants may cause poor analysis and even outright damage to the analytical instrument. For this reason sample extracts are usually subjected to some type of cleanup prior to analysis.

Removal of Fine Solids

The extraction of solid materials (e.g., soil, air filters) often results in the transference of fine particles from the sample to the extract. This may be especially noticeable during sonication where larger clumps are often broken down by the sound waves. The specifics vary by method and solvent, but there are three general types of filtration used to remove these particles.

Gravity filtration simply uses a funnel and filter paper. The extract is pulled by gravity through the paper and retained. This is useful for samples that are relatively "clean" or with larger particles.

Vacuum-assisted filtration uses filter paper in a Buchner funnel placed on a side-arm Erlenmeyer flask, which itself is connected to a vacuum line. The resultant vacuum

in the flask pulls the extract through the paper. This is useful for dirtier extracts or those with smaller particles. Either case results in the paper clogging more quickly and requiring more effort to get the sample to pass through.

Filter disks (such as MilliPore [Billerica, MA], Whatman [Kent, UK]) are used where the extract volume is small and the solvent will not affect/dissolve plastic. Here, 1–5 ml of extract is drawn into a syringe and a filter disk is fitted to the end. The plunger is depressed and the extract forced by pressure through the disk. This is useful for minimally dirty samples, but has the advantage of very small pore sizes resulting in the removal of more and smaller particles than the filter paper methods.

Now that the extracts are cleaned they are ready for the next step, drying, if needed.

Removal of Moisture

Often referred to as "drying," removal of excess moisture is very important. As important as it is analytically, it may be as simple as passing the sample through a quantity of anhydrous sodium sulfate (Na_2SO_4; or similar water-absorbing compound). There are occasions where cleanup and drying are done in a single step. That is, filter paper is placed in a funnel, Na_2SO_4 is added, and the extract passed through. In this way it is both dried (by the Na_2SO_4) and cleaned (by the filter paper).

Sample Extract Volume Reduction and Solvent Exchange

In almost every extraction procedure, there is an excess of solvent used. This is done to maximize the potential collection of the target compounds from the original matrix. Thus, extractions are often performed three or more times. The reason for this is that no extraction solvent is 100% efficient. Consider the liquid–liquid extraction of water with methylene chloride to look for organochlorine pesticides. The first shaking/mixing of water sample and solvent may cause only 90% of the target compounds to preferentially associate with the solvent. When the solvent is drawn off, 10% of the target compounds will still remain in the sample. If we assume 90% efficiency for each mixing (as an example only), 1% will remain after a second extraction, and only 0.1% after the third. In this manner we increase the effectiveness of the extraction method by performing it multiple times. The downside of this is that we use more solvent, and classical liquid–liquid extraction procedures may be left with 200+ ml total volume after extraction and cleanup. This significantly dilutes the concentration of the target analytes.

Reducing the volume increases the analyte concentration and the subsequent sensitivity of the analytical instrument. As most organic solvents are fairly volatile (boil at low temperatures), reducing the extract volume by boiling/evaporation is relatively easy. The two most common procedures utilize heat and boiling or gas/air blow-down.

Boiling evaporation: There are many different potential configurations for this. In essence, the extract is placed in a flask to which heat is applied. A column (e.g., 3-ball Snyder) is usually placed above the flask to better control the evaporation and reduce analyte loss. As the heat is applied the solvent boils, vapor escapes, and the volume is reduced. The target analytes are of significantly higher boiling point and are retained in the flask. This technique is not suitable to volatile organic compounds as they would evaporate as well. These flasks are usually shaped so that the decreasing liquid volume collects in a small volume where it is easily measured and collected. Heat is provided by a water bath or electric heating element.

Gas blow-down: The extracts are placed into suitable tubes and then into a warm water bath. The tubes usually have narrowed, marked bottoms to estimate volume. Instead of heating the extract to drive off the solvent, a stream of inert gas (e.g., nitrogen) is directed across the surface. This reduces the vapor pressure of the solvent and considerably increases the evaporation rate. This technique is very popular and may be more easily automated and used for more samples than boiling evaporation.

It is during this volume reduction step that the solvent may be exchanged for another. We often find that the best solvent for extraction is not the best for the actual analysis. For example, methylene chloride is a great solvent for liquid–liquid extraction because it has a good affinity for many organic compounds, but poor dissociation into water (it forms a nice layer). However, it can play havoc with many analytical instruments, particularly those sensitive to electronegative compounds, such as an electron capture detector. While the extract is being reduced in volume, the desired solvent is therefore added and the volume reduced again. Repetition of this procedure results in the replacement of the original extracting solvent with the one desired for analysis. The final volume desired for the extract to be ready for analysis is usually 0.5 or 1.0 ml.

Volume reduction allows for the sample's components to be highly concentrated, often 1000×. Consider a 1-liter water sample with 10 μg/liter of atrazine. We can extract this with CH_2CL_2 three times, blow-down the solvent with nitrogen, and have a final volume of 1.0 ml. By this process we have taken the 10 μg of atrazine from 1 liter and placed it into 0.001 liter. This concentration allows for lower detection limits.

Sample Extract Analysis

Unlike analyses for biologic organisms, individual chemical analysis methods are fairly limited in the range of compounds to which they are suitable. They can only detect a compound's presence to a certain low level that is dependent on method, instrument, and analyst and can only accurately measure to a concentration close to its calibration range. To this point we have collected a sample, extracted it, and prepared the extract for analysis. All that remains is to separate the individual analytes for detection and determine the amount of each present. We will now discuss two of the most widely used separation methods, gas chromatography (GC) and high performance liquid chromatography (HPLC). In conjunction with these two methods we will discuss the detectors most commonly associated with each.

Gas Chromatography

Chromatography in its simplest sense may be described as the separation of components as they travel through an interactive medium. It consists of two immiscible phases: the stationary phase and the mobile phase. The stationary phase is usually immobilized within a column or fixed on a support, and the mobile phase is passed through it. The phases are chosen so that the analytes of interest have different solubilities in them, resulting in different migration rates. The differences in phase solubility and migration rates between sample analytes cause them to travel at different speeds (separate) as they pass through the column, ideally exiting one at a time where they may be accurately detected.

GC utilizes an inert gas (mobile phase) for carrying volatilized sample extracts through a narrow-bore column (stationary phase). One of GC's major weaknesses is the requirement that the extract be volatilized and pass through the column in a gaseous state. This limits analyses to those compounds that are thermostable (stable at higher temperatures) and of sufficient volatility that they will become gaseous within the instrument's operating range. Still, GC may be called one of the main workhorses of the chemical analysis world and is composed of four main components.

The injection port volatizes the extract and provides a route of entry of the extract onto the column. A tiny volume (e.g., 0.5–2 μl) of extract is taken up by a microsyringe and injected through a rubber septum into the injection port. Much use is made of autosampling devices that are programmable and do everything—from handling the sample extracts through extract draw and injection and syringe washing. The port is kept heated well in excess of the extract's boiling point, and the extract is vaporized and mixed with the carrier gas. Upon introduction, the gas carries the vaporized extract to the column.

The oven is thermostatically controlled and contains the column through which the extract components separate as they travel. The oven's temperature is tightly controlled (to within 0.1°C) and able to rise/ramp at a rapid rate. The use of liquid nitrogen allows the oven to attain low temperatures. Often the oven begins the analysis at a fairly cool temperature (e.g., 50°C), allowing the analytes (which were vaporized in the injection port) to condense in the first part of the column. The temperature is then raised in a programmed manner, allowing the analytes to be carried through the column by the carrier gas at increasing, but different, rates. The increasing rate shortens analysis time and the different rates allow analyte separation.

Compounds emerge from the column in what is called the elution order, and this order is dependent on the column. There are two general types of column: packed and capillary. Packed columns are usually made of glass or steel, have a 3.18- or 6.35-mm inner diameter, are 1–3 m long, and are packed with a stable, inert support such as kieselguhr (diatomaceous earth) or silica beads that contain the stationary phase. These are not used much in PHLs as they are not well suited for trace level analyses. Capillary columns are much better suited for this task and have a significantly different structure. They are usually made of fused silica or steel, have a 0.10- to 0.53-mm inner diameter, are 12–100 m long, and have internal coatings that act as the stationary phase (rather than a packing). The narrowness of the column ensures significant contact between the gaseous analytes and the coating. Of the two general types of internal coating used, polysiloxanes are the most widely used. The different coating materials incorporate different functional groups, which affect how they interact with analytes. For example, the saturated hydrocarbon phase

squalane has a defined polarity of zero and analytes are eluted in order of increasing boiling temperatures. Different phases have different interactions and elution order may be more/less dependent on such factors as boiling point, polarity, and functional groups.

Carrier gas flow rate, temperature, and time are very important parts of optimizing the analytical method. Using a slow carrier gas flow and slow temperature ramp (e.g., from 50° to 250°C at 1°/min) would likely result in a very distinct and clear separation of compounds. However, it would take almost 3.5 hours for a simple analysis! Alternatively, one could increase the carrier flow rate and increase the ramp to 40°/min. While the analysis would only take ~5 minutes, it is likely all the compounds would come shooting into the detector as a single, undefined blob. This highlights how every method of analysis must be optimized by the analyst to produce the best separation for the selected target compounds in the shortest time possible.

A detector is just that; it detects or responds to a target analyte. It is this particular component that registers a compound when it exits the column. Without a detector, separation is useless. Also useless is the wrong detector for a particular class of analytes. Just as different extraction methods are used for different target compounds, so too different detectors are utilized for different classes of compounds. Part of the complexity of chemical analysis is seen in that incompatible extraction methods may use the same analysis detector, and the same extract may also be analyzed by different detectors for different compounds. We will now describe different detectors in use and for which compound types they are most useful.

Electron capture detector (ECD): ECDs utilize ^{63}Ni foil. This isotope of nickel is a beta emitter and produces a very localized cloud of electrons that ionizes a provided flow of gaseous nitrogen, resulting in an electric current between two electrodes. Compounds containing an electronegative element/component (e.g., Cl^-, F^-, nitro groups) eluting from the column absorb some electrons and decrease the current strength. This is very well suited for compounds with a high affinity for electrons, such as organochlorine pesticides.

Flame ionization detector (FID): FIDs utilize a hydrogen-fed flame to combust compounds as they emerge from the column. The combustion creates ions and charged particles that allow the passage of a weak current between the detectors electrodes. This is an excellent detector of organic compounds, especially hydrocarbons.

Photoionization detector (PID): PIDs utilize a special lamp to photoionize compounds. The ultraviolet (UV) lamp emits photons, and when their energy exceeds the first ionization energy of the target analyte, the analyte is ionized and the electron detected. This is therefore selective to certain hydrocarbons as each has different ionization energies, and only the ones within the range of the lamp will be detected. This is fairly sensitive to low levels of specific compounds (e.g., benzene) and is used for some trace level analyses.

Nitrogen phosphorus detector (NPD): NPDs utilize a flame and a rubidium salt bead to ionize/detect compounds containing nitrogen or phosphorus. The salt acts as a catalyst for the decomposition of nitrogen- and phosphorous-containing compounds. These ions then register in the collector electrode.

High Performance Liquid Chromatography

HPLC utilizes liquids (mobile phase) for carrying soluble sample extracts through a narrow-bore column (stationary phase). One of HPLC's strengths is the ability to analyze those compounds that are thermally labile (decompose at high temperatures) or of high polarity or high molecular weight. As such, it is a good complement to the weaknesses of GC. It is also quite useful for water-soluble compounds that are not easily extracted. The water sample may be analyzed with little preparation as would be required for GC analyses. There are four major components common to HPLC systems: pumps, injectors, columns, and detectors.

In a GC, the carrier gas is forced through the column by the pressure exerted by the gas tank from which it originates. There is no need for additional pressure. HPLC, on the other hand, needs pumps to provide substantial pressure. The mobile phase is initially drawn from containers at atmospheric pressure (~14.7 pounds per square inch [psi]) and must be pushed through a very densely packed column. Pressure at the injector may attain 20,000 kPa (~2900 psi). Without this high pressure, flow through the densely packed column would be intolerably slow. In order to maintain consistent carrier flow, pumps are also augmented with devices to dampen their pulses and may be configured to account for different mobile components of varying compressibility. Solvents are also degassed before they enter the column as even small numbers of dissolved bubbles interfere with separation and reduce column life.

Similar to GC, an HPLC injection is made swiftly, though in this case to minimize the disturbance to the mobile phase. Also similar is the extensive use of automation. Unlike GC, there is no heat or vaporization involved. Also different is the volume of sample injected. It is usually much larger than GC and on the order of 20–100 μl.

HPLC analyses are most often performed at ambient temperature, so an oven is seldom required. The columns are often thermally controlled, though, to maintain consistency between analyses and over time. The columns themselves are stainless steel tubes 3–15 cm long. These tubes have inner diameters ranging from < 1 to 4.6 mm. The packing material is retained in the tubes via porous disks at each end. By far the most widely used packing material is spherical silica gel particles of 2–5 μm in size. To increase consistent separation properties over time and analyses and reduce the gel particle's inherently strong polarity, they are often treated with alkyl monochlorosilane for a monomeric phase, or alkyl di- or trichlorosilane for a polymeric phase. These make the column material less polar, more robust, and allow for finer separations. The alkyl chains thus bound are anywhere from 8 to 18 carbons in size, but other functional groups are becoming more useful as well. These include aminopropyl and benzyl groups and dipolar ligands. These result in intermediate polarity and are useful for separating small polar molecules such as sugars and peptides.

In HPLC, the mobile phase conditions are critically important for separation. In fact, the degree of interaction between these phases determines the analyte retention times. In *normal phase* chromatography, the stationary phase is polar (e.g., silica beads) and the mobile phase less so. In *reversed phase* chromatography, the stationary phase is non- or weakly polar (e.g., bonded silica beads) and the mobile phase more so. The elution order of compounds is often reversed if the phases are changed. For example, in reversed phase systems, hydrocarbon compounds are strongly retained and polar compounds much less so. In fact, they go through so fast that they are often difficult to separate. To address this problem, analysts often use an elution gradient. Just as a GC may change its oven temperature to fine tune separation, an elution gradient changes the mobile phase composition over time. Thus, the mobile phase may start with a water/acetonitrile mix of 80/20 and end with 40/60. The changing polarity allows for better separation of compounds with similar, but not identical, polarity. Mobile phases that do not change in concentration are termed *isocratic*.

Detectors employed for the detection of compounds eluted from HPLC systems are just as specialized, though not as varied, as they are for GC. The most widely used detectors are based on the optical properties of the analytes. Refractive index detectors measure the change in the mobile phase's refractive index (angle at which incident light is bent) because of the presence of the analyte. This is not very robust or sensitive and is subject to variations due to minor temperature and other changes. It is not often used in PHLs.

Ultraviolet/Visible Detectors

Ultraviolet/visible (UV/VIS) detectors measure an analyte's absorption of one or more selected wavelengths in the UV/VIS spectrum as it passes between a source and detector. This absorbance is with respect to the mobile phase alone, which may be split into a reference cell for comparison. The region of the spectrum ranges from the near UV (185–400 nm), includes the visible (400–700 nm), to the very near infrared (700–1100 nm). In absorbance, one or more of the analyte's outermost electrons captures the UV/VIS photon, resulting in a change in the molecule's energy. Given that a molecule is composed of different components and bonds, there are opportunities for a molecule to absorb multiple photons of differing wavelengths. We find that functional groups of organic compounds often absorb photons of a characteristic wavelength. These functional groups are referred to as chromophores. Amine ($-NH_2$), for example, absorbs at 195 nm and absorbance at this wavelength indicates an amine-containing compound. The concentration of the analyte can then be determined by measuring the compound's absorbance at a specific wavelength and applying the Lambert-Beer law, which relates analyte concentration to detector response. Because not all compounds of potential interest absorb in these wavelengths, they may be subjected to chemical derivatization (addition of a chromophore) before passing through the detector.

The photons are provided by lamps. UV may come from a deuterium arc lamp and VIS from an incandescent lamp with tungsten filament. Alternatively, a xenon arc lamp may itself provide the entire UV/VIS spectral range. For monochromatic analyses (those involving only a very narrow range of wavelengths at a given time) the light is split by a grating before passing through the sample. The light then impacts the detector, which reads the absorbance for this narrow range. This strategy is used for "fixed" wavelength spectrometers. Alternatively, for a scanning spectrometer, the grating can turn,

allowing for a sequence of different "snapshots" of the sample with different wavelength ranges. For other analyses, the light impacts the sample first and the reflected light is then dispersed by a grating to impact a photodiode array (PDA) that is able to read all the wavelengths simultaneously.

Fluorescence Detectors

Fluorescence detectors measure the light emitted by a compound. The detector source emits light (photons) of specific energies. These photons cause the target molecules to enter an excited state, and the return to its ground state is accompanied by the emission of light (fluorescence) of a different wavelength. Detection of this resultant light is made with a spectrofluorometer. The same range of wavelengths used for UV/VIS analyses are utilized here, with the exception that the absorbance of photons is not measured. The intensity of the resultant fluorescence is directly proportional to the concentration of the analyte. Approximately 10% of organic compounds can fluoresce, so they are often subject to chemical derivatization (addition of a fluorescent chemical group) for detection. Fluorescence detection is quite sensitive and often used for trace level analyses.

Similar to some UV/VIS techniques, a source produces photons with a xenon arc lamp. With a fluorescence ratio fluorometer, the photons are then passed though an excitation monochromatic lens that selects a narrow band (15 nm) for passage through the sample. This allows for more specific analyses with only those absorbing in the specified band responding. The resultant light is then passed through a chromatic lens to narrow the band of wavelengths received by the detector. Spectrofluorometers, on the other hand, use two monochromatic lenses, which allows for full capture of the entire spectral band. They can also look for all emission wavelengths associated with a fixed excitation wavelength, or all excitation wavelengths associated with a fixed emission wavelength. This produces a much wider range of compounds potentially identified, but also much less selectivity for specific compounds or classes. The choice between these methods is often made on the basis of need, expense, and instrument availability.

It should also be noted that these detectors are not solely utilized by HPLC. HPLC is a separation method and UV/VIS and fluorescence are methods of detection. They are utilized in other analytical venues but happen to be well suited to the HPLC system since the analytes are in solution. This contrasts with the detectors utilized in GC. Their method of working depends on the analytes being in a vapor/gaseous phase.

It is also important to note that none of these detectors provide actual identification of any compound. They simply register when a compound enters the detector and compares its time (and some characteristics) to a standard run under identical conditions. The analyst will usually take a solution containing known amounts of the target compounds and analyze them first. From this, the analyst will determine each compound's retention time (time required from injection to detection) and signal strength (to calculate amount in the sample). A standard solution for organochlorine pesticides might contain heptachlor epoxide. If it takes 5:57 for the compound to be detected when the standard solution is analyzed, the analyst will then look for a peak at that same time in the sample analysis. However, a corresponding peak is not automatically determined to be heptachlor epoxide, it could be another unknown compound with similar charateristics. Such positive results must therefore be confirmed by a subsequent analysis by either a different column/detector or mass spectroscopy.

Mass Spectroscopy

Mass spectroscopy is not really a method of analysis in and of itself. In fact, a mass spectrometer (MS) is more properly termed a detector and is quite often hyphenated with the two separation methods described previously (resulting in GC-MS and HPLC-MS or LC-MS), where the MS replaces previously discussed detectors such as ECD and UV/VIS. Because of its versatility and increasing ease of use and sensitivity, it is one of the most widely used analytical methods.

The basic mechanism for MS analysis is the isolation of the sample analyte, its ionization/breakup into multiple pieces, and the subsequent subjugation of these pieces to electromagnetic fields under high vacuum. By studying how these forces act upon the ions, one may determine their mass-to-charge (m/z) ratio. This ratio is specific to specific ions, and individual molecules are split into characteristic ions. By measuring the relative abundances of different ions and their charges, one can not only quantitate the concentration of the analyte, but also identify it by "reassembling" the pieces. This is similar to putting a puzzle together. A given set of ions can usually be only reassembled into one compound, just as a set of puzzle pieces come together to form only one picture. This is in direct contrast to the detectors mentioned before. There, they were measured when an

analyte emerged from a column and perhaps some information was gathered about what type of chemical bonds were present, but the determination of a specific compound could only be made by reference to a standard.

To achieve this ability, there are a number of steps past the analyte separation that must be accomplished. First, the eluting analytes are subjected to vacuum. Because the MS analyzes ions in the gaseous state, liquids associated with sample separation (mobile phase such as methanol) must be removed. Its concentration relative to the target analytes is usually several orders of magnitude greater, and its signals would overwhelm those from the target analytes. Once the mobile phase is removed, the analyte molecules are broken up into pieces (ionized) in the instrument's ionization chamber. This is done through the use of a beam of electrons (electrical ionization) or a molecule such as ammonium (chemical ionization). The ions are then focused and accelerated by a series of electronic lenses. This increases their kinetic energy as they enter the analyzer. This part of the instrument filters the ions according to their mass-to-charge (m/z) ratio. Filtering allows ions to selectively escape and then impact the detector. The detector does not measure the size of the ion itself, just the number of impacts and thus the concentration. Each analyte has a statistical distribution of fragment ions. Their detection in known ratios leads to compound identification.

Let us consider a simple example. A water sample is analyzed for the presence of organochlorine pesticides, and atrazine happens to be present. The sample extract analytes are separated by GC. When atrazine ($C_8H_{14}ClN_5$) enters the MS, it is ionized by a beam of electrons. The resultant pieces range anywhere from the smallest (H^+) to the largest ($C_8H_{13}ClN_5^-$). They are accelerated and enter the analyzer, which releases them one at a time based on m/z ratio. The detector then registers the amount of each ion as it emerges. The data can be recorded in different ways. The normal-mass spectrum produces peaks of differing widths, which lead to the determination of the ion masses. Alternatively, all the ions may be recorded in order of their nominal mass and the peaks are relative to each other in height. The most abundant ion has the highest peak (base peak) and is set at 100% intensity. All other peaks are set relative to it. This is the most common method used in MS. In either method, identifying the compound from the data is done by either putting the pieces together to determine the original molecule or comparing the results to a library of fragmentation spectra. Most instruments have software to assist in this process, and there are many libraries available for free or purchase. MS detectors are generally much more complex in design and operation than other detectors already discussed. We will mention two of the most commonly used detectors very briefly. Those interested in further detail should consult the additional resources at the end of the section for more detail.

Quadrupole: Quadrupole analyzers are quite often used in PHLs. A quadrupole is a set of four parallel metal rods that act as paired electrodes. They are finely machined to form a hyperbolic cross-section in their interior. The rods are subjected to electric currents that induce powerful magnetic fields. These fields control the movement of ions that enter them. The fields are changed by altering either the radio frequency or the amplitude of the applied voltages. In either method, the systematic "scan" through a frequency or amplitude range allows ions in increasing m/z to escape and impact the detector.

Ion trap: Ion trap analyzers also use electrodes to trap ions in space, but the design and operations are substantially different. Instead of four parallel bars, there are two end caps on either side of a toroidal trap (donut-shaped). The trap is both a source and filter. The analytes are ionized by an electron beam upon entering the filter. The radio frequency is then increased to move them into the central part of the filter. Increasing the radio frequency destabilizes the paths of the confined ions, and they escape in order of increasing m/z. They then pass through small holes in one of the end caps where they impact the detector. The ions may also be individually chemically ionized before impact with the detector, greatly increasing the amount of data produced and increasing the analytical ability of the instrument.

Inductively Coupled Plasma Mass Spectroscopy

Inductively coupled plasma mass spectroscopy (ICP-MS) is a specific application of the MS detection technique and has excellent sensitivity. A plasma is a gas that has enough ions (positive and negative) to conduct electricity. In ICP, a burner composed of quartz is placed within an induction coil powered by a radio frequency. Argon gas is supplied and ions in the gas accelerated back and forth as the radio frequency changes. As atoms and ions collide, they produce more ions (sustaining the flame) and great heat (usually 6000–10,000°C; equivalent to the surface of the sun). Aqueous samples

are nebulized (made into a fine mist), introduced to the flame, and atomized. At this temperature, all chemical bonds are broken and the resultant elements are in a free, gaseous state.

At this time a large proportion of the elements are also ionized by collisions with other elements. A small proportion of these ions then pass through a series of electrically charged cones into the attached MS. The cones have very small openings (e.g., ~1 mm and ~0.4 mm) to allow the MS to retain a vacuum. Often this is a quadrupole design and the ions are released to the detector in order of increasing m/z ratio. The strength of the resultant signal is proportional to the element's concentration. This analytical technique is used most often for the determination of lead in environmental or clinical samples and multiple metals in drinking water.

Atomic Absorption Spectroscopy and Flame Emission Spectroscopy

Atomic absorption spectroscopy (AAS) and flame emission spectroscopy (FES) are conceptually two of the simplest methods of detection, but like ICP-MS, their use is limited to the identification of individual elements, not the compounds in which they may be found. They are based on the principle that each element's electronic configuration and ground state are unique. Each element absorbs light only at very specific wavelengths (corresponding to an electron's promotion to a higher-state orbit) and releases light at similarly specific wavelengths (corresponding to an electron's return to a lower-energy or ground state). By measuring the wavelength absorbed (AAS) or emitted (FES) and their intensities, one may determine what elements are present in the sample and their concentration.

A complication to this analysis is the binding of elements into molecules. This inhibits the absorbance and release of light and creates structural interferences. To overcome this, samples are flash heated to an excess of 2000°C where all chemical bonds are broken and all elements are free. This heating is accomplished in one of two ways. The aqueous sample may be nebulized and introduced at a constant rate to a flame produced by a combustible gas (e.g., acetylene). Alternatively, the sample may be placed within a graphite tube. When a sufficient current is applied, the resistance of the graphite to current results in heat to volatize the sample. In each case the resultant vapor is passed through an optical path between a light source and the detector(s).

In AAS analysis, the light is emitted from the source, passes through the flame (or graphite tube), is split by the monochromator, and impacts the detector. The monochromator selects which specific wavelengths correspond to the target elements of interest. If there are no target elements present, the light intensity incident on the detector is equal to that emitted from the source. A reduction in intensity indicates the element's presence. In FES, the element's electrons are in an excited state because of collisions at the high temperature. As the electrons return to their ground state, they release photons of characteristic energy (wavelength) that are dispersed by wavelength with a grating and registered by a photomultiplier tube (a device that detects ions and produces a current as a result of ion collisions). This analytical technique is often used for the determination of lead in blood samples as part of lead poisoning monitoring in children.

Quality Control

Quality control is important for any analysis, but especially so for chemistry. Because of the large number of steps typically required to prepare a sample for analysis and the critical dependency on sample, extraction, and analytical conditions for accurate analysis, chemical analysis methods have more built-in quality control measures than either microbiology/virology or radiology. As for many factors involved with chemical analyses, quality control requirements differ by method and sample type. The following provide a good example of what types are typically required and what purpose they serve.

Internal Standard

Internal standard (IS) is a known amount of a compound(s) added to a sample, its extract, or standard solutions to measure the responses of the target analytes. By adding a known amount of IS directly to the extract (or sample for some analyses), you should see the same response for each analysis. A decreased or increased response indicates a problem with the analysis. For Method 508.1 the IS is pentachloronitrobenzene. We add the same amount to all extracts and standards and expect to see it detected at the same level each time (say "5.0"). If a sample extract is analyzed and the IS is only 2.5, we know there is a problem with the analysis. The septum might have cracked or the injection needle faulted. This also indicates that the result for any target compound might be only half of what is actually present. The IS is a check on each analysis to look for such problems.

Surrogate Analyte

This is a compound added to all samples before extraction to measure how well the procedure works. If 10 μg of surrogate is added, but only 7.0 μg detected in the end, we can say that the extraction efficiency is only ~70% and the results must be qualified. In fact, low surrogate results may invalidate the entire analysis. Method 508.1 uses 4,4-dibromobiphenyl as a surrogate. An "appropriate surrogate" is one for which the chemistry (properties) of the surrogate and the target analyte(s) is similar.

Laboratory Reagent Blank

The laboratory reagent blank (LRB) is an aliquot of laboratory water or other sample matrix that is treated the same way as all other samples, from extraction through analysis. The purpose is to detect any contaminants that might be present in the solvents, glassware, etc. For example, a sample might show a result corresponding to dieldrin. If the LRB also has that same result, further work would need to be done to confirm that the sample result is not due to laboratory contamination.

Instrument Performance Check

Instrument performance check (IPC) is a solution of specific surrogates, IS, and target analytes directly analyzed by the instrument without prior sample preparation or extraction. It is used to measure how well the instrument performs versus specified requirements. For example, the instrument might be required to have a specific amount of separation between two compounds, or a specific ratio of response between two peaks. If the criteria are not met, the instrument is considered unable to perform the analysis and adjustments must be made.

Laboratory Fortified Blank

The laboratory fortified blank (LFB) is identical to the LRB with the exception that known quantities of each target analytes are also added prior to extraction. The purpose of the LFB is to show how well the method works for each target analyte and if the laboratory can perform the method well. For example, if 5.0 μg of dieldrin is added to the LFB, but only 2.5 μg detected in the extract, then we know there was a problem with the extraction procedure. We might not notice this problem otherwise and all reported values would be incorrect. It is common for a method to have different extraction efficiencies for different analytes. Using the LFB shows, for example, that a method may be 98% efficient for extracting heptachlor, 95% efficient for heptachlor epoxide, but only 76% efficient for atrazine.

Laboratory Fortified Sample Matrix

Laboratory fortified sample matrix (LFM) is the same as the LFB except that a duplicate of one or more samples is "spiked" with the same target compounds as the LFB. This is done to see if there are components of the matrix that affect extraction or analysis. For example, if we add 5.0 μg of dieldrin to the LFM, but get back 2.5 μg (and the LFB got back 5.0 μg) we know there is something about the sample itself interfering with the analysis. By comparing the results of the sample and its spiked duplicate, one can get a better idea of how the sample matrix is affecting the target analytes.

Quality control measures are obviously quite substantial. They also vary by analyte, sample type, and analytical instrument. The take-away item for this portion of the chemical analysis section is that the analysis of the sample itself is only a small part of the analytical picture. For the circumstance where there are only a couple of samples to be analyzed, it is often the case that there are more quality control measures, extractions, and analyses than there are samples. A typical analysis order for the instrument may thus look like this:

1. IPC
2. Reagent blank
3. Standard 1
4. Standard 2
5. Standard 3
6. Standard 4
7. Standard 5
8. LRB
9. LFB
10. Sample 1
11. Sample 2
12. LFM
13. Sample 3

There is much more that could be discussed, such as the use of duplicates to determine reproducibility,

continuing quality assurance to ensure defensible data, standard curves for relating analyte concentration and detector response, and reporting limits to determine method sensitivity. These are beyond the scope of this book.

Chemical analyses may be considerably complex compared to microbial analyses. They often require equipment dedicated to the analysis of a relatively few numbers of compounds. This makes chemical analysis a potentially expensive proposition, one that not all PHLs are willing to make. It is much easier to train a microbiologist to be a sort of jack-of-all-trades that can perform analysis for many different organisms with a somewhat limited array of equipment. Many of the chemical methods, by contrast, are highly individualized and the analysis unique to that compound class. Laboratories often find that a single chemist can retain proficiency in only a small number of methods, with the result that a larger staff is needed. In addition, chemical analysis instrumentation is generally much more expensive to purchase and maintain. Some state PHLs no longer maintain any except the most basic chemical analysis capacity (e.g., NO_2/NO_3/Fl) and contract with private laboratories for the rest.

Discussion Questions

1. Samples submitted for analysis almost always need to be collected by a local health department or other authorized official. Why might samples collected by homeowners and other private citizens be unacceptable?
2. Sample extraction is the attempt to remove the target compound(s) of interest from the sample. Why is this necessary and why might it be difficult, especially for food and soil samples?
3. What are some of the potential health hazards associated with performing liquid–liquid extractions?
4. Describe how liquid and gas chromatography are similar and different in terms of compound separation.
5. What must a compound be able to do to be detected by UV/VIS spectrometry?
6. When considering the analysis of a sample for an unknown element (metal), would ICP-MS or AAS be more useful, and why?
7. Why is mass spectroscopy performed under vacuum?
8. If the recovery of one of four target compounds from both the LFB and LFM was on the order of 30%, the recovery for the others exceeded 90%, what conclusions might you draw?
9. Go on the Internet and find three good sources for detailed information on some or all the techniques described in this chapter. They cannot be government-sponsored (.gov) or listed under Additional Resources. Briefly describe what they provide and why they are good sources of reliable information.
10. Many chemical analysis quality control measures are built into the extraction and analysis and require specific levels of result for the final sample analysis to be valid (e.g., IS must be ± 10%). What reasons might be given for a lack of similar requirements with microbial analyses?

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4

The Basics of Radiological Analyses

Introduction

The purpose of this chapter is to impart to the reader a better understanding of what is involved in an analysis for a radioactive element or compound. These analyses are performed relatively infrequently in public health laboratories (PHLs), but they are an important component of public health, especially in areas where radionuclides are likely to be a source of contamination. While many of the analyses are simpler than those for microbiologicals and chemicals, the potential for harm to the analyst is potentially greater. Radiation poisoning rarely causes acute illness that may be noticed by the analyst, but some types may penetrate several inches of shielding and create significant risk for lung cancer if inhaled. Proper care must therefore be taken to minimize personal exposure at all times from sample receipt to ultimate disposal. As done in Chapter 3, this chapter is divided into sections based on the flow of work associated with sample analysis. The detail presented is purposefully basic with the intent to impart the general ideas and techniques involved in sample analysis. References for further study are provided at the end of the chapter.

A material (element) is termed radioactive when its individual atoms spontaneously undergo decay. In these materials, the original atomic nucleus (parent) is inherently unstable. In order for the atom to become stable, it must lose energy and drop into a lower energy state. It does this by releasing ionizing particles and/or radiation. The new atom (daughter) is therefore more stable, though it may itself decay into another product. This process of continuing decay is called a "chain" and may consist of anywhere from 1 to more than 14 steps.

The instability of the nucleus is based on quantum-level forces, which fluctuate over time. It is therefore impossible to predict when any individual atom will decay, but the rate of decay for a large number may be statistically determined. These rates vary by element and are relatively constant over time. The decay rate measurement most often used is the *half life* of a material. That is the length of time required for half the parent nuclei to decompose to the immediate daughter nuclei. This time varies by element from fractions of a second to millions of years. Half life does not vary by the amount of material, so the half life of 1 gm of uranium-238 (^{238}U) is equal to that of 1 kg.

Activity, on the other hand, does vary by amount and indicates the number of decays per time period. The activity of our kilogram of ^{238}U is therefore 1000 times greater than that of the single gram. The SI (International System) measurement for activity is the becquerel and one Bq is defined as one decay per second. Because of the large number of decays per second commonly observed, measurements on the order of giga (10^9) and tera (10^{12}) becquerel are often used. Sometimes the unit of curie (CI) is used and is equivalent to 3.7×10^{10} Bq. By far the most common and readily recognizable decay products are *alpha* and *beta* particles and *gamma* radiation. There are other products as well, including positrons, neutrons, and positive and negative neutrinos. These are not usually of public health concern and will not be considered further here.

Alpha particles: These are identical to helium nuclei (i.e., consisting of two protons and two neutrons),

carry a positive charge, have energies typically between 3 and 7 million electron volts (MeV), and are denoted by the Greek symbol α. In spite of their size and energies, alpha particles do not travel far when emitted. Indeed, they are typically stopped within a few centimeters of air caused by collisions with other molecules. They are also stopped by a single sheet of paper or the outer layer of dead skin cells. Danger from external exposure is therefore generally insignificant. However, alpha emitters pose great health risk if ingested or inhaled. The size and energies of the particles are sufficient to cause significant damage to cellular DNA when there is no intervening barrier to stop them (e.g., air, skin). It is for this reason that homes are inspected for radon (an alpha emitter), which is an odorless gas and is associated with lung cancer. An alpha emitter, americium-241, is used in many smoke detectors.

Beta particles: These are electrons (or positrons) with a negative (or positive) charge, have energies in the KeV to MeV energy range, and are denoted by the Greek symbol β. Beta particles are much smaller than alpha particles and consequently have a smaller mass-to-energy ratio. They travel several feet in air and penetrate skin. However, they are still stopped by a sheet of aluminum or a plexiglas shield. Because of their smaller size and subsequent decreased ability to cause damage, they are not considered as dangerous as alpha particles in the event of internal exposure. Beta emitters are used in medicine and form the basis of positron emission tomography (PET) scan.

Gamma rays: These are electromagnetic waves with frequencies exceeding 10^{19} Hz and are denoted by the Greek symbol γ. Gamma rays do not have mass, but have great amounts of energy and penetrative power. Like all electromagnetic waves they have a dual nature, exhibiting properties of both light and particles. Light properties include a measurable wavelength and diffraction in a prism. Particle properties include the assignment of a discrete amount of energy released as a photon. Gamma rays are only stopped by substantial shielding such as lead blocks/sheeting or several feet of concrete. They are used in medicine to treat cancers and sterilize equipment. Their sterilization capabilities are also utilized to preserve food and spices.

Ionizing radiation can have mild to severe effects on the body depending on the radiation type, its energies, its route of exposure, and its activity. In general, radiation causes cellular damage because of direct impact (alpha and beta particles) and/or through the disruptive adsorption of energy (gamma rays). Damage such as skin lesions may be minor and heal over time. More severe effects include significant organ damage and the development of cancers because of genetic mutations. Ionizing radiation cannot be seen, felt, tasted, heard, or smelled so physical analyses must be done to determine their presence or absence. It is because of their potential for harm that they are tightly regulated, and they are a public health concern because of their potentially unknown presence.

Finally, it is worth noting that radiological analyses are performed relatively infrequently by state laboratories, and such analyses that are performed are usually simple in nature. In many states, the responsibility for radiological analysis resides within a department of nuclear safety, environmental health, or similar entity. This may cause conflict when it comes time to prepare for, or respond to, a radiological emergency involving humans. Whereas the responsible agency may have experience analyzing environmental samples for ionizing radiation, they may have no experience translating those methods for use on clinical samples. They may also be ill equipped to perform a large number of analyses in a short time frame (which PHLs are called on to do for other analyses on a frequent basis).

Sample Types and Collection

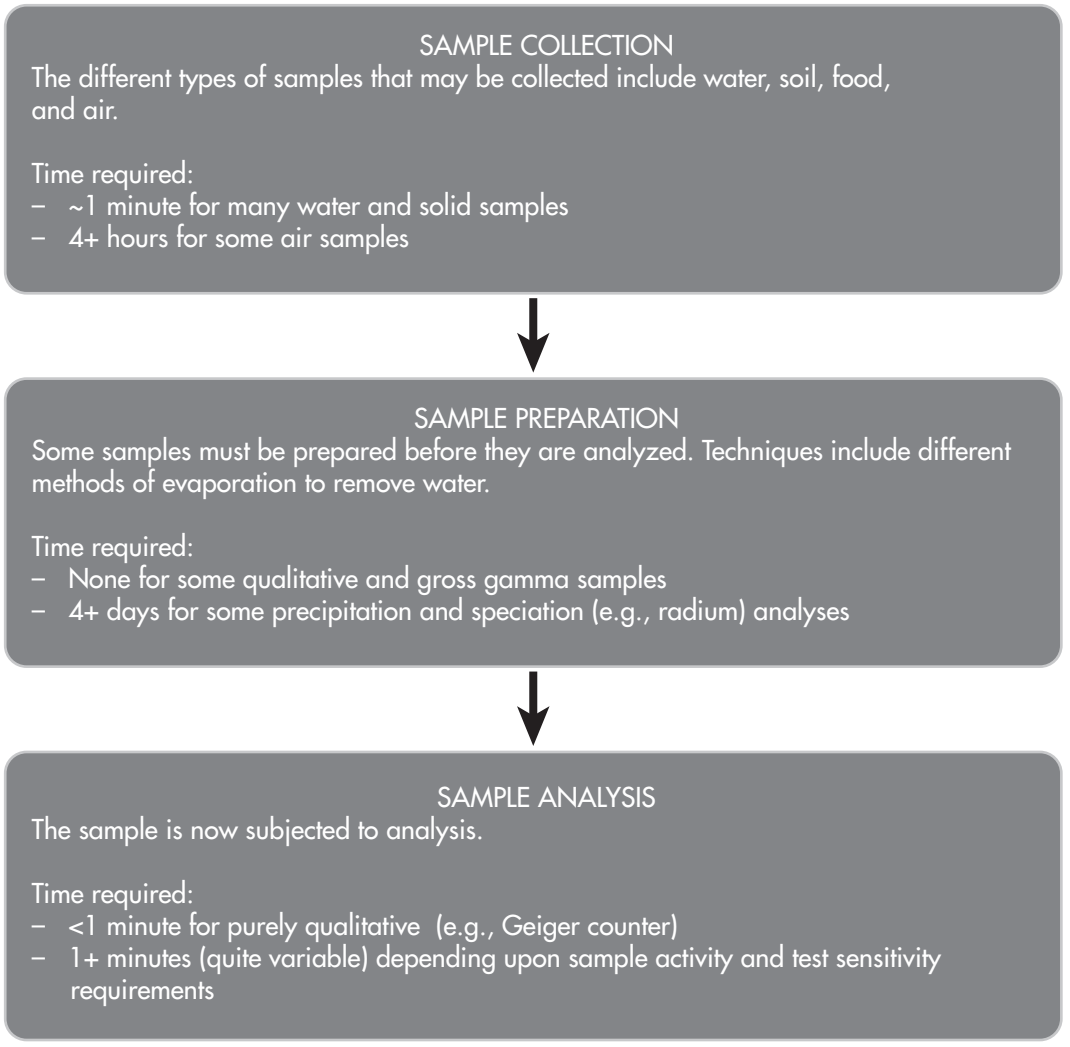
Water

Water samples are the most likely to be found in a PHL. They are analyzed in compliance with the US Environmental Protection Agency's (EPA) regulations concerning drinking water limits (and are discussed in more detail in Chapter 6). Other water samples may be collected as part of site remediation (e.g., at nuclear waste sites), accidents, or monitoring during legitimate use. In contrast to samples collected for chemical analyses, these samples are fairly consistent across analytical methods and may simply specify that the sample collected be of sufficient volume for analyses and be preserved to pH ≤ 2 (or delivered to the lab within 5 days). There are no specifications for container type, time limits for analysis, nor is thermal preservation required.

Food

Food is not routinely analyzed for radioactivity. There is a method for the analysis of food for polonium-210 but PHLs do not typically retain the capabilities for this analysis.

Figure 4-1 Flowchart showing the steps from sample collection to analysis for radiological compounds.



Air

Air samples are collected as part of environmental and workplace monitoring. These usually consist of the use of filters through which air is pumped at a defined rate for a measured time interval. There are other systems, such as in-line stack monitoring, that are used in industry and government but not usually encountered in PHLs. An example is American National Standards Institute (ANSI) *Guide to Sampling Airborne Radioactive Materials in Nuclear Facilities*, which provides specifications on proper sample collection utilizing filters.

Wipes and Soil

Wipes are often employed to search for surface contamination in areas where radionuclides are used. Typically, a gauze pad is wiped over a defined area and placed in a vial. Solvent may be added to the gauze to increase absorption of the target(s) to the pad. Response to a leak or spill would entail "wipe areas" of different sizes varying by agency (Occupational Safety and Health Administration [OSHA], Nuclear Regulatory Commission [NRC], Department of Energy [DOE]) and analytical method. Soil samples are usually collected in glass jars with no required preservation. In comparison to microbiological and chemical analyses, the guidelines for radiological sample collection, preparation, and analysis are more diverse and less standardized.

Sample Preparation

As is the case for some chemical analyses, there are occasions where sample preparation is not necessary. Such is the case for many mechanisms devised to detect the

presence of ionizing radiation. For example, the standard Geiger counter is used to measure beta and gamma radiation by simply passing the wand near the sample. This method cannot give a quantitative analysis of the activity of the sample, or of specific isotopes, but is a good first-glance look at whether a material contains any beta or gamma emitters (alpha particles are too weak to penetrate the counter's detection chamber window; some are fitted with a window made of mica, which will pass alpha particles). There are also multiple types of passive detectors that are usually worn (as a ring or on the lapel) and are chemically altered when in the presence of high-energy beta and gamma radiation. These usually undergo a color change to indicate exposure, or are sent to a laboratory for analysis. The remainder of this section will deal with the analysis methods most likely to be used in a state laboratory.

Evaporation

Some aqueous samples are evaporated prior to analysis. As noted in the introduction to this section, alpha and beta particles are relatively easily stopped, and their presence in the midst of a volume of water may be difficult to detect. By allowing the water to evaporate, the residue containing the radionuclides is retained for analysis without the interference of the original water molecules. Typically, a sample containing 100–200 mg of total solids is evaporated to dryness via hot plate and drying oven.

Leaching

Soils and sludge are usually leached with acid before analysis for gross alpha and gross beta analysis. This allows the radionuclides to be dissolved away from the soil, which interferes with analysis by blocking both particles. The sample is mixed with nitric and hydrochloric acids, allowed to sit for a period of time, the acid and soil separated with the acrid retained, and the acid evaporated to dryness.

Precipitation

Samples may also be chemically treated to selectively retain specific nuclides. We tend to view radionuclides as sources of radiation (which they are), but they are also subject to specific chemical reactions based on their structure in the same manner as any other element such as carbon or sodium. One can therefore perform chemical reactions in a sample that will only affect the analyte of interest. An example of this is the precipitation of radium in drinking water. The addition of a barium chloride solution and acid to an aqueous sample causes any radium present to precipitate out. The precipitate is then redissolved prior to analysis.

Ashing and Drying

Some solid sample types, such as filters, may be ashed prior to analysis. However, this is not always the case, and newer technologies (e.g., gas proportional counting) allow the wipe to be placed directly against the detector without prior preparation. Ashing, specifically wet ashing, is useful for some analyses, and may be coupled with other procedures to determine specific isotopes, which gross methods cannot do. Through this procedure the wipe or filter is treated with a series of strong acids to dissolve away and remove all carbon, with only the radionuclides remaining.

Sample Analysis

Unlike the case for most chemical analyses, there is no requirement for compound separation before analysis. In fact, there is also no need for any type of sample delivery to the detector more complicated than simply placing the sample in a vial and placing that in the instrument, or placing the solid sample next to the detector. From that point the detector will analyze all the constituents present. Also, unlike chemical analyses, the time required for detection is variable and may be quite lengthy. Detection happens essentially instantaneously for chemical compounds as they impact the detector, but quantitative radiological detectors need to be in proximity to the sample for a time period sufficient to achieve the desired sensitivity. This varies by method, isotope, and amount of isotope in a given sample.

Geiger Counters

Geiger counters work with a Geiger–Müller (GM) tube, which is filled with an inert gas (e.g., helium) and through which voltage is passed. The walls of the tube act as a cathode and a wire passed through the middle of the tube acts as an anode. When radiation particles or photons pass into the gas, they impact some of the molecules and cause them to ionize. These ions in turn impact other molecules and induce them to ionize in a cascading effect. The ions are drawn to the chamber's cathode and anode where their impact is registered, accompanied by the familiar "click." Most Geiger counters have a glass window through which radiation may pass,

but is too thick to allow passage of alpha particles. Some counters are fitted with mica windows that do pass alpha particles but are much more fragile. The Geiger counter is used for qualitative purposes only as it does not distinguish between particle types, their respective energies, or report activity in comparison to a standard.

Proportional Counting

Gas flow proportional counting works in much the same manner as a GM counter and is used for the detection of alpha and beta particles. There is a chamber of gas (in this case usually 90% argon and 10% methane; referred to as P-10) in which incoming radiation may ionize, and there is a voltage between an anode and cathode. However, the voltage and configuration of the chamber are set such that incoming and newly generated electrons maintain sufficient speed to continuously generate new ions as they travel (Townsend avalanche). Alpha particles impact the gas molecules and cause them to ionize. Beta particles also cause some gas molecules to ionize but can be directly detected as well. These ions are directed to the anode and cathode where they register as a pulse. Through these control measures, the kinetic energy of the incoming particle may be measured and the pulse size differentiates between alpha and beta particles.

For analysis, the sample is dried in a planchet and placed in the instrument's sample chamber. This chamber is in close proximity to the counter window and any alpha and beta particles are counted. The sample remains in the chamber until enough time has passed to meet method sensitivity requirements. This time must be calculated based on requirements and sample considerations, such as total dissolved solids (which hinder detection).

Liquid Scintillation

Liquid scintillation is also used to detect alpha and beta particles and involves mixing the sample with a scintillation cocktail. The cocktail consists of an aromatic solvent, such as toluene (which readily interacts with ejected particles), and a fluor. Solvent molecules absorb energy from the particles and transfer it to the fluors. They, in turn, release that energy as a flash of light. This is detected by a photomultiplier in the scintillation counter, and the sample's activity (number of decays per time frame) measured. The method cannot distinguish between different alpha and beta emitters. Also, the efficiency of the process is rather poor compared to other detection methods. Low energy particles, samples containing some elements (e.g., chlorine), or those that are brightly colored may have a diminished response because of "quenching." That is, the particles may fail to transfer energy to the solvent (or the solvent to the fluor) and/or light emitted by the fluor is reabsorbed by the sample before it reaches the detector. Efficiencies may be as low as 30%. This may be offset by corrective calculations and sample preparation.

A variation of this theme is used to measure alpha emitters that are in the gas phase. Here, the sample is placed into a scintillation cell. The cell's interior is coated with a fluor agent and is placed in a counter.

Gamma Spectroscopy

There are two types of detectors most widely used for analysis of gamma photons, and they are based on different technologies. Each has associated strengths and weaknesses and the choice of one over the other is made based on the analytical need or method requirements. Both rely on a photon from the source (sample) interacting with the detector material, and take advantage of the fact that gamma ray photons emitted as part of the decay process are unique, in terms of energy, to the radionuclide from which they come. This interaction between gamma photon and detector material ultimately produces an electric signal based on the photoelectric effect, the Compton effect, and/or pair production. This voltage pulse is then both shaped by a multichannel analyzer (MCA) into a Gaussian or trapezoidal shape and converted from analog to digital. The converter also sorts the pulse by height and assigns them into specified channels in the spectrum. The number of these channels can usually be set by the operator (typical number of channels ranges from 512 to 16,384; powers of two). The choice of channel number depends both on the system resolution and energy range under investigation.

The detector thus produces peaks associated with gamma photons from a specific isotope. The horizontal position of the peak (from less to increasing energy) is determined by the photon's energy and the peak's area by the gamma ray's intensity. The width of the peak is determined by the instrument's resolution, and high-resolution detectors can separately identify the photons with similar energy from two isotopes. The resolution is usually expressed as the full width at half maximum (FWHM). This is the width of the peak at half the highest point. The FWHM may be expressed in absolute terms (electron volts, eV) or relative terms (percentage of

width by height). The efficiency of the detector reflects the ability of the detector to interact with an individual photon. Some escape the detector and none are 100% efficient. Thus, larger detectors are generally more efficient than smaller ones. Lower efficiency detectors therefore take longer to produce a spectrum than more efficient ones.

Scintillation detectors use a crystal that emits light (scintillates) when it interacts with a gamma photon. The intensity of the light produced is proportional to the energy of the impacting photon. The light then interacts with a photomultiplier that converts the visible light photons into an electric signal and amplifies it. The most common detectors of this type are made of thallium-activated sodium iodide crystal (NaI(Tl)). When a photon impacts the detector's atoms, some become excited and emit their own photons. These then strike a photocathode that releases an electron. The released electron then impacts a dynode and causes the release of several more electrons. This sequence continues until the electrons impact the last dynode and register a voltage pulse across external resisters. By this process of "photomultiplication," the original photon of high energy is transformed into multiple electrons of lower energy that can be measured. The intensity/number of these final electrons is dependent on the energy of the original photon. The detectors therefore measure the number of gamma photon impacts from a sample, and also the energy of each one. In this way they can differentiate between the different gamma emitting radionuclides. Advantages of this crystal type include the relative ease with which large crystals may be manufactured and the generally more intense bursts of light produced compared to other crystals.

Semiconductor detectors use a fundamentally different system. In essence, incoming photons impact electrons in a semiconductor. The movement of electrons results in an electric signal that is then carried to the MCA, which consists of an analog to digital converter (ADC) and a counter. Much more so than crystals, semiconductors are finely sensitive to the energy of the impacting photons and thus have greater resolution. On the other hand, they require cryogenic temperature to work properly and so are not as convenient to use. They are also not as sensitive as scintillation detectors. The most common detectors of this type use lithium-drifted germanium (Ge(Li)) semiconductor material. The germanium version is recommended by the EPA because of its higher photon resolution and large size availability.

For analysis, a liquid sample is poured into a specified container and placed in the counting chamber until instrument-sensitivity requirements are met. Solid samples may be simply placed in close proximity to the detector.

Quality Control

Quality control is important for analysis, but not as rigorous as for chemical analyses. Quality control requirements differ by method and sample type. A good example of what types are typically required and what purpose they serve is given here.

Blank

The blank is an unused planchet or sample container analyzed at least once per 10 samples. It is done to show that there is no sample contamination or "memory effect" from one sample to the next.

Background Samples

This is a sample (e.g., tap water) having the approximately same amount of total dissolved solids as the collected samples and is treated the same. There is at least one of these run with every 20 samples, and two per batch.

Duplicate Samples

One out of every 10 samples is to be treated and analyzed in duplicate. This is a check of the method's and operator's consistency.

Quality Control Check Sources

This is a dilution of radium from a different source than that used for the calibrations and is a check of the calibration source's accuracy. There are three of these run with each batch of 30 samples.

Laboratory Fortified Blank (Spiked Samples)

The laboratory fortified blank (LFB) is identical to the blank with the exception that known activities of the target radionuclides are also added prior to analysis. Samples may also have known activities added. The purpose is to show that the procedures and equipment are operating properly.

Compared to chemical (and microbiological) analyses, many radiological methods are relatively simple.

This belies the complexity behind the instrumentation, but reflects the level of expertise required by the analyst. One must still be suitably trained in the safe handling and preparation of these samples, and the proper use of sensitive instruments, but many of the pitfalls associated with contamination and resolution are minimized. On the other hand, great care must be taken to ensure the personal safety of the analyst and other laboratory staff because of the potential for nondetectable but cumulative exposure. Radiological analyses fulfill an important niche in public health but are unfortunately rarely recognized away from know sources of contamination such as waste sites, nuclear facilities, and certain geologic formations.

Discussion Questions

1. Of the three types of ionizing radiation, which might be the most difficult to work with, and why?
2. Of the three types of ionizing radiation, which might be the easiest to detect and measure, and why?
3. There are several different elements that are alpha emitters. Given the difficulty associated with identifying a particular radionuclide versus simply measuring gross alpha activity, why would a laboratory bother to identify the specific isotope(s) present?
4. Some molecular biology laboratories use radiolabeled probes (i.e., probes incorporating ^{32}P or ^{35}S). The half life of some of these is measured in days and weeks rather than years. How might a laboratory safely dispose of such probes when their use is complete?
5. Go on the Internet and find two good sources for detailed information on some or all the techniques described in this chapter. They cannot be government-sponsored (.gov) or listed in Additional Resources. Briefly describe what they provide and why they are good sources of reliable information.

Additional Resources

Knoll, G. F. (2000). *Radiation detection and measurement*. Hoboken, NJ: Wiley.

L'Annunziata, M. F. (2007). *Radioactivity: Introduction and history*. Philadelphia, PA: Elsevier Science.

Occupational Safety and Health Administration. (2009). Radiation—safety and health topics. Retrieved November 12, 2009, from http://www.osha.gov/SLTC/radiation/index.html.

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US Environmental Protection Agency. (2009). Clean Water Act Analytical Methods. Retrieved November 11, 2009, from http://www.epa.gov/waterscience/methods/.

US Environmental Protection Agency. (2009). Radiation protection. Retrieved November 12, 2009, from http://www.epa.gov/radiation/.

US Environmental Protection Agency. (2009). SW-846 Online. Retrieved November 1, 2009, from http://www.epa.gov/osw/hazard/testmethods/sw846/online/index.htm.

5

Clinical Testing

Michael Petros and David C. Jinks

Introduction

This is the first of four chapters devoted to discussing the actual tests that public health laboratories (PHLs) perform. The chapters vary somewhat in terms of length, organization, and level of detail. This is because of multiple reasons, including differences in the frequency of disease occurrence, the numbers of samples submitted for analysis, the regulations and agencies that have oversight for sample types, and the relative importance of individual organisms and diseases to the public's health. As was mentioned previously, these chapters are not meant to be exhaustive of all that PHLs do. They are designed to provide the reader a suitable background for why a particular type of sample may be analyzed, the organisms and/or compounds that are the targets of analysis, and the strategies and methods commonly employed for their analyses.

There are several ways in which all the tests a PHL performs could be divided. For the purposes of this text, we decided to divide them based on sample type. Thus, we find chapters discussing clinical (obtained from humans), water, food, and air samples. The regulations and methods associated with these sample types often differ. For example, the US Food and Drug Administration (FDA) approves tests used for the diagnosis of disease in humans and the Clinical Laboratory Improvement Amendments (CLIA) oversees clinical laboratory operations. Food samples, on the other hand, may be analyzed by methods provided by the US Department of Agriculture (USDA), water samples analyzed by methods from the US Environmental Protection Agency (EPA), and air samples from the Occupational Safety and Health Administration (OSHA). Oversight for these sample types varies, but may be based on specific compounds or tests, an individual's performance on a proficiency test, and/or the proficiency of the laboratory as a whole to correctly perform a specific analysis.

For this chapter we will examine nine often-recognizable components of PHL clinical testing. That is, each section will discuss a type of test that is either its own section within a laboratory (e.g., newborn screening) or a recognized activity within a larger section (e.g., influenza within a virology section). The tests were chosen to represent both the breadth of expertise required by PHL staff and the great majority of samples submitted and analyses performed. Analytical techniques include the differential culturing and staining for *Escherichia coli* O157:H7, the dissection of animals for rabies virus, the examination of stool samples for *Cyclospora*, and sophisticated instrumental analysis of blood for elemental lead. While the numbers of samples vary considerably by test, those submitted for analysis for sexually transmitted infections, newborn screening, and blood lead accounted for 85% of all samples received by the Illinois Department of Public Health (IDPH) laboratories from July 2007 through June 2008. These proportions certainly vary by PHL, just as which tests they offer vary, but clinical tests are often the "bread and butter" of PHL activity and represent their greatest impact on individual health.

Tuberculosis

Overview of Tuberculosis

Tuberculosis (TB) is the disease caused by infection with a member of the *Mycobacterium tuberculosis* complex.

While the terms infection and disease are often used interchangeably for many conditions, it is less appropriate here. This is because only ~10% of those with TB infection actually progress to active TB disease with symptoms.[1] The remainder have what is termed latent TB (LTBI), which does not cause disease and cannot be spread. The bacteria do not multiply in the body as the immune system can prevent its spread. Those with compromised immune systems, such as individuals with human immunodeficiency virus (HIV), are at increased risk of developing active TB. This is characterized by such symptoms as bad cough over 3 weeks (possible with blood), chest pain, weakness, loss of appetite and weight, and chills and fever. TB is spread through the air (e.g., in droplets expelled when someone with active TB coughs or sneezes).

There were 13,299 cases of TB reported in the United States in 2007 at rate of 4.4 per 100,000 persons. This is a 3.3% decrease in cases from 2006, and a 50% drop from the peak period in 1992. Cases among foreign-born (not born in the United States) individuals accounted for 58% of cases in 2007 compared to 31% in 1993, and the case rate (number of cases per 100,000 persons) is almost 10 times higher than for US-born individuals. The proportion of patients with primary multidrug-resistant TB (MDR TB) was 1.1%, and two cases of extensively drug-resistant TB (XDR TB) were reported.[2] It is estimated that almost one third of the world's population (~2 billion people) are infected with TB, resulting in ~2 million deaths annually. Over 9 million people become ill with TB each year, and it is the leading cause of death for those infected with HIV.[3]

Vaccine

The bacille Calmette–Guérin (BCG) is a vaccine for TB. However, it is not often used in the United States because of its variable effectiveness in adults, the potential for causing a positive skin test for TB (false positive for infection), and the generally low prevalence of TB. It is used in other countries with higher prevalence of TB to prevent childhood tuberculous meningitis and miliary disease (where the disease is disseminated into the body and presents multiple small nodules). In the United States, BCG use is considered for:

- Children who test negative but are continually exposed to adults who are incompletely treated for TB infection or who are infected with drug-resistant strains, and;
- Healthcare workers who work with a large number of patients infected with drug-resistant strains or work in areas where transmission of infection is poorly controlled.[4]

The vaccine is also specifically contraindicated for those who are, or might become, immunocompromised and those who are pregnant.

Treatment

LTBI treatment in high-risk individuals is part of the overall strategy of TB elimination, and does much to prevent active disease if the regimen is completely followed. Not all individuals with LTBI undergo treatment, and they may be monitored for future progression to active TB. Those with active TB, and individuals with LTBI and a high risk of developing active TB, are subject to extensive treatment regimens with such drugs as isoniazid, rifampin, and/or pyrazinamide. Treatment regimens typically last many months. Incomplete treatment contributes to the development of drug-resistant strains of TB. MDR TB is resistant to at least isoniazid and rifampin, the two most commonly used for treatment. XDR TB is resistant to these, fluoroquinolones, and at least one of three injectable drugs (e.g., kanamycin). Further discussion of treatment is beyond the scope of this section, but more information may be found at http://www.cdc.gov/mmwr/PDF/rr/rr5211.pdf.

Sidebar 5-1 Tuberculosis Elimination[5–7]

In order to address this in the United States, the Centers for Disease Control and Prevention (CDC) has taken the lead of the TB elimination effort. The goal is the elimination of TB in the United States by 2010 and, although total numbers of cases and case rates are declining, as of this writing it would appear the goal will not be met. The elimination plan is centered around six goals based on recommendations made by the Institute of Medicine (IOM) in its report *Ending Neglect*. These goals are:

1. *Maintain control of TB* through the maintenance of an active program of TB diagnosis, treatment, investigation, and prevention.
2. *Accelerate the decline* of TB through the use of targeted testing and resources, rapid recognition and response, and advanced analysis techniques.
3. *Develop new tools* for the diagnosis, treatment, and prevention of TB.

4. *Reduce the global burden of TB* by increasing US participation in worldwide identification, treatment, and prevention activities.
5. *Mobilize and sustain public support* by engaging different public and private partners in sustaining health communication campaigns focused on TB elimination.
6. *Track progress* toward the goal of TB elimination using collected and reported data and share such data with partners, target audiences, and interested parties.

Globally, the Stop TB Project is hosted by the World Health Organization (WHO) and has the continuing targets of reducing the global burden of TB by 50% relative to 1990 levels and the elimination of TB as a public health concern by 2050 (less than one case per million people). To do this, the Project has the goals of increasing access to accurate diagnoses and effective treatment regimens, increasing the supply and affordability of quality medications, devising new strategies to address MDR TB and HIV-related TB morbidity, and promoting the development of new treatments, tests, and vaccines.

The posters shown in Figures 5-1 and 5-2 are examples of promotional materials created by the CDC. Note that one highlights March 24 as World TB Day. This day is observed each year to commemorate the date in 1882 when Dr. Robert Koch announced the discovery of *Mycobacterium tuberculosis*. The designation of this day provides an opportunity for those working in TB control to discuss issues concerning TB control and elimination with potential partners and to raise general awareness of the disease and its impact.

Organism Characteristics

M. tuberculosis is a Gram-variable, nonmotile, rod-shaped, obligate aerobe and is shown in Figure 5-3. It is a facultative intracellular pathogen preferring cells with higher oxygen content (e.g., lung tissue) and is also characteristically slow growing. It is not classified as either Gram-positive or Gram-negative as it does not possess the chemical compounds upon which the Gram staining technique is based. There are actually four relatively common *Mycobacterium* species known to cause TB in humans and animals that form the tuberculosis species complex. *M. tuberculosis* is primarily pathogenic in humans, whereas *M. bovis* may be transmitted to humans from infected animals, *M. africanum* is primarily pathogenic in Africa, and *M. microti* infects animals. It is important to be able to differentiate these species should they appear together in a sample. Other species are ubiquitous in nature and approximately 15 mycobacterium other than tuberculosis (MOTT) are potentially pathogenic to humans. Examples include *M. kansasii* and *M. abscessus*.

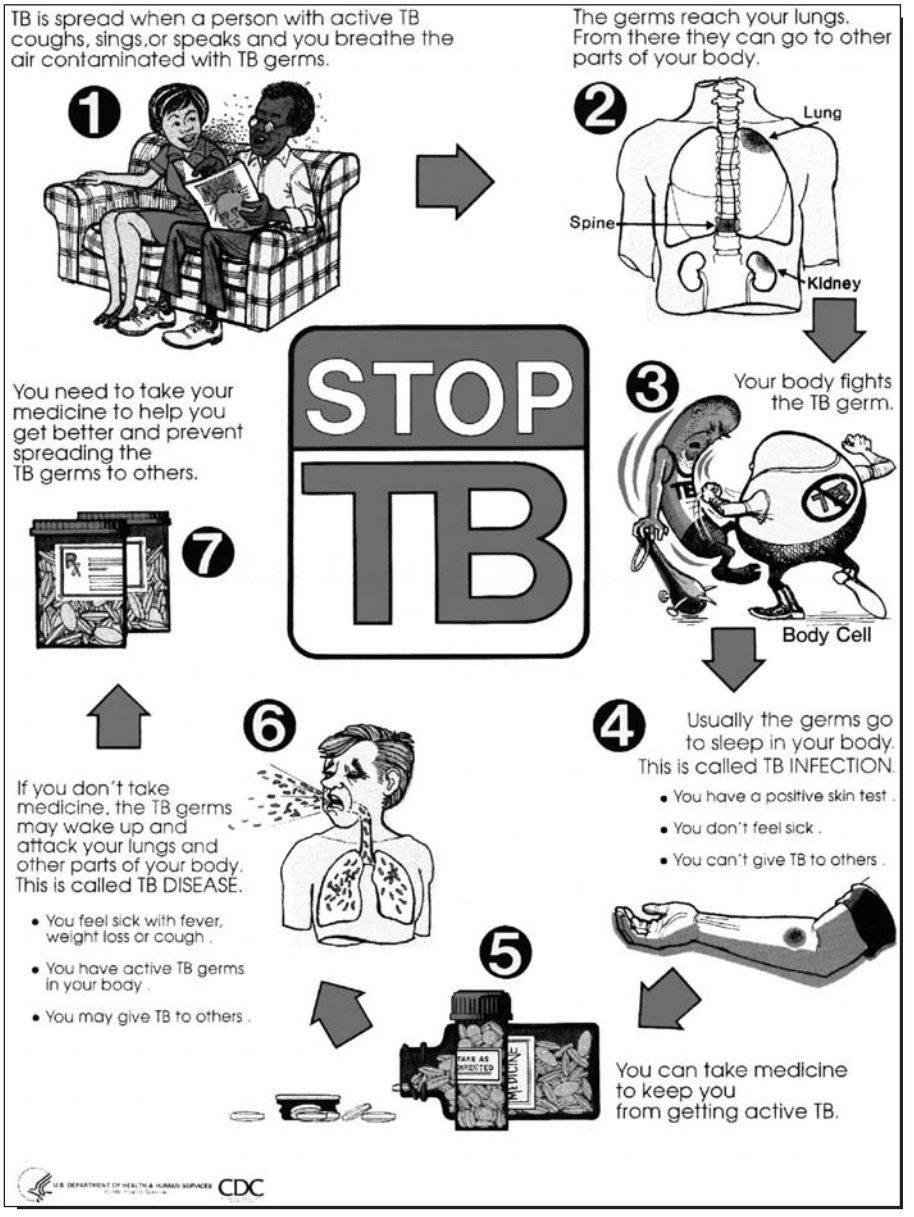
Sample Collection and Analysis

Sample Collection

It is important to note that analysis for TB presents a level of potential harm to the analyst not often seen with other samples. Most other infectious organisms tested for in a PHL are either not transmittable via aerosol, are treatable with common antibiotics, or both. The risk is therefore relatively small for a chance of infection via contact with a sample and laboratories typically operate in a biosafety level 2 (BSL 2) environment, with many activities occurring in BSL 1. However, given the nature of TB transmission (via aerosol) and the potential for exposure to MDR and XDR strains, the CDC recommends the use of a BSL 2 facility for all nonaerosolizing operations and the use of a biosafety cabinet (BSC) for any aerosol-generating operations. If the sample(s) is from a known or suspected source of XDR TB, BSL 2 facilities using BSL 3 practices are highly recommended. Finally, the culturing and propagation of known XDR TB strains requires the use of BSL 3 facilities and enhanced practices.[8] Details of laboratory biosafety levels are discussed in Chapter 10.

Although there are many potential types of samples collected for analysis, sputum samples are the most prevalent. As mentioned previously, *Mycobacterium* spp. prefers higher oxygen content conditions for growth and samples from the lung area are much more likely to contain detectable numbers of such organisms. There is the potential risk of exposure to the clinician collecting the sample as the patient must expel sputum into the collection container. While the sputum itself does not present a higher risk of exposure, the coughing that often accompanies sputum expulsion does. It is recommended that such samples be collected away from other people and outside if possible. Typically, samples are collected on three successive mornings when the sputum concentration is expected to be highest. However, circumstances may dictate the spot-morning-spot schedule where samples are collected on two clinic visits and the morning in between.[9] Other potential samples include bronchial wash, urine, wounds, stool (smear testing only because of excessive flora), cerebrospinal fluid (CSF), blood, bone marrow, and other bodily fluids.

Figure 5-1 A Stop TB poster from the CDC's Tuberculosis Elimination. (Courtesy of CDC/Division of Tuberculosis Elimination.)



Onsite Testing

There are two commercial tests available to determine if a person is infected with TB. One is the Mantoux tuberculin skin test (TST). This test consists of an intradermal (into the skin) injection of tuberculin fluid (0.1 ml tuberculin purified protein derivative [PPD]) into the forearm. The individual then returns after 48–72 hours for observation. Interpretation is based on the size of any apparent induration and the individual's personal risk of infection. An induration as small as 5 mm may be considered positive for those at increased risk of infection, while an induration of 15 mm is considered positive for anyone. False positives are possible and may be associated with poor technique, previous vaccination with BCG, or infection with nontuberculosis mycobacterium. False negatives may be associated with very recent or old infection, overwhelming infection, poor technique, and other live virus vaccinations or infections.[10]

The QuaniFERON®-TB Gold test is an FDA-approved test for the detection of latent and active TB infection in blood. A blood sample is collected and mixed with two synthetic peptides that represent two proteins

Figure 5-2 A World Tuberculosis Day poster promoting TB elimination. (Courtesy of CDC/Division of Tuberculosis Elimination.)

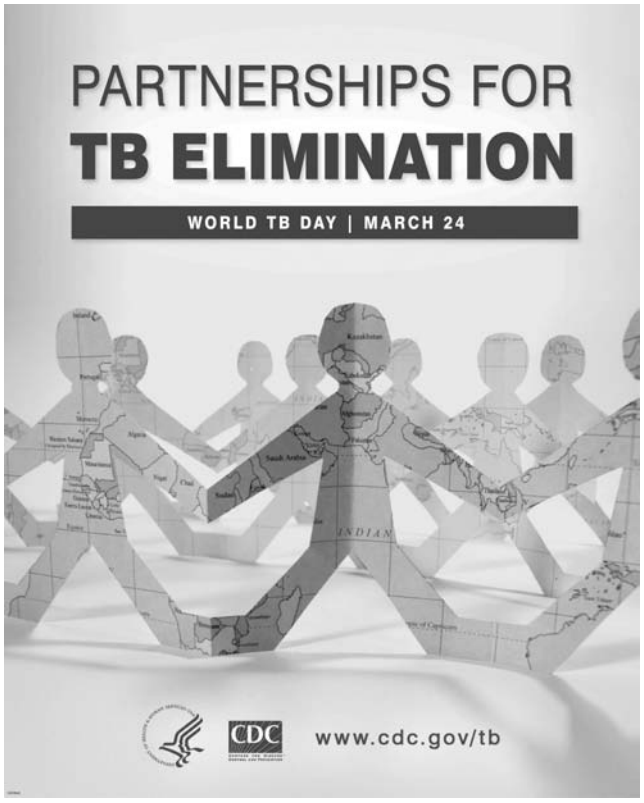
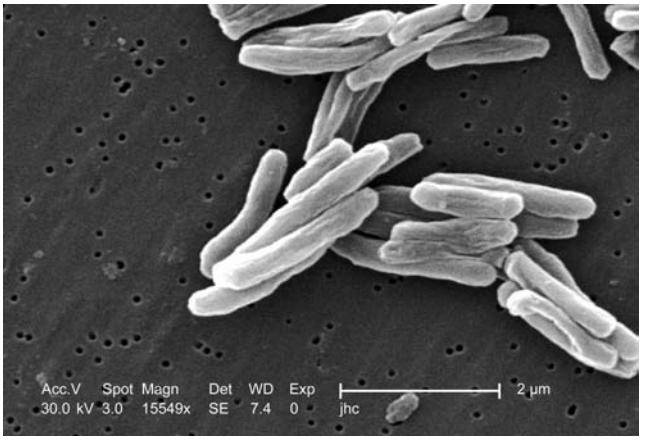


Figure 5-3 Scanning electron micrograph of *Mycobacterium tuberculosis* (15,549×). (Courtesy of CDC/ Dr. Ray Butler.)

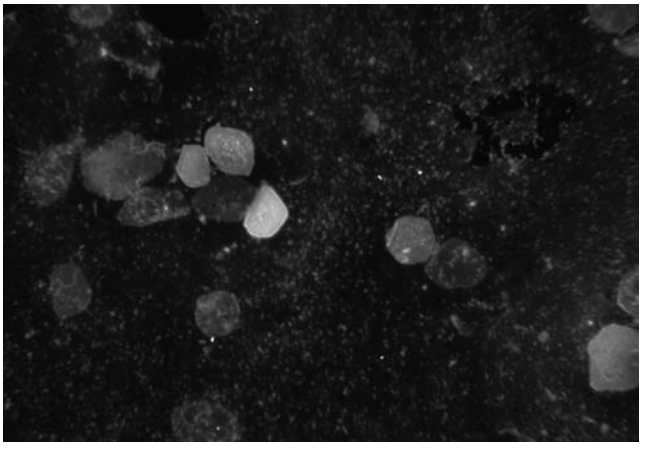


expressed by *M. tuberculosis*. White blood cells, which react to these antigens (indicating infection), release interferon-gamma (IFN-gamma) in response. After 16–24 hours of incubation, the amount of IFN-gamma released is measured. This test is not subject to many of the weaknesses of the TST, and not affected by prior vaccination with BCG. Positive tests should be confirmed by further clinical evaluation (e.g., chest radiographs) and laboratory culture.[11] Neither of these tests are regularly performed at state PHLs, but they may be useful for screening or in areas where laboratory services are not available.

Rapid Acid-Fast Bacilli Smear

Sample smearing and staining is usually a first step in *Mycobacterium* spp. analyses. There are several techniques in use. As mentioned previously, Gram staining is not useful in this instance, but a couple of acid-fast stains are used. These are acidic dyes that react with mycolic acids contained in the cell membranes of some organisms. *Mycobacterium* spp. contain these compounds and are therefore considered acid-fast bacilli (AFB). One popular technique uses fluorescent chromophores as part of the acid stain. Auramine-rhodamine stains react with the mycolic acids in the *Mycobacterium* spp. cell membrane and subsequently fluoresce reddish-yellow under ultraviolet (UV) illumination (Figure 5-4). Another method that is less specific is the Kinyoun technique using carbol fuchsin as the primary stain and methylene blue as

Figure 5-4 Sputum smear of *Mycobacterium tuberculosis* using fluorescent acid-fast stain. An image bank of full-color photos is available online at http://www.jbpub.com/catalog/9780763771027/. (Courtesy of CDC/Ronald W. Smithwick.)



the secondary stain. This is a more traditional acid-fast technique and the slide is subsequently viewed using light microscopy. *Mycobacterium* spp. appear as reddish rods. Ziehl–Neelsen (Z–N) stain uses the same stains as Kinyoun, but requires the application of heat to allow the carbol fuchsin to penetrate. It is therefore not used as often as Kinyoun. An example of Z–N staining is shown in Figure 5-5. A disadvantage of both methods is the tendency for other bacteria to stain as well. It is therefore not well suited for sputum samples, but performs quite well on isolates. Commercial kits containing staining reagents are available.

Primary Culture

Samples submitted for the analysis of *Mycobacterium* spp. must be grown so that isolates may be used for identification and drug susceptibility. Two types of solid culture media are used. Middlebrook 7H11 is agar-based and has become the dominant media used. Lowenstein-Jensen is egg-based and has been largely supplanted by Middlebrook 7H11. The use of Middlebrook 7H11 is preferable as growing times are reduced and there is less overgrowth by other bacterium. *Mycobacterium* spp. generally are slow growing and colonies may take 4–6 weeks to appear. Examples of cultures grown with both media are shown in Figures 5-6 and 5-7.

Samples are inoculated into Middlebrook 7H11 two-sectored plates for primary culturing. One side of the plate allows general growth and the other is selective for *Mycobacterium* spp. by the addition of antibiotics. Samples may also be subjected to automated analysis by inoculation into mycobacteria growth indicator tubes (MGIT). These tubes contain Middlebrook 7H9 broth supplemented with a PANTA antibiotic mixture (to inhibit non-*Mycobacterium* growth). The MGIT are incubated in the Becton–Dickinson (BD) BACTEC 960 instrument. This instrument both incubates the tube(s) and observes growth by a fluorescent reaction in the tubes associated with oxygen depletion. This instrument may observe detectable levels of growth within 2–3 weeks, significantly quicker than plate cultures.

Figure 5-5 ***Mycobacterium tuberculosis*** **observed using Ziehl–Neelsen stain. An image bank of full-color photos is available online at http://www.jbpub.com/catalog/9780763771027/. (Courtesy of CDC/Dr. George P. Kubica.)**

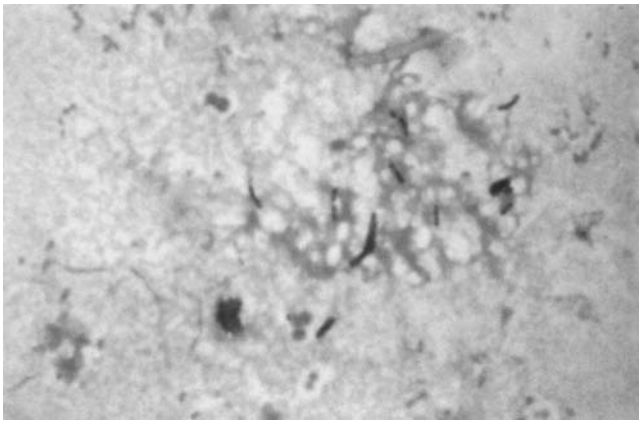


Figure 5-6 **Close view of** ***Mycobacterium tuberculosis*** **colonial morphology on Middlebrook culture. (Courtesy of CDC/Dr. George P. Kubica.)**

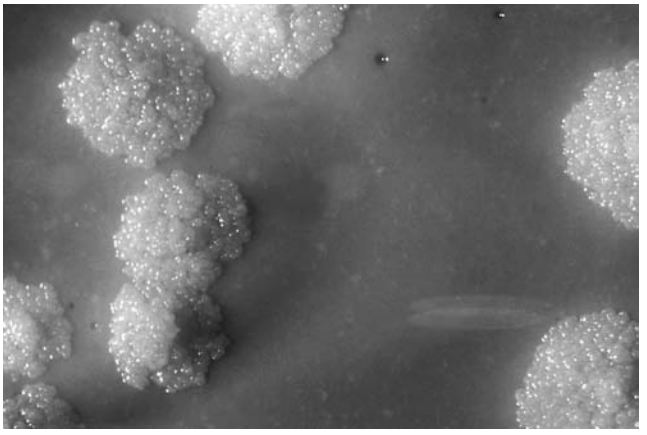
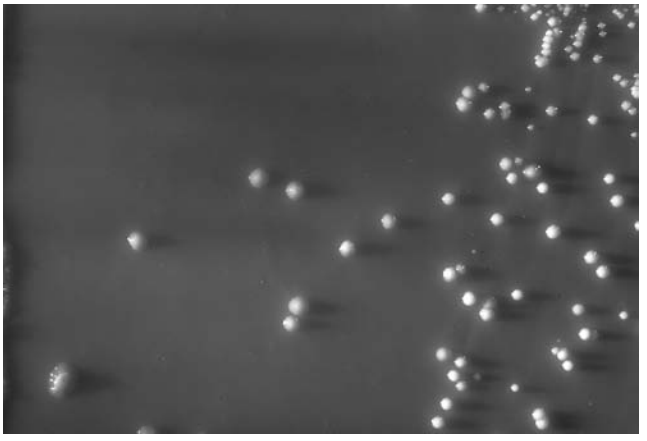


Figure 5-7 **Close view of** ***Mycobacterium*** **spp. colonial morphology on Lowenstein-Jensen culture. (Courtesy of CDC/Dwight Lambe.)**



Identification

Once colonies are grown on media they are subjected to confirmatory analysis. The CDC has made available a standardized method for the identification of *Mycobacterium* spp. that uses high performance liquid chromatography (HPLC) to resolve species-specific mycolic acids. In brief, a suspension of bacteria is treated to release these membrane-bound acids that are then extracted and chemically converted into *p*-bromophenacyl esters (which absorb in the UV range). They are then separated by reversed-phase HPLC using a methanol-dichloromethane gradient and detected via UV absorbance. The method can identify in excess of 22 species.[12] There are also multiple, that is, not standardized, methods of analysis using species-specific compounds and gas chromatography. Techniques include the detection of tuberculostearic acid via gas chromatography (GC) with a flame ionization detector (FID) or mass spectrometer (MS), the conversion of mycocerosic acids into pentafluorobenzyl esters with analysis by GC electron capture detection (ECD), and the chemical treatment of a culture sample and analysis by gas-liquid chromatography with FID.[13–16]

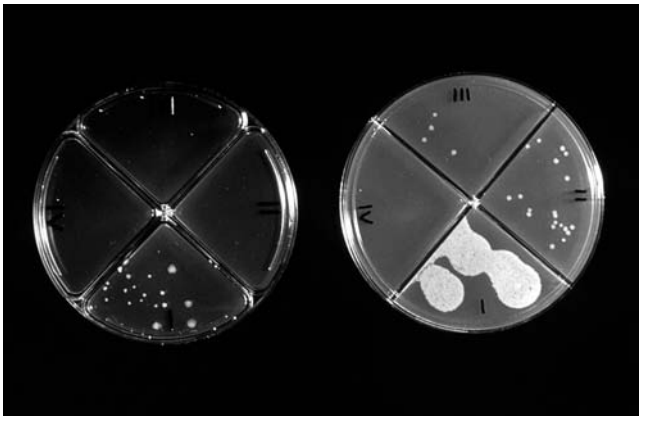
There is also a polymerase chain reaction (PCR) kit that has been FDA approved for use (Amplified MTB; Gen-Probe, San Diego, CA). The CDC recommends that it be used only for swift screening and not a substitute for traditional identification methods (e.g., acid-fast staining and culturing). The sensitivity and specificity for the test varies considerable from lab to lab for unknown reasons. The CDC also recommends testing the first sputum sample, the first smear-positive sputum sample, and other samples as indicated. Laboratories are advised to use this test only upon physician request, only on specific samples, and to deny this test if there is insufficient sample to perform this test in addition to other established methods.[17]

Finally, there are biochemical reactions and tests that may be performed to identify TB species. However, these have become rather antiquated and are seldom used. Those interested in which tests are available and how they are used should consult the CDC reference, *Public Health Microbiology: A Guide for the Level III Laboratory*.

Drug Susceptibility

A critical component of *M. tuberculosis* analysis is the testing of an isolate's drug susceptibility. As briefly described previously, there are increasing numbers of infections with drug-resistant strains. Knowing that an individual is infected with such a strain will not only guide treatment for the individual, but also provide information for epidemiologic purposes. The test itself is relatively simple to perform and there are several options.

Figure 5-8 Example of *Mycobacterium tuberculosis* drug-susceptibility testing. (Courtesy of CDC.)



One option is the inoculation of Middlebrook agar plates to which antibiotics have been added. Isolates from the primary culture are streaked onto the plates and observed for growth (compared to antibiotic-free control agar). Often, two or four different antibiotics may be tested on one plate by splitting it into halves or quarters (Figure 5-8). Such plates are commercially available. Most laboratories only test susceptibility to the first-line antibiotics isoniazid, ethambutol, rifampin, and pyrazinamide. Growth in antibiotic-containing sections indicates resistance. As needed, cultures may be sent to the CDC for full susceptibility testing at their Mycobacteriology Laboratory Branch. A second option is the further use of the MGIT system. Growth obtained 1–2 days after a MGIT tests positive, or an isolate obtained and diluted in saline from the primary agar culture, is inoculated into a series of MGIT tubes. The tubes contain various defined concentrations of the first-line antibiotics, or no antibiotics as a positive control. Growth in an antibiotic-containing tube indicates resistance to that antibiotic.

Reporting

Unlike almost any other analysis conducted by a PHL, the time required for full-sample analysis and results reporting is often measured in weeks, rather than hours or days. The CDC recommends reporting acid-fast smear analysis

within 24 hours of sample receipt, initial identification of *M. tuberculosis* within 14 days, and drug-susceptibility test results within 21 days (for first-line drugs). Analysis for resistance to the other drugs may take an additional 4–6 weeks depending on growth times and if the sample was referred to another laboratory. One can therefore see how the speed of PCR analyses may be of great benefit with identification in as little as 2 days. While these still need to be followed up by culture for drug susceptibility, they allow epidemiologic investigations and healthcare and treatment planning to begin much sooner.

Recent Innovations

The IDPH laboratory in Chicago has made some important innovations in the use of nucleic acid analysis regarding TB. They have developed a PCR protocol for the detection of *M. tuberculosis* in sputum samples from a paper written by Desjardine et al.[18] The test is used to screen individuals whose TB infection has already been established and analyzed and who have obtained medical treatment. A negative result indicates that the medication has been effective in controlling the infection such that the individual is no longer infectious (does not expel TB organisms in sputum). A commercially available DNA kit is used to extract the DNA, which is subsequently analyzed with a laboratory-developed PCR protocol. This test is used as a screen of treatment effectiveness only and samples are not further cultured or tested.

Another innovation by the IDPH laboratory is in the use of PCR to identify *Mycobacterium* spp. directly from sputum. It has recently begun using a molecular-based testing algorithm for the speciation of *Mycobacterium* spp., replacing standard testing procedures such as HPLC, biochemical reactions, and GC. Acid-fast cultures are first tested using the Innogenetics Line Probe Assay (INNO-LiPa) that is able to identify the *M. tuberculosis* complex (MTC) and 16 clinically relevant nontuberculous *Mycobacterium* in a single test. The assay is based on nucleotide differences in the 16S-23S ribosomal RNA spacer region. Cultures identified as the MTC are further tested using an in-house developed PCR assay that distinguishes among *M. tuberculosis*, *M. bovis*, and *BCG bovis*. Mycobacterium cultures that cannot be identified by the LiPa are tested by sequence analysis of the 65-kilodalton heat-shock protein gene (*hsp65*) for final identification.[19] This test is faster, cheaper, and more specific than more traditional methodologies.

TB testing has been, and will likely remain for the foreseeable future, an important aspect of PHL work. For example, the IDPH laboratories received 10,383 mycobacterium samples from July 2007 through June 2008 (unpublished data). The overview of TB showed that the majority of the cases diagnosed in the United States are from those who are not citizens. The nature and ease of global travel means that there will continue to be an influx of TB into the United States and we must therefore remain vigilant. TB elimination abroad faces tough challenges with logistics and funding, and the inconsistency and incompleteness of many treatments contribute to the increasing numbers of infections with multiple and extremely drug-resistant strains.

Influenza

Overview of Influenza

Influenza is a common, contagious respiratory illness that is endemic throughout the world. Most presentations are mild, but some may be severe and even fatal. There are several different terms used when referring to influenza infection, and each has distinct meaning. *Seasonal flu* is that which infects humans on an annual basis, with anywhere from 5 to 20% of Americans becoming ill. Children are typically two to three times more likely than adults to become ill, and the elderly are also at increased risk. Although the majority of people recover without treatment, there are approximately 200,000 hospitalizations and 36,000 deaths annually.[20] *Pandemic flu* refers to a virulent strain that spreads rapidly from person to person and is identified worldwide. Finally, *avian* (or bird) *flu* refers to those strains that infect primarily waterfowl and do not usually infect humans. However, these may infect other animals (e.g., pigs) that can also be infected with human strains. Pigs are thus considered intermediate hosts.[21]

The season for illness in the United States runs from late fall through winter, though there are exceptions. A community outbreak usually begins suddenly and peaks within 3 weeks. Subsidence occurs within another 3–4 weeks unless another wave is introduced from outside. Because schools are places where large numbers of people are congested for extended periods of time, children are especially infectious and families with children experience more illnesses than families without children. Common symptoms include dry cough, headache, tiredness, high fever, sore throat, runny nose, and muscle aches. Some of the serious complications include shaking chills, chest pain while breathing, productive cough, bacterial pneumonia, ear and sinus infections, dehydration, and worsening of

existing chronic conditions. Infected individuals are capable of spreading infection up to 1 day before symptoms appear to 5 days after becoming ill. Transmission occurs when an infected person sneezes or coughs, expelling small droplets that are then inhaled or touched (followed by hand-to-mouth action) by someone else. Treatment is currently available through four medications: amantadine, rimantadine, zanamivir, and oseltamivir. Recently, the CDC has recommended against taking amantadine or rimantadine. While these two drugs have the benefit of being effective against both A and B influenza types, there are concerns of developing resistance by influenza A.[22,23]

Diagnosis on the basis of symptoms alone is difficult if the presence of influenza in the local community has not been established. Initial symptoms may resemble those of other infectious agents such as *Mycoplasma* pneumonia, adenovirus, rhinovirus, and *Legionella* spp. Once identified in the community, however, providers seldom wait for laboratory results for a diagnosis and treat individuals presumptively. Health officials do monitor select clinics (sentinel sites) and some samples are still collected and sent for analysis to provide surveillance on current influenza strains and their extent. The CDC recommends that testing be done initially to determine the presence of influenza in a given population, but later reserved for patients at increased risk for complications, or where such testing would significantly guide treatment regimen.

Sidebar 5-2 The 1918 Pandemic[24,25]

The deadliest and most widespread infectious disease epidemic in the past 100 years was the 1918–1919 "Spanish" influenza A H1N1 pandemic. This epidemic presented in three waves through the world population and is estimated to have killed up to 50 million people. In the United States, influenza killed an estimated 675,000 individuals. This is 10 times the number of US fatalities in WWI, and half of those were caused by influenza and not combat. Approximately 25% of the United States and 33% of the world's population (~500 million people) were at one time infected. The pandemic was also notable for its severity, with a case fatality rate in excess of 2.5% compared to less than 0.1% for others, and its appearance in three distinct waves in a single year. The mortality rate in India was especially severe, reported at ~5%. The impact of this pandemic continues as all influenza A pandemics since then have been caused by descendants of the 1918 strain.

Somewhat counterintuitively and different from any other known outbreak, some of the individuals at greatest risk for serious morbidity and death were those usually considered at lowest risk: young and healthy adults. Historically, a graph of influenza mortality rate versus age shows a "U" shape with the very young and elderly at increased risk of death (with rates in excess of 2%). The 1918 pandemic showed a distinctive "W" pattern. While the very young and elderly were still at increased risk, there was a middle peak as well corresponding to 25–34 year age group. Fatality rates for this group were ~1%, compared to ~0.2% for those aged 5–14 and 0.4% for those aged 45–54. This is more than 20 times greater than rates for previous years for this group and represented almost half of all influenza deaths. The cause for the relatively severe pathogenicity in this otherwise healthy age group is still unknown but under investigation. Research continues, and public interest is high as it is estimated that the emergence of a virus with pathogenicity similar to the 1918 strain would likely kill in excess of 100 million worldwide.

Organism Characteristics

Influenza is a disease caused by an infection with viruses belonging to the orthomyxovirus group. They are enveloped, single-stranded RNA viruses and there are three major serotypes (A, B, and C). Types A and B are comprised of eight separate segments of RNA and usually have a round shape. Type C influenza virus has seven RNA segments. There are two major surface proteins on an influenza A virus that protrude from the surface and give it a distinctive "spiked" appearance. Hemagglutinin (HA) assists the virus in attaching to and infecting a host cell. Neuraminidase (NA) enables new viruses to exit their host cell and spread within the respiratory tract.[21] Type A viruses are found in a wide variety of animals and humans and are responsible for most serious illnesses and epidemics. Type B circulates widely in humans, may cause an epidemic, and causes a milder illness. Type C is similar to type B, but also circulates in some animals and does not cause epidemics.

While all three types occur in various strains (existing and newly emerging), only type A is subtyped according to the type of surface proteins present. Type A is also the only type to infect birds. To date, scientists have identified 16 different HA types and 9 NA types.[26]

Strain nomenclature is based on type, the place where it was first isolated, a lab identification number, the year of discovery, and, in the case of influenza A viruses, its types of HA and NA. An example is A/Hong Kong/156/97 (H5N1), which refers to the Hong Kong flu discovered in 1997 with H5 and N1 types.

Avian influenza A strains are further classified as either low or highly pathogenic (LPAI or HPAI). This is based on specific molecular and pathogenesis criteria. Most LPAI viruses cause mild illness in poultry, but HPAI may cause severe illness and death. Contrary to the general rule, the H5N1 causes no illness in ducks though it is otherwise considered a HPAI. The avian influenza subtypes H5N1, H7N7, and H7N3 have been associated with HPAI. Human infection with these strains has ranged from relatively mild to severe (H5N1 and H7N7). Humans have been infected with LPAI (e.g., H9N2) with very mild to moderate illness.[26]

Avian influenza usually refers to influenza A viruses found in birds, but they occasionally infect humans as well. Birds host all known influenza A subtypes. In recent years, avian influenza has come to be synonymous with the H5N1 strain. This is highly contagious among birds and can be deadly to them. There have been cases of transmission to humans, and these are thought to be the result of close contact between humans and birds and bird waste (e.g., poultry farms and markets). This virus has also caused the largest number of detected cases and severe disease among humans of all avian viruses. The overall case fatality rate for known infections is in excess of 50%, though this varies considerably through time and geographic area. Human-to-human transmission is so far quite rare and limited to very close family contact. There are three known A types circulating in humans as of this writing (H1N1, H1N2, and H3N2) and it is thought that some of the genetic components came from birds.[22] The three prominent subtypes known to infect both birds and humans are: H5, H7, and H9. A transmission electron micrograph (TEM) of the novel H1N1 first identified in the United States in April 2009 is seen in Figure 5-9. Figure 5-10 shows finer structure details in a group of unspecified influenza virions.

Influenza type B viruses are usually only found in humans and are not further classified into subtypes. Illnesses caused by infection with B viruses range from mild to severe (fatal), epidemics are generally less severe, and they do not cause pandemics. Likewise, type C viruses are not subtyped, do not cause epidemics, and cause only mild illness.

Figure 5-9 Transmission electron micrograph of novel H1N1 (in tissue) with visible surface morphology. (Courtesy of CDC/C. Goldsmith and D. Rollin.)

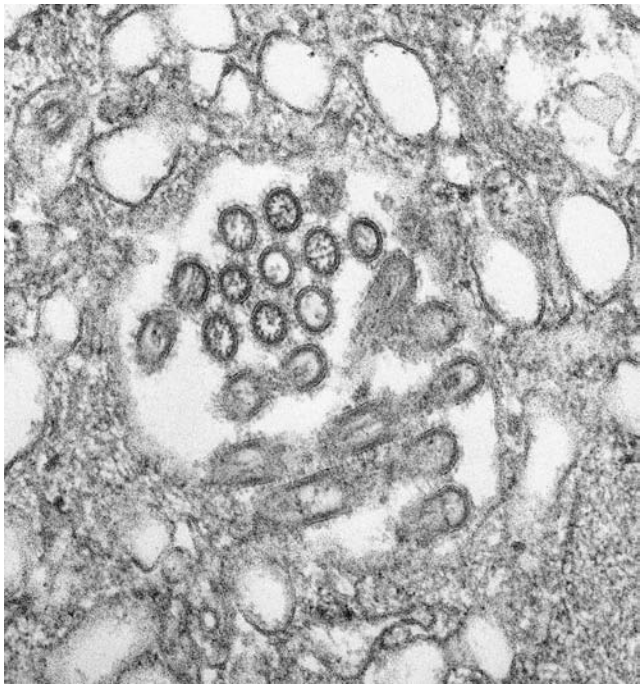
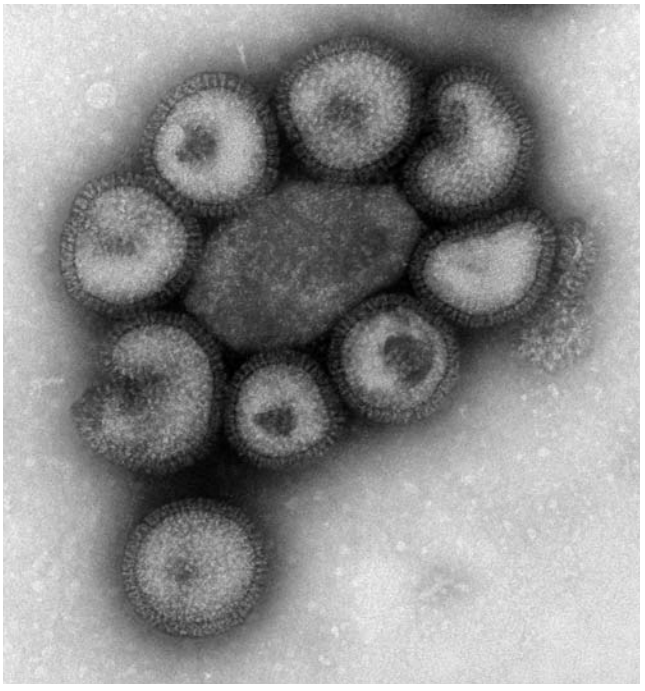


Figure 5-10 Transmission electron micrograph of influenza virions showing fine structural details. (Courtesy of CDC/Dr. F. A. Murphy.)



Genetic Drift, Shift, and Reassortment

There are a couple methods whereby a given influenza virus may significantly change. The first of these is simply *genetic drift*. This is a point mutation in the two genes that code for the surface glycoproteins hemagglutinin and neuraminidase. Of course, this mutation must not be lethal for the virus. This change may be significant enough so that a host's immune system may no longer recognize it as an invader. At this point, the virus attains a higher degree of virulence because of the change in appearance. Genetic drift occurs in both A and B types.[27] It is because of this drifting that new vaccines must be made each year because the body's immune system does not recognize the new virus, though the change may be quite small. Figure 5-11 illustrates this process.

Genetic shift is much more dramatic, and usually more significant. It occurs only in A viruses. In this instance, two different strains of A type influenza infect the same animal and their genetic material becomes mixed. This is also known as genetic *reassortment* because the basic genetic material (8 RNA strands) for each virus replicate in the same cell and mix as they become a new virus particle. This creates a potentially new, virulent virus. If the product is a new strain that is able to jump from one species to a new one, it is termed antigenic shift.[28] There is the possibility of a new pandemic if the new virus can be introduced/remain in humans, cause serious illness in humans, and be easily transmitted from person to person. There are three mechanisms for antigenic shift according to the National Institute for Allergies and Infectious Diseases (NIAID):

- Antigenic shift 1: An intermediate host acquires both an avian and a human strain of influenza A, the viruses infect the same cells, their genetic components mix, and the resultant new virus is able to be passed back to humans.
- Antigenic shift 2: An avian strain spontaneously jumps from birds to humans without genetic change.
- Antigenic shift 3: An avian strain spontaneously jumps to an intermediate host, and from there to humans, without genetic change.

Figure 5-12 illustrates this process.

Sample Collection and Analysis

Laboratory tests for influenza virus include reverse transcriptase (RT)-PCR, viral culture, immunofluorescence, serology, rapid antigen testing, and multiplex microbead assays. These tests are done for preliminary identification, confirmation analysis, and viral subtyping. There are multiple rapid tests that may be performed onsite in healthcare settings and homes for preliminary diagnosis. These tests do not meet the surveillance needs of confirmed identification and subtyping, but may be useful for initial screens and to guide individual treatments.

Sample Collection

Samples should be taken within 3 days of symptom onset and usually include nasopharyngeal aspirates and swabs, nasal and throat swabs, and nasal washes. Blood samples (3–5 ml) may also be used. Nasopharyngeal aspirates and blood samples are especially useful for laboratory analysis of avian influenza A.[29]

Onsite Testing

Rapid commercial tests are those that may be performed onsite in healthcare settings, or even in homes, within 15–30 minutes. The reliability of these tests varies, and not all are able to distinguish between virus types. They may detect A only, A and B without distinction, or A and B while differentiating between the two. None provide subtyping information, which is important for surveillance purposes. In addition, their sensitivity and specificity are generally poor compared to laboratory methods such as viral culture, leading to false negatives and false positives depending on the local prevalence. These parameters may also vary based on the type of sample used for analysis. In general, test sensitivities are in the range of 50–70% and specificities are 90–95%. Physicians and healthcare workers should be cognizant of the test's positive predictive value (PPV) and negative predictive value (NPV) and how these relate to the local disease prevalence when interpreting results.[30]

As mentioned previously, these tests are not routinely performed once influenza has been established in the community. From that point, diagnosis is usually based on the presentation of symptoms. Random testing may still be performed to guide treatment (e.g., the use of antivirals). However, the culture and RT-PCR laboratory tests are considered the reference standard against which all other tests are based. These two and other laboratory tests are performed continuously during and between outbreaks to survey the types of infection present and their extent.

RT-PCR is done via disruption of viral shells and analysis of released genetic material. The influenza RNA is transcribed into its corresponding complementary DNA using transcriptase and influenza-specific primers.

Figure 5-11 Chart illustrating the process of genetic (antigenic) drift. (Courtesy of National Institute of Allergy and Infectious Diseases: http://www3.niaid.nih.gov/NR/rdonlyres/A68ECEB4-8292-479B-B33F-FAEEDBAC7BBC/0/AntigenicDrift_HiRes.jpg.)

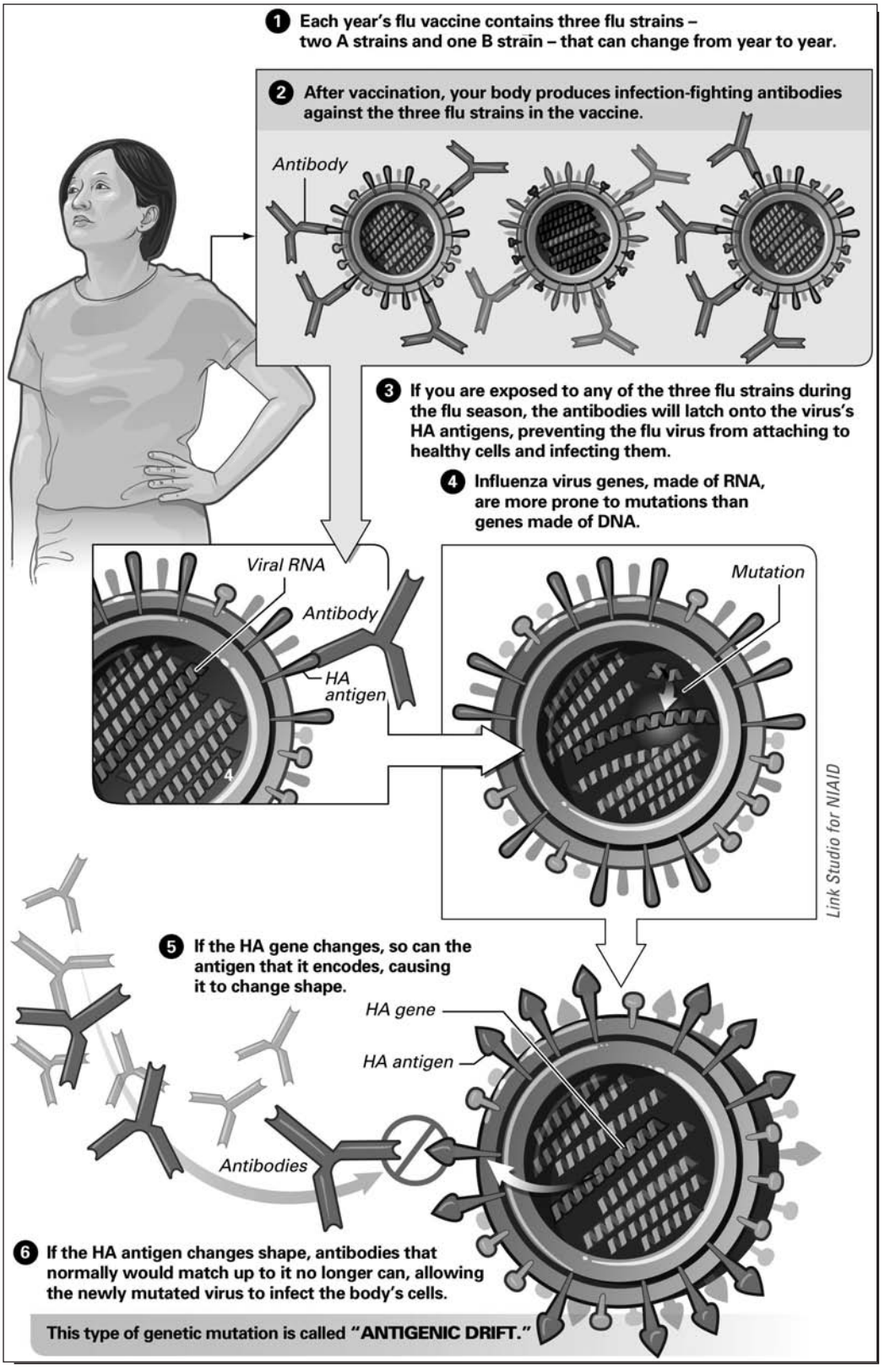
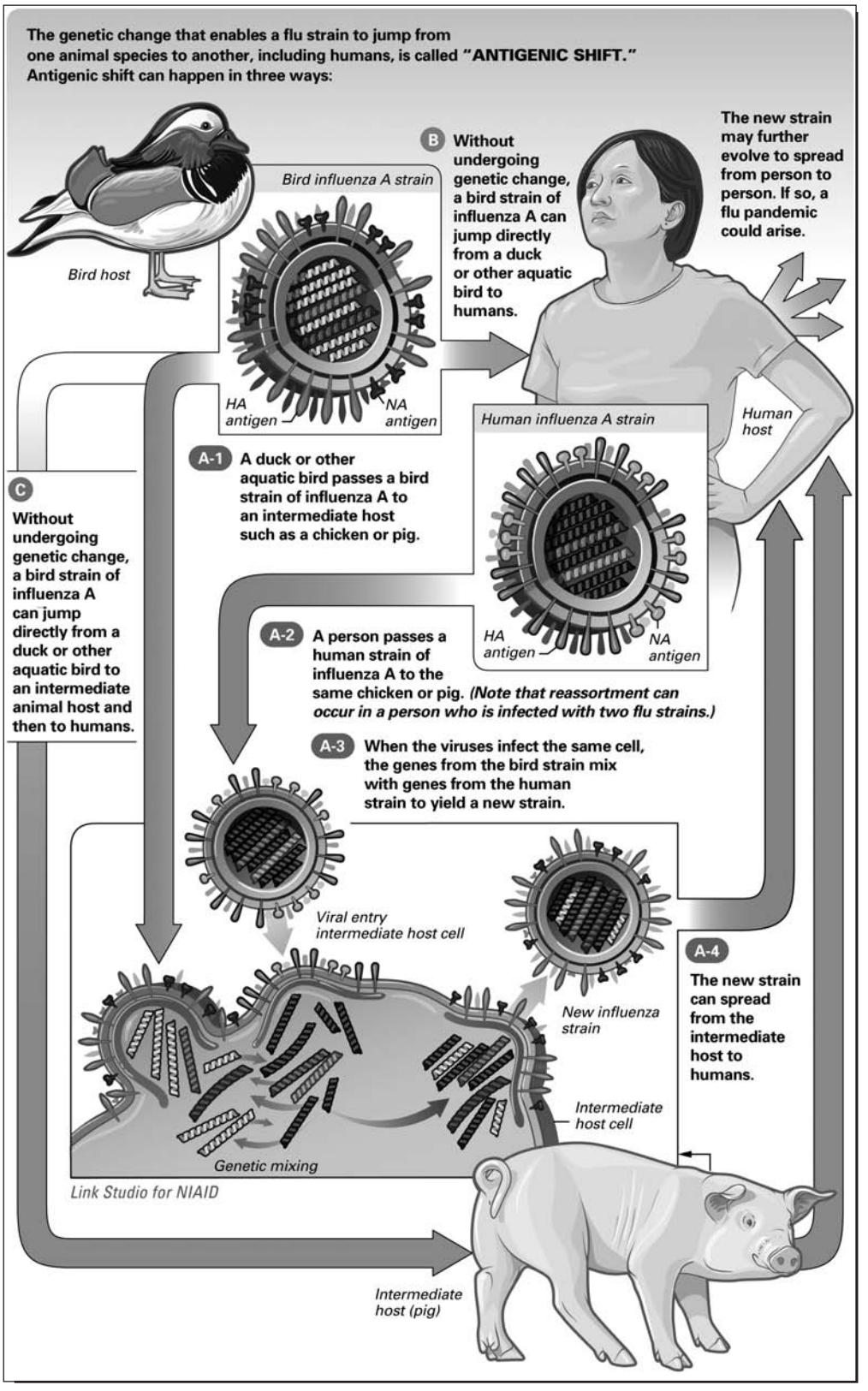


Figure 5-12 Chart illustrating the process of genetic (antigenic) shift. (Courtesy of National Institute of Allergy and Infectious Diseases: http://www3.niaid.nih.gov/NR/rdonlyres/3D377A8B-747F-480A-832E-02A8ED9D1B3C/0/AntigenicShift_HiRes.jpg.)



Presence of influenza corresponding to the primers results in the synthesis of cDNA, which is subject to amplification and detection. Further details of this, and other tests described, were provided in Chapter 2. Reagents for RT-PCR analysis may be obtained in commercial kits such as the QIAamp Viral RNA Mini Kit.

Viral culture is performed through the inoculation of cell lines with the suspected virus. Commonly used cell lines include primary rhesus monkey kidney and Madin-Darby canine kidney (MDCK) cells. Reagents for analysis may be obtained from the WHO (Influenza Reagent Kit), the CDC, or commercially. Viral replication may be detected by hemadsorption with guinea pig erythrocytes, and viral presence may be directly observed by immunofluorescence of infected cells. The influenza virus (A or B) isolated may be typed or subtyped by hemagglutination (HA) and hemagglutination inhibition (HAI) with specific reference antisera.

Immunofluorescence (IF) staining, both direct and indirect, may be performed on either infected cell lines (discussed in viral culture) or on clinical samples. Reagents may be obtained from the WHO (Influenza Reagent Kit), the CDC, or commercially and contain either fluorescein conjugated type-specific antibodies or unlabeled antibodies and a fluorescein isothiocyanate (FITC) conjugate.

Other test methods include *serology assays* that are based on the use of the HAI and enzyme immunoassays. *Neutralization* and *microneutralization* tests are less commonly used. *Microbead assays* (e.g., Luminex xTAG Respiratory Viral Panel) are also becoming increasingly useful, and samples may be tested for influenza and other viruses in a single analysis.

As with many other testing sections, those tasked with testing samples for influenza may develop testing algorithms designed to meet the current testing needs. For example, routine sample screening may consist of RT-PCR for both influenza A (universal; swine and human) and B. A sample negative for A and B may be referred for viral culture and analysis for other respiratory viruses such as adenovirus and respiratory syncytial virus (RSV). On the other hand, a sample positive for influenza B is reported as such, while a positive for influenza A is further tested for the presence of human H1 or H3. However, these testing patterns are very much open to change as the current influenza strains change, reagents are available, and time and instrument constraints because of high sample volume. An example is the emergence of novel (swine) A H1N1 in 2009. For the IDPH laboratory in Chicago, they changed the screening test from universal A and B to universal A and swine A (dropping B). A universal A positive but swine A negative was followed by analysis for human H1 and H3. A universal positive and swine positive was followed by analysis for swine H1. As time passed and there were few cases of human A, the screening test was altered again to test for swine A and swine H1 (dropping universal A). A sample negative for these two would be followed by a test for universal A. As the number of sample submissions have dropped, they have again incorporated universal A and B into the screening tests. This situation is an excellent example of how PHLs must often change their practices to adapt to current situations.

Sidebar 5-3 WHO Influenza Surveillance Network[31,32]

The WHO conducts constant influenza surveillance worldwide through its Global Influenza Surveillance Network, allowing it to recommend influenza vaccine content twice each year. Because of the constantly shifting nature of influenza, vaccines must be tailored each year to the predominant strains. The Network was established in 1952 and is comprised of the WHO, national influenza centers (NICs), and WHO collaborating centers (CCs). There are currently 122 institutions in 94 countries that are recognized as NICs, and they collect samples from patients with flulike illness. There are four in the United States (California, Georgia, Michigan, and New York). This number and distribution provide a global scope of sample collection, and each year they collect ~175,000 samples and submit ~2000 virus isolates to CCs.

The five CCs (one each located in Australia, Japan, the United Kingdom, and two in the United States—at the CDC in Atlanta, GA, and St. Jude Children's Research Hospital in Memphis, TN) provide antigenic and genetic analyses for influenza change surveillance as well as provide serological analyses to determine the effectiveness of vaccines to induce a satisfactory immune response. During September 28 to November 29, 2008, for example, WHO and US National Respiratory and Enteric Virus Surveillance System collaborating laboratories tested 24,657 samples for influenza. Only 282 were positive for influenza A (of those subtyped, 112 were H1 and 16 were H3) and an additional 83 for B were positive for influenza A. The CDC antigenically characterized 30 influenza isolates during the 2008 to 2009 flu season. Twenty were A H1N1, three were A H3N2, and seven were B. Twenty-seven of these were found to be antigenically related to the 2008–2009 vaccine.

Influenza testing remains one of the critical capacities of PHLs nationally. While the actual numbers of samples submitted may fluctuate widely on an annual basis, the need for capacity is consistent. For example, the IDPH laboratories received only 1085 samples for influenza analysis from July 2007 through June 2008. In the first half of 2009, after novel H1N1 become a widespread concern, the laboratories were receiving upward of 600 per week (unpublished data). While the current strains of influenza are unlikely to be as deadly as the 1918 outbreak, the potential remains for a similar strain to emerge. Influenza surveillance and testing capacity are therefore considered a preparedness response on the same par as anthrax and plague.

Rabies

Overview of Rabies

Rabies is a viral disease most often transmitted through the bite of an infected animal. Interventions for this disease have proven quite effective where there are sufficient resources. The cost of these programs exceeds $300 million annually and is largely spent on dog vaccinations. Because of these high costs, effective programs are unaffordable in most of the developing world. Thus, in excess of 90% of all human exposures (and 99% of deaths) worldwide are caused by bites from infected dogs.[33] There are 2 to 3 deaths annually in the United States and approximately 55,000 worldwide. It is also estimated that 10 million people receive postexposure prophylaxis (PEP) annually.[34]

In the United States in 2006, more than 92% of all animal cases occur in wildlife, whereas the majority of cases were in domestic animals prior to 1960. Greater than 113,000 animals were tested in 2006, with 6940 positive cases. Hawaii is the only state that is rabies free. Raccoons are the most frequently infected species, accounting for 37.7% of animal cases. They are followed by bats and skunks at 24.4% and 21.5%, respectively. Domestically, cats were four times more likely to be infected than dogs (4.6% and 1.1%, respectively).[35,36]

Transmission occurs when saliva from an infected animal is passed to one uninfected, most frequently through a bite. The virus may be either directly introduced into the peripheral nervous system or indirectly after replication in nonnervous tissue. In either case, uptake into the peripheral nervous system is important for disease progression. This may take anywhere from days to months after initial exposure and, in rare cases, as long as 2 years. The virus is then transported to the central nervous system (CNS), where it disseminates and replicates swiftly. It is through this dissemination and replication, followed by propagation to other peripheral nerves, that symptoms begin to appear. Factors such as the viral dose, virus variant, and site of exposure affect the timing and severity of symptoms.[37]

Rabies infection, if left untreated, causes acute encephalitis in warm-blooded hosts. The initial symptoms may be nonspecific and flulike, such as malaise, fever, and headache. Symptoms progress to include cerebral dysfunction, anxiety, and confusion. Final symptoms include delirium, hallucinations, insomnia, and fear of water (hydrophobia). However, current treatments that include injections of immune globulin and vaccine (in a series) are quite effective in preventing disease progression if given prophylactically (i.e., after suspected exposure but before symptoms appear). Treatment involves vaccination (for those not previously vaccinated) and administration of human rabies immune globulin (HRIG).[38] Before 2005, rabies infection was uniformly fatal if there was no treatment given before the onset of symptoms.

Sidebar 5-4 The Milwaukee Protocol[39,40]

In 2005, a 15-year-old girl sustained a laceration while handling a bat within a building. While the wound was washed, no medical treatment was sought. Within a month she began exhibiting symptoms characteristic of rabies infection, including fatigue, paresthesia of a hand followed by diplopia, nausea and vomiting, blurred vision, weakness of the left leg, and gait abnormality. The symptom progression led to questioning which revealed the potential rabies exposure. Rabies antibodies were found by the CDC in her CSF and serum. Given the probable failure of antiviral therapy, treatment also included a novel antiexcitatory component. Literature searches had shown that death was the result of secondary processes associated with infection, not any primary process, and that the immune system was capable of clearing the virus. A therapy was therefore designed to protect the brain from injury while allowing the immune system to work. The patient was put into a drug-induced coma, which was hoped to reduce the inflammatory response and allow the body's

immune system time to effectively combat the infection. Ribavirin was administered as there was some evidence it might penetrate the CNS and prevent rabies myocarditis. Neither rabies vaccine nor rabies immune globulin were administered. Drug volumes were tapered in response to patient physiologic cues, and she blinked her eyes when drops were administered on the 14th day of hospitalization. She continued to regain control and was removed from isolation on the 31st day and discharged on the 76th day after rehabilitation. While she is able to be self-sufficient and attend school, she did sustain neurological impairment and it is unknown to what extent she will fully recover. She was the first person known to have survived rabies disease without having been previously vaccinated or having received PEP. At 27 months postexposure, the patient retains some gait difficulties, has fluctuating dysarthria, and intermittent sensations of cold in the feet. While she cannot play sports to the level before exposure, she has successfully completed high school while taking advanced courses, can drive, plans to attend a local college, and has no noticeable mood disorders.

Organism Characteristics

The virus itself belongs to the order *Mononegavirales*, family *Rhabdoviridae*, and are shaped like bullets. There are three genera in this family, and rabies belongs to the genus Lyssavirus. Rabies is an RNA virus, approximately 180 nm long, 75 nm in diameter, and enveloped. The genome is approximately 12,000 nucleotides long and encodes for five proteins.[41] One may observe the characteristic bullet shape of rabies virions in Figure 5-13.

Sample Collection and Analysis

Sample Collection

As the virus preferentially concentrates and rapidly replicates in the CNS and brain, commonly collected clinical samples are inadequate for this specific analysis. Instead, brain issue is collected, requiring the death of the animal subject. PHLs do not typically analyze human samples, though some, such as saliva and CSF, may be collected for research purposes.

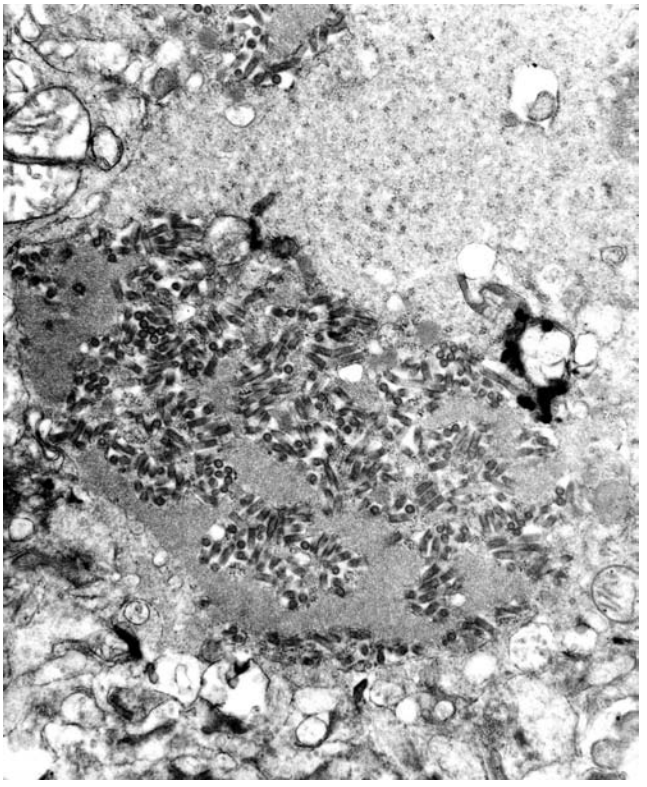
Sample Analysis

The most economic, rapid, and reliable test is the direct fluorescence antibody (DFA) currently in use by all or most state PHLs. The *Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing* relies on DFA staining of brain tissue from the potentially infected animal. Portions of the brain (preferably the brain stem, cerebellum, and/or hippocampus) are removed and spread on microscope slides. They are then incubated with fluorescently labeled rabies antibodies and washed. Using a fluorescent microscope, tissue containing viral proteins will fluoresce. There is no fluorescence in the absence of the virus because the antibodies will not bind and be washed away (in theory, at times some remains as background).[42,43] There is the potential for nonspecific background staining given the nature of the sample. There are therefore criteria for result interpretation and additional tests possible to confirm a potentially negative result.

Criteria for Result Interpretation

A positive rabies result requires the identification of apple green fluorescence of intracytoplasmic inclusions and,

Figure 5-13 Transmission electron micrograph of rabies virions (bullet shaped) within infected tissue. (Courtesy of CDC/ F. A. Murphy.)



for a negative result, the samples must be clearly negative showing no specific staining or rabieslike characteristics. Other examined specimens that do not meet either criterion are reported as inconclusive (nondiagnostic), or unsatisfactory if the specimen is unsuitable for testing. However, in most cases, the specimens can be tested and reported as positive or negative.

The burden is when acceptable specimens cannot be clearly ruled out as positive, negative, or unsatisfactory and need to be reported as inconclusive. Stain impressions that fall under the inconclusive category are difficult to diagnose and usually present nonspecific fluorescence with inclusion morphology not typical of rabies but with possible fluorescing bacteria that might mask small amounts of rabies-specific staining. This type of specimen is repeated with a specificity negative control reagent (SNCR) that targets agents other than the rabies virus and can be a useful tool to resolve inconclusive results. Figures 5-14 through 5-17 show examples of these difficult analyses.

In the rabies laboratory the nonspecific fluorescence is troublesome to interpret for the trained and untrained technologist alike, and this unwanted florescence can be present at one time or another in any specimen. There are several potential reasons for this type of undesired staining, such as the cross-reactive binding of antibodies unrelated to antigen recognition, the nonspecifically bounding of Fc receptors such as those present on Gram-positive cocci bacteria (staphylococcus, streptococcus), and some parasites (e.g., schistosomes).

Whatever the reason, this binding can be strong and difficult to block and the source of the problem should be investigated. The SNCR repeat test produces several result outcomes to confirm or rule out specific

Figure 5-14 Micrograph showing a tissue impression with suspicious (nonspecific) fluorescence and no viral morphology inclusions or distribution characteristic of a rabies infection. (Courtesy of Illinois Department of Public Health, Division of Laboratories.)

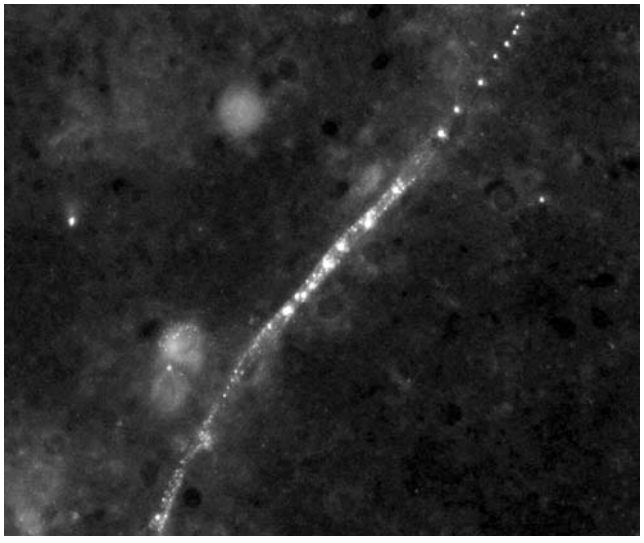


Figure 5-15 Same specimen tested with Specificity Negative Control Reagent (SNCR) and showing nonspecific fluorescence. (Courtesy of Courtesy of Illinois Department of Public Health, Division of Laboratories.)

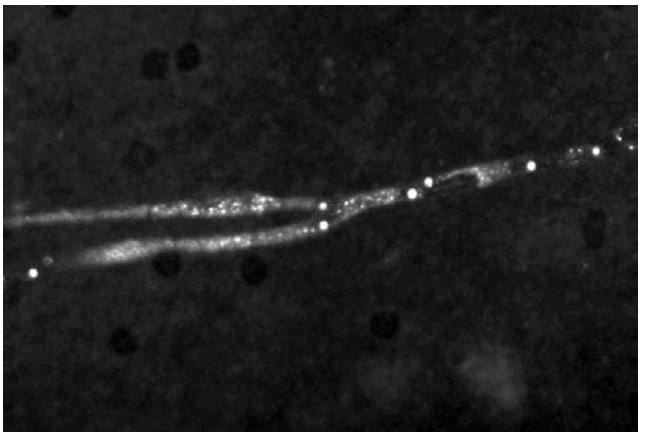


Figure 5-16 Micrograph of a Brown bat specimen positive for rabies virus by DFA showing specific fluorescence and characteristic inclusions. (Courtesy of Illinois Department of Public Health, Division of Laboratories.)

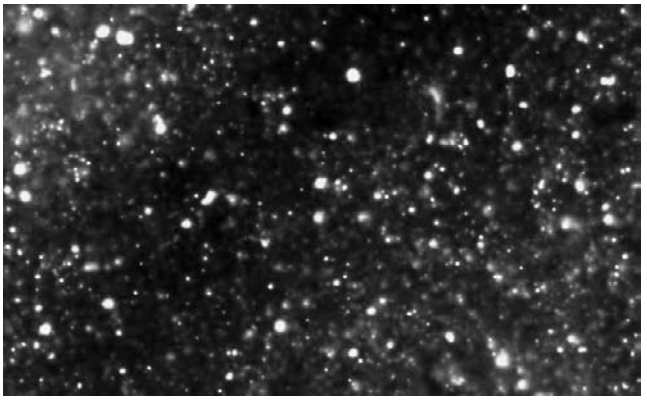
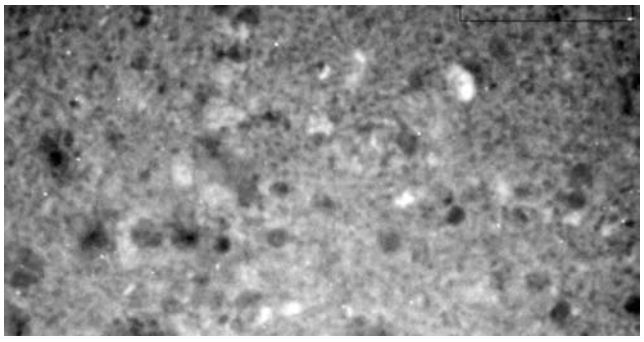


Figure 5-17 Same specimen tested with Specificity Negative Control Reagent (SNCR) showing no nonspecific fluorescence. (Courtesy of Illinois Department of Public Health, Division of Laboratories.)



or nonspecific fluorescence, and the observed SCRN inverse reaction occurs as a result of the reagent targeting agents other than the rabies virus and highlighting the nonspecific fluorescence. The different result outcomes of the SNCR repeat test may be overwhelming to new and inexperienced employees, and is critically important that all rabies result examinations be evaluated and confirmed by a second, more experienced technologist.

There are other tests available for rabies, but these are done for research rather than diagnostic purposes (e.g., electron microscopy, RT-PCR). Likewise, there are several tests available for human samples. These have the benefit of being done before death, but no single test is sufficient to diagnose infection. Saliva may be tested by RT-PCR or virus isolation, and serum and spinal fluid may be tested for rabies virus antibodies. Direct examination for the viral antigen may be performed on skin follicle biopsies as well. Specialized tests such as rabies antigenic monoclonal typing and DNA sequence techniques have become useful tools for the phylogenic analysis of bats and terrestrial rabies variants. These techniques are most directly associated with the epidemiology, outbreaks, and crossover of geographic boundaries by recognized rabies variants. From these, the most commonly used by PHLs is the antigenic monoclonal typing. The test is less costly than DNA sequence analysis, requires no additional equipment than what is needed for the standard DFA test, and can provides rapid results. DNA sequencing can be costly, labor intensive, requires specialized equipment, and is not usually performed at PHLs for rabies.

Sidebar 5-5 Impact of Test Results as Observed by IDPH Laboratory Staff

The rabies laboratory carries the responsibility and associated factors that come when performing animal autopsies and reporting results. The test results have a direct impact on healthcare decisions and may be handled in a detached manner like most other analyses. On the other hand, many people have a fear of rabies infection such that multiple assurances must be made. Animal autopsies occasionally involve pets who are considered parts of their owner's families. Laboratory staff may therefore have more contact with this family, and in a more personal manner, than almost any other type of analysis as they try to balance the needs of a thorough analysis with minimizing the disfigurement, which is an inherent part of sample acquisition. Examples from the IDPH laboratory include:

> "The mother who witnessed her five year old being attack by a bat and after receiving rabies negative results embarked on a personal laboratory investigation requesting laboratory records that would provide assurance that no possible human error occurred at the time of the specimen examination."
>
> "The couple who drove five hours to the lab to pick up their pet for funeral services and burial at the pet cemetery."
>
> "The gentleman who requested minimal dissection intrusion on his cat, who subsequently paid $5000 for taxidermy services, and who currently displays his beloved pet in his living room."

In general, people fear rabies. They do not really know what it is, but often think it is untreatable and most likely to be fatal. While unchecked infection may indeed be fatal, PHL testing of suspect animals, combined with prophylactic treatment, may do much to allay public fears. It is because of this testing that there are so few deaths in the United States each year, and few people inappropriately treated. The immediacy of need and critical nature of correct result interpretation mean that PHLs must maintain high degrees of both proficiency and readiness. The IDPH laboratory in Springfield received 4634 samples for rabies analysis from July 2007 through June 2008 (unpublished data). This volume requires both well-trained staff and a high capacity for swift and accurate analysis.

West Nile Virus and Arboviruses

Overview of West Nile Virus and Other Arboviruses

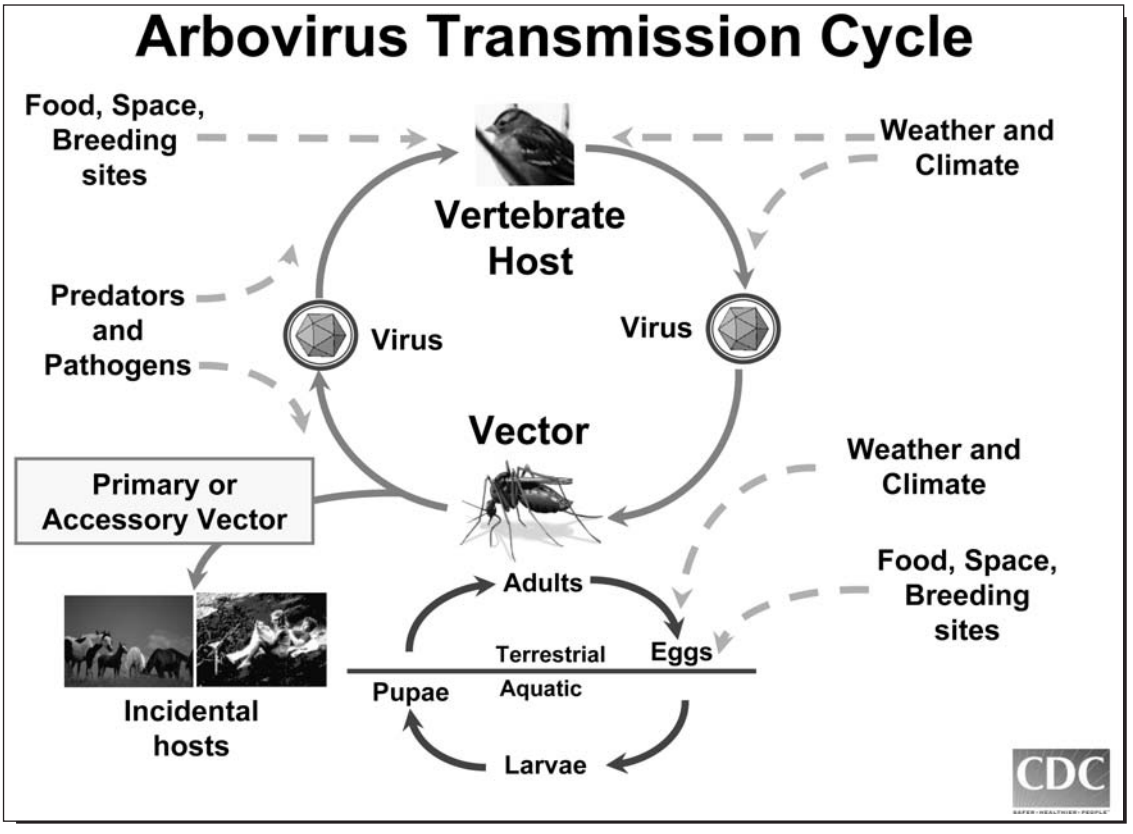
Contrary to what the name might suggest, arboviruses have nothing to do with trees with the possible exception that many birds happen to live in them. *Ar*thropod-*bo*rne *viruses* are in fact defined as those requiring a blood-sucking arthropod vector to facilitate transmission between two vertebrate hosts. The most well known arthropod is the mosquito, though ticks, psychodids (gnats), and ceratopogonids (biting midges) may also perform this function. The transmission cycle of arboviruses between their primary hosts, possible vectors, and human recipients is complex and illustrated in Figure 5-18. While multiple viruses exist, only three families have the ability to cause encephalitis (inflammation of the brain) in humans: *Togaviridae* (genus *Alphavirus* only), *Flaviviridae*, and *Bunyaviridae*. Arboviruses have a global distribution, but there are four that cause the majority of illness in the United States: eastern equine encephalitis (EEE), western equine encephalitis (WEE), St. Louis encephalitis (SLE), and La Crosse encephalitis (LAC). These have been joined by West Nile virus/encephalitis (WNV) as an illness of concern after its first identification in the United States in 1999.[44]

Arboviruses as a whole cause anywhere from 150 to 3000 confirmed cases annually in the United States. This varies considerably because of weather and other factors. The actual number of human infections is likely much greater as many people are either asymptomatic or present with mild symptoms such that illness cause and confirmation are not always performed. However, there is significant chance of permanent damage or death associated with some infections. EEE may cause permanent neurologic damage in 30% of clinical cases and has a fatality rate of 30%. The rates for SLE are 10% and 5%, respectively. Annual costs are ~$150 million with the majority going for surveillance and vector control activities (e.g., mosquito spraying).[45]

Eastern Equine Encephalitis

EEE is an alphavirus that was first identified in the 1930s. Occurrences are focused on the East and Gulf coasts and some select Midwestern areas, and there are 5 to 15 cases confirmed annually in the United States

Figure 5-18 Illustration of the arbovirus transmission cycle. (Courtesy of CDC.)



(254 total confirmed and probable cases reported from 1964 to 2007).[46] EEE is normally transmitted between the mosquito *Culiseta melanura* and birds in swampy areas. This mosquito species prefers to feed on birds, and it is suspected that the virus is transmitted to humans via another vector such as *Aedes sollicitans*. For those that will develop apparent illness, symptoms of infection usually develop within 4–10 days and include sudden fever, muscle pains, and headache. More severe disease may present with seizures and coma, with death occurring in approximately one third of clinical cases. Patients younger than 15 or older than 50 are at greater risk of severe disease. Almost one half of those who recover will have sustained permanent brain injury.[44,47] Figures 5-19 and 5-20 show identifying characteristics of *Culiseta melanura* (an important EEE vector) and a view of EEE virions.

Western Equine Encephalitis

WEE is an alphavirus that was first identified in the 1930. Occurrences are focused on the western part of the United States with 640 confirmed and probable cases reported from 1964 to 2007.[46] It is normally transmitted between the mosquito *Culex tarsalis* (Figure 5-21) and birds, though there are other common mosquito vectors. The majority of infections are either asymptomatic or present as a mild flu. Serious symptoms appear suddenly and include headache, fever, nausea and vomiting, anorexia, and malaise with an altered mental state. While death occurs in only ~3% of cases, 5 to 30% of young victims may be left with permanent damage.[44,48]

St. Louis Encephalitis

SLE is a flavivirus and the most common mosquito-transmitted pathogen in the United States with 4667 confirmed and probable cases reported from 1964 to 2007.[46] It is distributed throughout the country, though more concentrated in the Midwest. The virus is maintained via a bird–mosquito cycle with several *Culex* species participating. Less than 1% of all infections become clinically apparent. Thus, the average of 193 confirmed cases annually is most likely a severe underrepresentation of the actual disease burden. Symptoms of illness present within 5–15 days of infection and range from mild fever to meningoencephalitis with a fatality rate of 5 to 30%.[44,49] A TEM of SLE in tissue is shown in Figure 5-22.

Figure 5-19 Illustration of physical characteristics of *Culiseta melanura* an important vector of Eastern equine encephalitis. (Courtesy of CDC.)

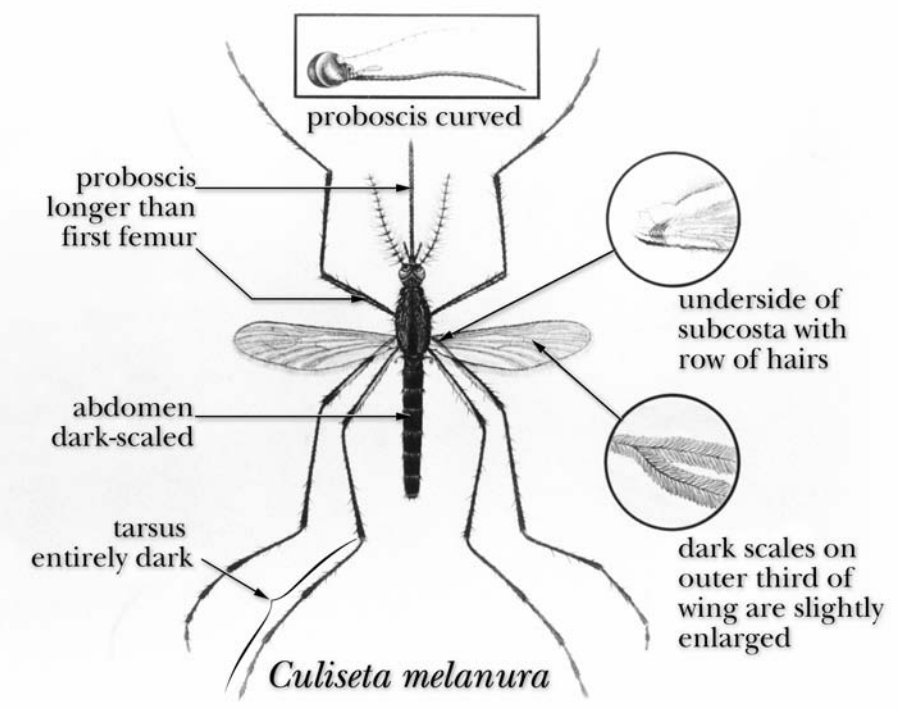


Figure 5-20 Transmission electron micrograph of Eastern equine encephalitis virions. (Courtesy of CDC/Dr. Fred Murphy; Sylvia Whitfield.)

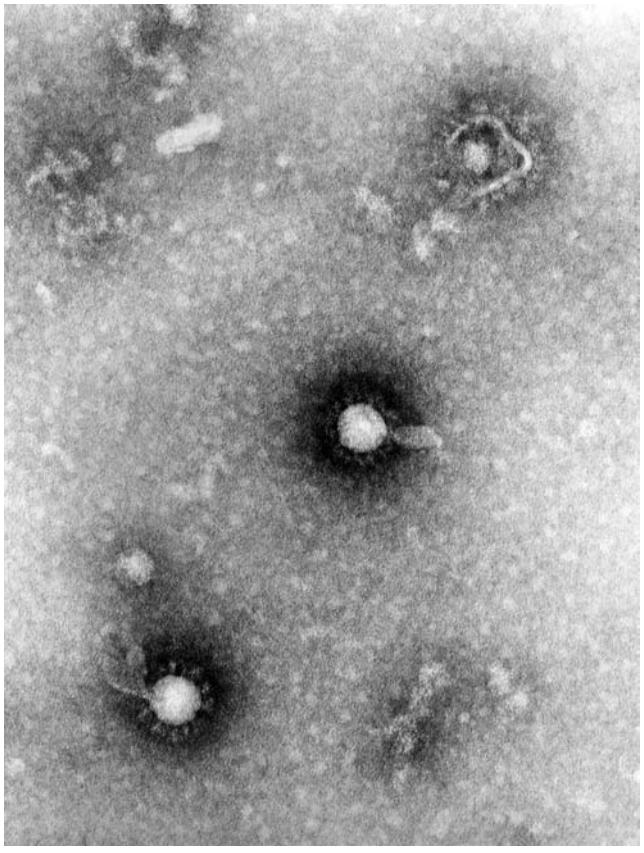


Figure 5-21 Photo of *Culex tarsalis* showing distinctive light-colored bands (A and B) and one of two bilateral silver scutal marks (C). (Courtesy of CDC; Photographer: James Gathany.)

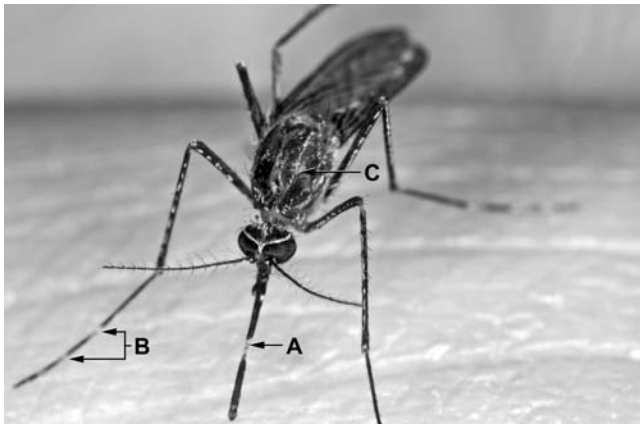
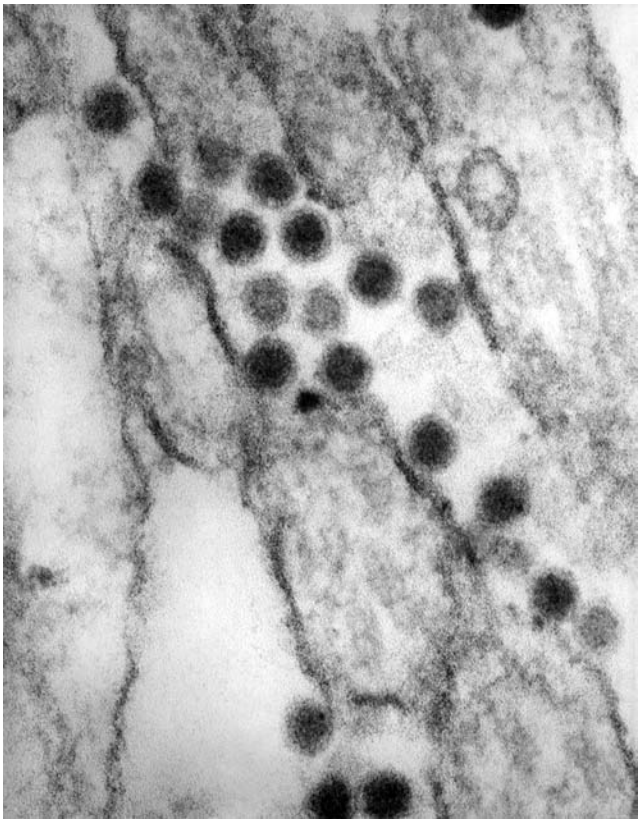


Figure 5-22 Transmission electron micrograph (negatively stained) showing St. Louis encephalitis virions. (Courtesy of CDC/Dr. Fred Murphy; Sylvia Whitfield.)



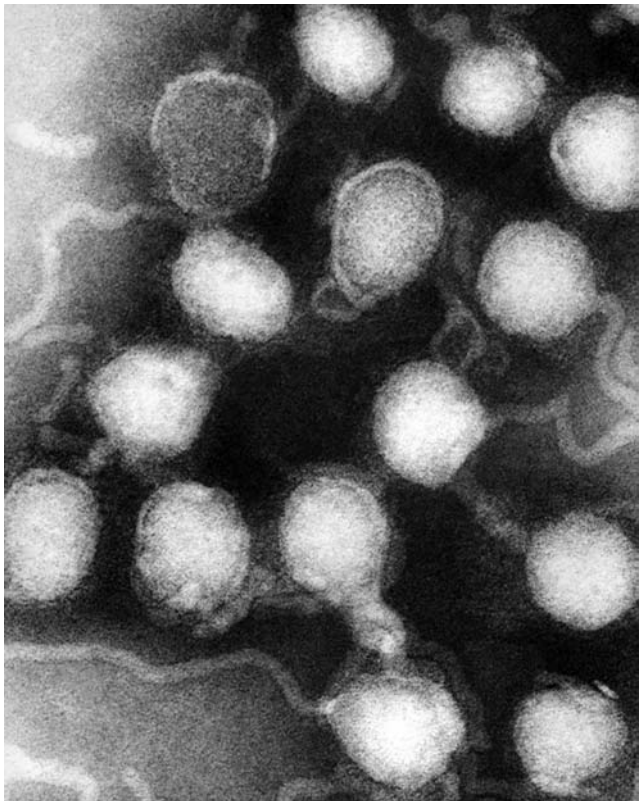
La Crosse Encephalitis

LAC is a bunyavirus discovered in La Crosse, WI, in 1963. Historically, there have been an average of 70 cases reported annually, most cases occurred in the Midwest. The virus is transmitted by the mosquito *Aedes triseriatus* with chipmunks and squirrels as the natural hosts. While the majority of infections are asymptomatic, illness usually presents with such symptoms as headache, fever, nausea and vomiting, and lethargy. More severe disease may present with seizures, coma, and paralysis; death may occur in $< 1\%$ of clinical cases. There may also be neurologic complications after recovery.[44,50] A TEM of SLE in tissue is shown in Figure 5-23.

West Nile Encephalitis/Virus

WNV is a flavivirus and quite closely related to SLE (indeed there is cross-reactivity in many tests). WNV

Figure 5-23 Transmission electron micrograph (negatively stained) showing La Crosse encephalitis virions. (Courtesy of CDC/Dr. J. Obijeski.)



was first identified in 1937 in the West Nile district of Uganda. The first positively identified outbreak in the United States occurred in New York in 1999 and has since been found in most states. It is not known how WNV was introduced in this country. The virus may infect a wide range of vertebrates, and *Culex* spp. are the primary vector along with *Aedes* and *Anopheles*. Upward of 80% of those infected will remain asymptomatic; up to 20% will develop relatively mild symptoms such as headache, fever, aches, nausea and vomiting; and < 1% will develop severe illness with symptoms including high fever, stupor and disorientation, stiff neck, tremors, convulsions, and coma. There were 1356 cases of WNV reported in 2008, with 687 (51%) reporting as neuroinvasive (encephalitis or meningitis) and 624 (46%) reporting as WN fever (nonneuroinvasive).[44,51,52] Figures 5-24 and 5-25 show identifying characteristics of *Culiseta quinquefasciatus* and a view of WNV virions.

Sidebar 5-6 West Nile Virus in the United States[53]

WNV was first identified in the United States in 1999 and has since become the leading cause of arboviral encephalitis. Data from 2007 show that viral infection of humans or animals expanded into 19 new US counties, and recurred in 1148 where it had been previously reported. Animal cases are important for tracking how the virus spreads into new areas and animal cases identified through surveillance activities precede human cases in that area. Surveillance and case data are reported to the CDC through ArboNET. Data consist of both human (patients, blood donors) and animal (mosquito surveillance and mammals) and are used to track disease spread and annual activity.

WNV infection in humans was widespread in 2007, with 3630 cases reported from 775 counties in 44 states. Of these, 34% were neuroinvasive disease, 65% were WN fever, and the last 1% were unspecified. The states with highest rates (cases/100,000 persons) were North Dakota (7.7), South Dakota (6.2), Wyoming (4.6), Montana (4.0), and Colorado (2.2). In terms of surveillance, there were 2182 WNV-infected birds reported from 315 counties in 35 states and Puerto Rico. There were also 507 reported cases in nonhuman mammals (93% equines). Finally, 8215 mosquito pools from 371 counties in 39 states, the District of Columbia, and Puerto Rico were positive (77% were composed of *Culex*).

This report shows how WNV has spread across the United States in a short period of time to become endemic. The numbers of cases in 2007 are comparable to 2006 and are fairly steady (with annual variations caused by a number of factors). Consistent testing of samples, for both diagnostic and surveillance purposes, is important to guide public health actions at the local and national level.

Arboviruses cause illness in humans by invading the CNS and causing encephalitis. There are currently no human vaccines available for the viruses discussed here (though there is one available for Japanese encephalitis). There are also no known effective antiviral medications, and antibiotics are of no use for viral infection. Treatment is therefore supportive. Much energy is spent on prevention, with two main strategies consisting of encouraging

Figure 5-24 **Illustration of physical characteristics of *Culex quinquefasciatus* an important vector of West Nile virus. (Courtesy of CDC.)**

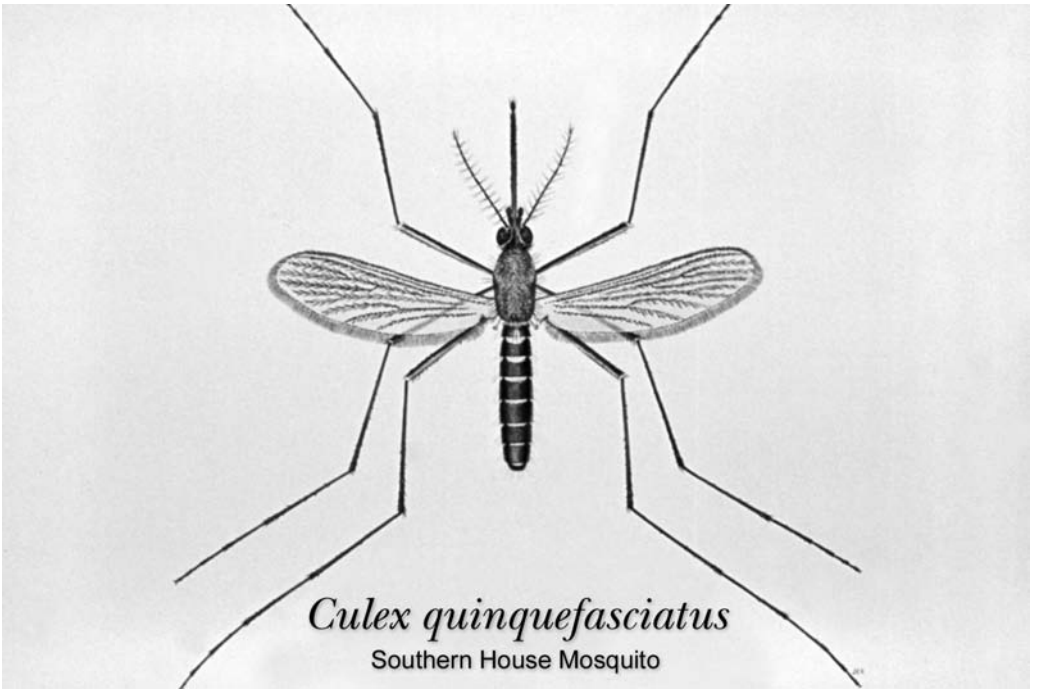
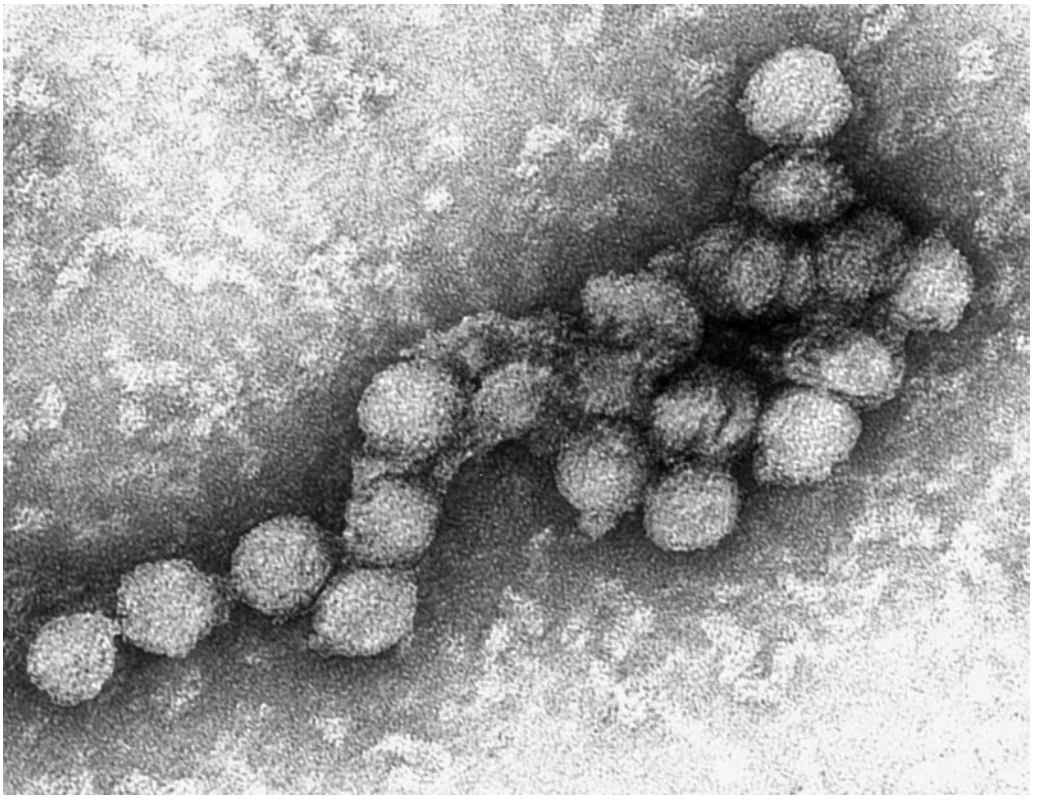


Figure 5-25 **Transmission electron micrograph showing West Nile virions. (Courtesy of CDC/P. E. Rollin; Photographer: Cynthia Goldsmith.)**



the use of personal protective habits and community-scale interventions such as mosquito control spraying. Other arboviruses known to cause illness in humans include California, Japanese, Powassan, Venezuelan, and tick-borne encephalitis.

Organism Characteristics

Togaviruses are 65–70 nm long enveloped viruses containing positive-sense, single-stranded RNA ranging from 10,000 to 12,000 nucleotides in length within an icosahedral nucleocapsid. Flaviviruses are 40–60 nm long enveloped viruses containing positive-sense, single-stranded RNA ranging from 10,000 to 11,000 nucleotides in length within an icosahedral nucleocapsid. The envelopes of both toga- and flaviviruses contain a hemagglutinin and lipoproteins. Bunyaviruses are 90–100 nm long enveloped viruses containing negative-sense, single-stranded RNA forming a three-part genome (large, medium, and small components) ranging from 11,000 to 19,900 nucleotides in total length and forming a helically shaped and elongated nucleocapsid.[54,55]

Sample Collection and Analysis

Sample Collection

Typical samples submitted for analysis include human serum and CSF. Two milliliters of sample are adequate for both sample types. Tissue samples (e.g., brain, spinal cord, spleen, liver, kidneys, blood) collected postmortem are capable of analysis. Human testing is usually based on antibody detection, except for fatal cases where postmortem samples may be tested for viral RNA. Mosquitoes and dead birds may be sampled for surveillance purposes, and like all tissue samples, are homogenized in a lysate solution to release potential RNA into solution, which can be extracted and used for PCR analysis. These are not often performed at health department laboratories.

Sample Analysis

There are several tests performed that assist in not only the identification of the encephalitic agent, but also indicate the time of exposure. Thus, samples from both acute infection ($<$ 10 days after onset of symptoms) and from convalescence (2–4 weeks after onset) are collected. Immunoglobulin M (IgM) levels begin a rapid decline from peak levels after about 4 weeks. Immunoglobulin G (IgG) levels slowly rise to reach maximum levels approximately 8–9 months postinfection and may remain elevated for extended time periods. Multiple tests may then be performed for screening and confirming identification. *Serological assays* are performed on serum and CSF and look for virus-specific antibodies. Four methods commonly used include microsphere immunoassay (MIA), plaque reduction neutralization test (PRNT), IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA), and indirect fluorescent antibody (IFA). *Viral detection assays* are performed on acute CSF, tissues, blood, environmental specimens, and look for viral RNA. Two common methods are RT-PCR and viral isolation.

MIA uses flavivirus monoclonal antibody-coated microspheres to capture recombinant WNV and SLE antigens. These microspheres are mixed with sample (serum, CSF) and bind any complementary antibodies. The antigen–antibody complex is then mixed with a second fluorescently labeled antihuman immunoglobulin antibody that will detect IgM in the sample. Each microsphere is dyed to obtain a distinct fluorescent pattern. This is known as the spectral address. The instrument used is a modified-flow cytometer that consists of two lasers. One laser stimulates the microsphere to determine which arbovirus antigen was the target. The other laser activates the antihuman immunoglobulin to detect that there is a positive result.

PRNT is the confirmatory assay for positive IgM and IFA results. The basic method is the inoculation of confluent cells with virus and their subsequent overlay with agar. Following 72 hours of incubation, a second layer of agar containing neutral red is added, incubated for 24–48 hours, and observed for plaque reduction. Significant reduction (e.g., $>$ 90%) is considered positive for the virus.

MAC-ELISA uses an antibody capture method (versus antibody sandwich indirect method). The assay uses goat antihuman IgM to capture IgM antibody from human serum followed by reaction with WNV recombinant antigen, in turn followed by reaction with horseradish peroxidase (HRP) anti-flavivirus antibodies. The initial selection and separation of IgM allows for much lower detection levels (and earlier detection). This assay is followed by IgG indirect ELISA that is performed as an additional confirmation of the IgM assay and to show evidence of past infection if IgM is negative. These tests are sensitive to SLE and WNV only, cannot differentiate between the two, and must be verified by cross-species plaque neutralization on paired samples.

Sidebar 5-7 False-Positive Results from a Commercially Available WNV Kit[56]

Commercially available immunoassay kits (IgM ELISA) are often used by health providers and private laboratories for testing individuals for WNV. Samples testing positive are considered presumptive for neuroinvasive disease and must be confirmed by additional testing performed at the state PHL. In 2008, three state health departments notified the CDC that a number of immunoassay-positive individuals were negative for WNV when tested at the state laboratory. Retesting of samples provided by a commercial laboratory showed false-positive rates of 20% and 56% for two kit lots, while the product insert reported an expected false-positive rate of 2%. The kit lots have since been voluntarily recalled by the manufacturer. This case serves to highlight the vigilance PHLs and public health officials must pay to the consistency of test results and the value of test result surveillance.

IFA assays are tests for SLE, EEE, WEE, and LAC and use reagents available from commercial vendors. The assay follows the traditional indirect fluorescence method of using prepared arbovirus antigens to bind to any specific antibodies present in the sample. The conjugate is then exposed to antigen–antibody complex that will fluoresce under microscopy to reveal the presence of the specific arbovirus. This is also presumptive and must be confirmed.

RT-PCR follows the basic premise of PCR by using primers to identify target RNA, transcribe it into complementary DNA, and amplify the numbers of DNA pieces to detectable levels through consecutive polymerization. The addition of fluorescent agents that react with the target nucleotide sequence allows real-time detection as fluorescence increases with increasing nucleotide numbers. There are different techniques available (e.g., TaqMan and NASBA).

Virus isolation has some advantages for diagnostic evaluation in the laboratory. It is sensitive because of the natural amplification of the virus in tissue culture during the replication process. It is specific because only the virus will be amplified. However, virus isolation procedures are rarely performed for the detection of arboviruses because of the difficulty of technique and the longer time required (compared to RT-PCR).

As with many other testing sections, those tasked with testing samples for arboviruses may develop testing algorithms designed to meet the testing needs of their jurisdiction, conserve resources and reagents, or both. For example, the IDPH laboratory in Chicago screens all samples submitted for arboviral analysis for only WNV and SLE by the MIA test. These two are most commonly encountered in their jurisdiction, and reagents for EEE and WEE are in short supply. If the screen is positive for WNV, it is reported out. If it is positive for SLE it is repeated and confirmed by PRNT before reporting. The difference between the automatic reporting of WNV versus SLE is because of the high incidence of WNV in the jurisdiction. Samples collected from those aged older than 18 years are also screened for California encephalitis (CE) by enzyme immuno assay (EIA). CE usually occurs in children, and positives are repeated and confirmed by PRNT before reporting.

Arbovirus testing gained new prominence in the past decade with the emergence and spread of WNV across the United States. From its first detection in 1999, it is now found in almost every state. We also find collaborations between different testing agencies when they combine analyses of mosquitos, birds, nonhuman mammals, and people to describe the extent of infection in an area. The IDPH laboratory in Chicago received 3192 human arbovirus samples from July 2007 through June 2008 (unpublished data), and these were joined by public health partners with other surveillance data to show where the viruses were likely to infect others in Illinois.

Protozoan Parasites

Overview of Protozoan Parasites

In general, an organism may be considered a parasite if it draws shelter and/or nourishment from a host without contributing to the host's betterment. This is quite different from a symbiotic relationship where both parties draw some benefit (e.g., mitochondria within cells). Parasitic infection in humans is frequent and can cause significant illness. There are 350 to 500 million cases of malaria (*Plasmodium* infection) worldwide each year, with more than 1 million deaths, most of them children in sub-Sahara Africa. Even though malaria has been eradicated in the United States since the 1950s, there were 1337 reported cases in 2002 (1332 were acquired in other countries, however).[57] Also in the United States, *Giardia* causes an estimated 2 million infections annually, and *Cryptosporidium* ~300,000 while also being the most frequent cause of recreational water-related disease outbreaks. *Toxoplasma* causes an estimated 1.5 million infections in the

United States each year and is the third leading cause of food-borne disease-related deaths.[58]

There are three general types of parasite that can cause disease in humans. *Protozoa* are unicellular organisms that may be free-living or parasitic and are able to multiply in humans. Protozoa are usually invisible to the naked eye, and examples include *Entamoeba* and *Giardia*. *Helminths* are often recognized as worms, are multicellular, and also may be free-living or parasitic but cannot multiply in humans. They are usually visible to the naked eye, such as *Ascaris lumbricoides*, which may exceed 30 cm in length. *Ectoparasites* are the blood-sucking arthropods and include mites, ticks, and fleas. The rest of this section will discuss protozoa as they are most frequent target of analyses in PHLs.

Cryptosporidium Species

There are multiple species of *Cryptosporidium* that may infect humans. The most prevalent are *C. parvum* and *C. hominis*, although infections with *C. felis*, *C. meleagridis*, *C. canis*, and *C. muris* have been identified. *Cryptosporidium* are chlorine resistant and the standard levels of chlorine used in drinking and recreation waters may be insufficient to kill the organisms. It is thought that consumption of 1–10 organisms may be sufficient to induce infection.[59] The life cycle for *Cryptosporidium* is somewhat complex and is illustrated in Figure 5-26. In brief, oocysts are ingested and undergo excystation in the intestinal tract where the sporozoites are released and parasitize epithelial cells. These then undergo asexual multiplication, followed by sexual reproduction (producing male microgamonts and female macrogamonts). After fertilization of the macrogamonts by the microgamonts, two types of oocysts are developed, both of which sporulate. One is thin walled and remains in the host as a source of autoinfection and another is thick walled and excreted. The excreted oocysts are then a source of infection for others.[60]

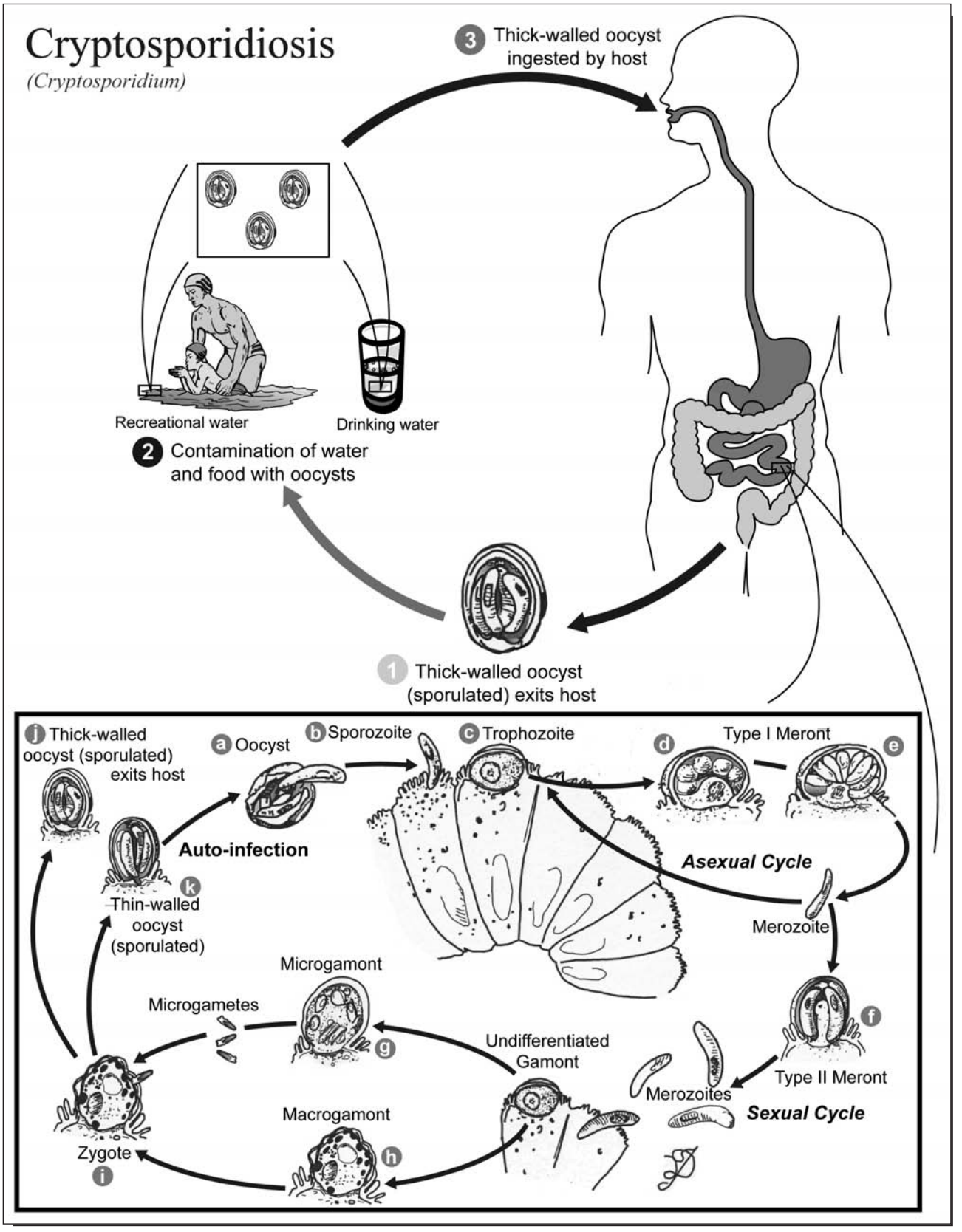
Cryptosporidiosis is the illness associated with *Cryptosporidium* infection and is a frequent source of infection in the United States, detected in approximately 2% of tested stool samples. This translates into ~300,000 new cases annually.[61] Serological surveys indicate that upward of 80% of the population has been infected with *Cryptosporidium* at some time.[59] The illness is characterized by watery diarrhea with other symptoms such as associated dehydration, stomach cramps, nausea and vomiting, fever, and weight loss. Symptoms usually begin within 7 days of ingestion (incubation typically 2–10 days) and illness may last 1–2 weeks. Most people do not become severely ill, though those with weakened immune systems are more susceptible to more severe symptoms. There is no effective drug treatment for cryptosporidiosis, though the FDA has approved the use of nitazoxanide for diarrhea.[60]

Sidebar 5-8 Cryptosporidiosis Outbreak in Utah[62]

The incidence of *Cryptosporidium* reports peaks in late summer and is associated with the summer swimming season. Thus, many state health departments are especially vigilant for this infection during this time period. In 2007, the Utah Department of Health received 1902 laboratory-confirmed case reports of *Cryptosporidium* during June to December 2007. This was compared to the mean of 16 annual reports from 2002 to 2006. Data available from 1506 patients showed that 80% reported exposure to a variety of recreational water venues in the 2 weeks preceding illness. Smaller numbers of patients reported water exposure and contact with persons ill with diarrhea (592), water exposure only (503), and contact with ill persons only (170). Furthermore, 136 patients reported swimming while ill with diarrhea.

Initial control measures included advising the public not to swim while ill with diarrhea, asking healthcare providers to request increased *Cryptosporidium* testing as indicated, and requesting recreational water facilities to hyperchlorinate their water if patients had swum there. (Hyperchlorination involved raising the free chlorine to levels beyond that which is safe for consumption or swimming, but should inactivate the parasite.) Continuing surveillance indicated these measures were inadequate, and secondary control measures were implemented. These included the banning of children younger than 5 years and those requiring diapers from public-treated recreational water venues, the hyperchlorination of such venues weekly with the additional posting of safe swimming guidelines (e.g., no drinking water), and other measures with child care programs. Incidence decreased soon after these measures were implemented, though this also coincided with Labor Day weekend and the closing of many parks. This case serves to highlight the importance of laboratory stool testing for disease surveillance and how decisions may be made based on such data.

Figure 5-26 Illustration of the life cycle of different species of *Cryptosporidium*. (Courtesy of CDC/ Alexander J. da Silva, PhD/Melanie Moser.)



Cyclospora cayetanensis

This is the only known species of *Cyclospora* known to infect humans. *C. cayetanensis* is an obligate intracellular parasite, meaning it must invade a host cell to survive. It is thought that the consumption of 1–10 organisms may be sufficient to induce infection.[59] The life cycle for *C. cayetanensis* is illustrated in Figure 5-27. In brief, oocysts sporulate in the environment *after* excretion. This produced two sporocysts, each containing two sporozoites that are ingested (e.g., via contaminated water) and excyst in the gastrointestinal tract. This action frees the sporozoites to parasitize epithelial cells where they undergo asexual reproduction, sexual development, and maturation into oocysts. These are shed in the stool, but unlike *Cryptosporidium*, which is immediately infective, these oocysts must first sporulate as described previously.[63]

Cyclosporiasis is the illness associated with *C. cayetanensis* infection and is most common in tropical and subtropical areas. There have been at least 11 identified food-borne outbreaks of cyclosporiasis in North America since 1990, affecting ~3600 people.[63] The illness is characterized by potentially severe watery diarrhea and sometimes explosive bowel movements with other symptoms such as associated dehydration, anorexia and weight loss, nausea and vomiting, fatigue, and mild fever. Symptoms usually begin within 7 days of ingestion and illness may last 10–12 weeks. The recommended treatment for *Cyclosporiasis* is the combination antibiotic trimethoprim–sulfamethoxazole.

Giardia lamblia

This protozoan was named *lamblia* in 1915, but many consider the name *intestinalis* to be more correct. The issue is under review by the International Commission on Zoological Nomenclature and both names are commonly used. We will use *G. lamblia* for this text. *Giardia* is a protozoan motile through the use of five flagella. It is thought that consumption of a single organism may be sufficient to induce infection.[59] The life cycle for *Giardia* is fairly simple (compared to other protozoa) and is illustrated in Figure 5-28. In brief, cysts are ingested and undergo excystation in the intestinal tract. This releases trophozoites (two each per cyst) that continue to divide by binary fission. As the trophozoites travel toward the colon they encyst. Excreted cysts are a source for continuing contamination for others.[64]

Giardia is the causative agent of giardiasis and is the most frequent cause of nonbacterial diarrhea in the United States with an estimated prevalence of infection of 2% of the population.[59] Symptoms usually begin within 7 days of ingestion (incubation typically 1–14 days) and illness may last 2–6 weeks, though many of those infected do not develop symptoms. Those that do present are quite similar to those for *Cryptosporidium* and include diarrhea, abdominal cramps, greasy stools, bloating and flatulence, weight loss, and occasionally vomiting. Drugs that can be prescribed for *Giardia* infection include metronidazole and tinidazole, in addition to those used to treat diarrhea.

Plasmodium Species

There are over 150 known species of *Plasmodium*, four of which known to be transmitted human to human. They are *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. While *P. knowlesi* has been identified in humans on occasion, it is not known whether or not it requires a nonhuman intermediate host as is still considered a zoonotic version. The life cycle for *Plasmodium* spp. is somewhat different from those discussed before because the oocysts are formed outside the human body (inside the mosquito vector). The life cycle is illustrated in Figure 5-29. In brief, an infected *Anopheles* mosquito injects sporozoites into a human during a blood meal. These enter the *exo-erythrotic cycle* by invading liver cells and maturing into schizonts, which then release merozoites. These then undergo asexual replication in red blood cells to form trophozoites, and may either continue the *erythrocytic cycle* by maturing into schizonts and releasing additional merozoites, or differentiate into sexual gametocytes (with microgametocytes male and macrogametocytes female). These are ingested by a mosquito during a blood meal, and enter the *sporogenetic cycle* in the gut. Here the gametes combine to form zygotes, develop into oocysts within the midgut wall, and later rupture to release sporozoites that travel to the salivary glands where they may be inoculated into the next human host.[65]

Malaria is the illness associated with *Plasmodium* infection and *P. falciparum* is the most prevalent species identified. *P. malariae* also has worldwide distribution, but is not as frequently a source of infection, and *P. ovale* and *P. vivax* have complementary, but slightly overlapping, distributions. The illness is characterized by fever and chills accompanied by headache, fever, chills, sweats, diarrhea, vomiting, weakness, and myalgias. Further clinical features vary somewhat by species, level of infection, and level of host immune response, but can include system illnesses such as splenomegaly, anemia, thrombocytopenia, hypoglycemia, and pulmonary and renal

Figure 5-27 Illustration of the life cycle of *Cyclospora cayetanensis*. (Courtesy of CDC/DPDx.)

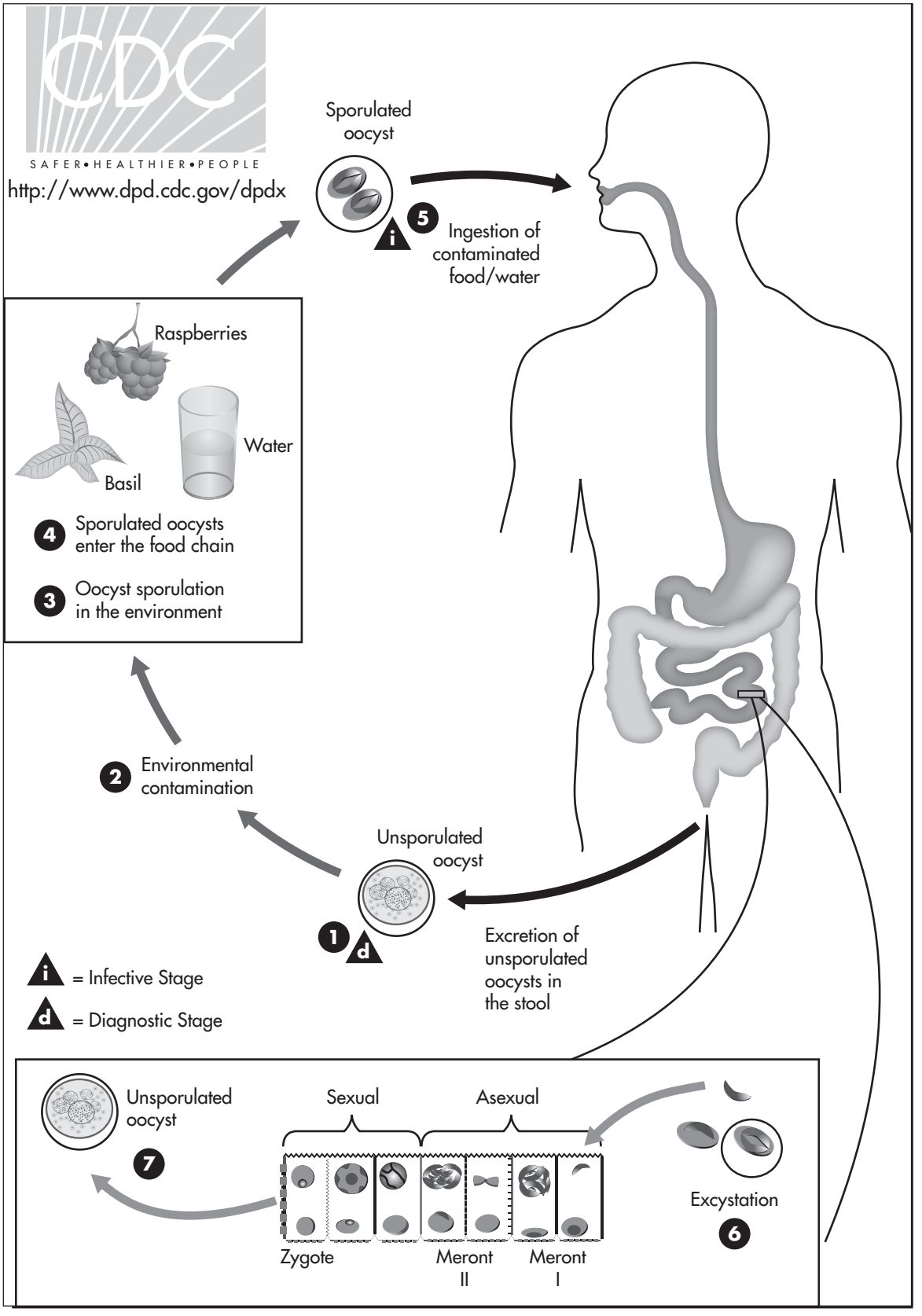


Figure 5-28 Illustration of the life cycle of *Giardia lamblia*. (Courtesy of CDC/Alexander J. da Silva, PhD/Melanie Moser.)

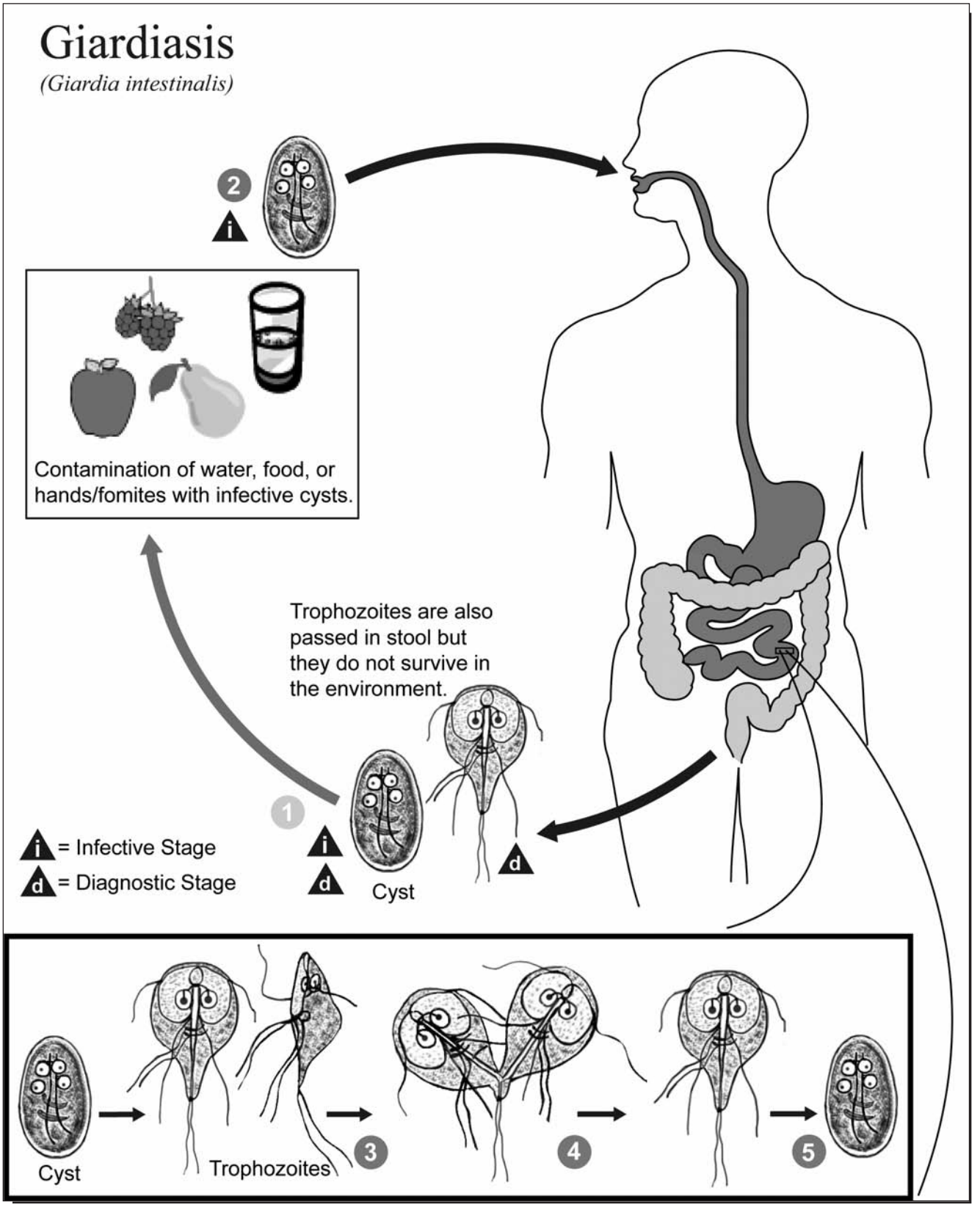
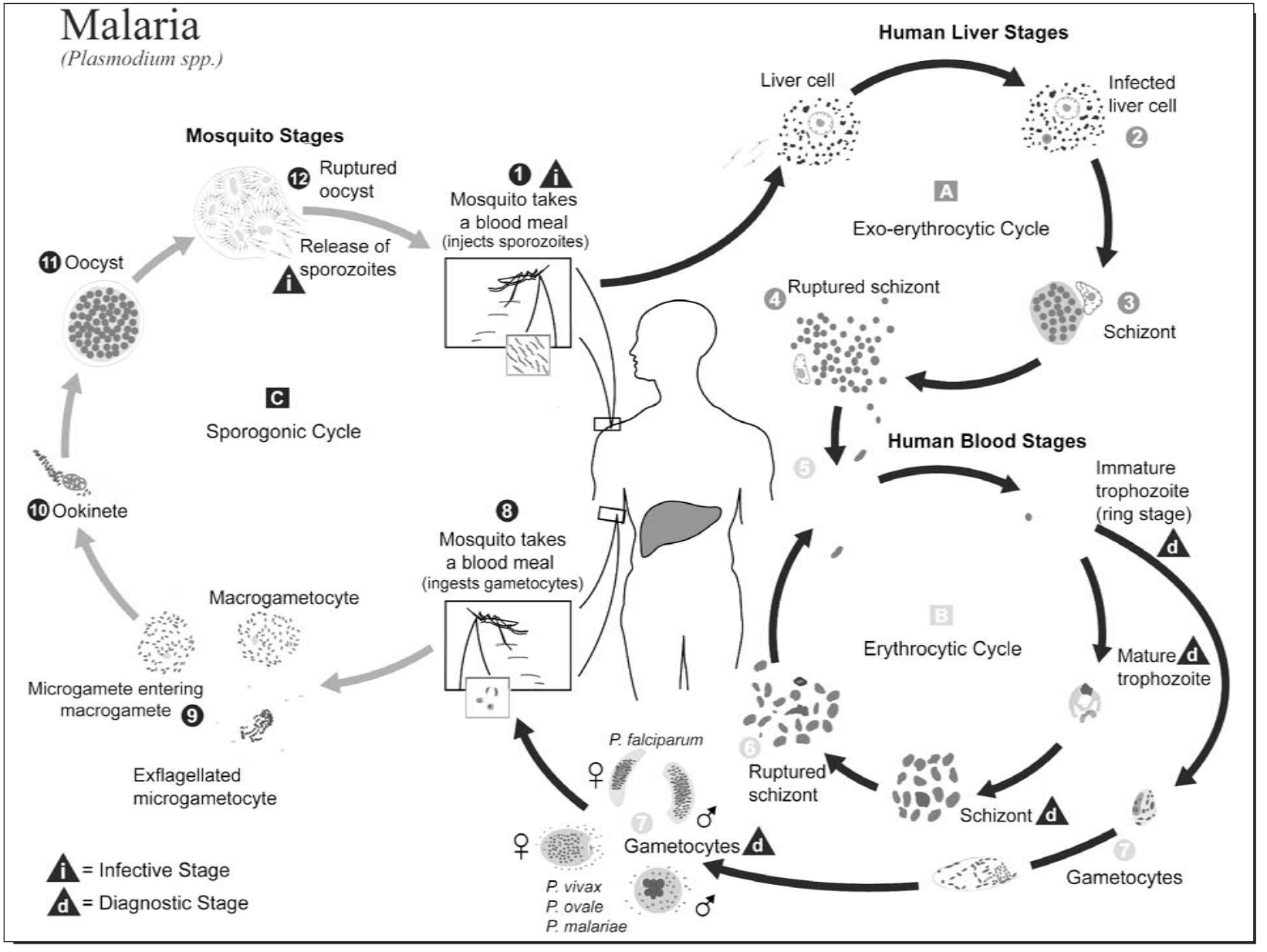


Figure 5-29 Illustration of the life cycle of different species of *Plasmodium,* which cause malaria. (Courtesy of CDC/Alexander J. da Silva, PhD/Melanie Moser.)



dysfunction. *P. falciparum* infection is particularly associated with severe and potentially fatal complications. Symptoms may begin within 7–30 days of infection once the erythrocytic cycle has begun (though *P. vivax* and *P. ovale* may form dormant hypnozoites in the liver that may cause illness months/years later) and progress to serious complications within 24 hours if untreated.

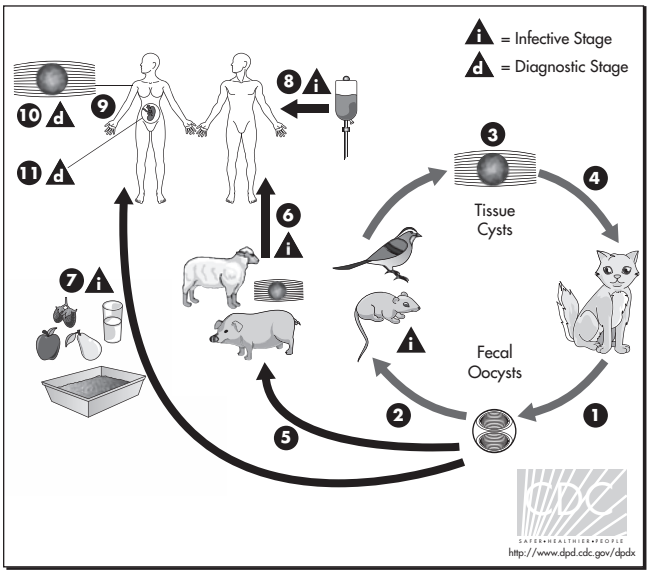
Sidebar 5-9 Malaria in the United States[66]

Malaria has not been considered endemic in the United States since the 1950s when eradication programs were implemented. However, many PHLs still test for the parasites, and identify persons infected more often than most might think. Data concerning malarial infection and laboratory analysis is collected through the National Malarial Surveillance System and the National Notifiable Disease Surveillance System. In 2007, the CDC received reports of 1505 cases of malaria in the United States (compared to 1564 in 2006), one of which was transfusion-related and another of which was fatal. Of these, the infecting species was identified for 1051 cases, with *P. falciparum*, *P. vivax*, *P. malariae*, and *P. oval* identified in 43.4%, 20.3%, 2.0%, and 3.5% of cases, respectively. The highest rates were found in those returning from travels in West Africa. All cases were reported as being imported with the exception of one blood transfusion case. The country of residence was known for 997 cases, and 734 were US residents and 263 were from other countries. While malaria is not considered to be endemic in the United States, and reported cases are almost exclusively acquired outside the United States, surveillance and testing is still required for those who travel abroad and for visitors from other countries.

Toxoplasma gondii

T. gondii is the only species of *Toxoplasma* known to infect humans, and its only definitively known hosts are domestic cats (and their genetic relatives). Humans are only intermediate hosts and cannot pass the infection. The life cycle for *T. gondii* is fairly simple compared to other protozoa and is illustrated in Figure 5-30. In brief, unsporulated oocysts are excreted by a cat and sporulate within 1–5 days. Intermediate hosts such as rodents, birds, or farm animals consume these oocysts where they transform into tachyzoites, become localized

Figure 5-30 Illustration of the life cycle of *Toxoplasma gondii*. (Courtesy of CDC/DPDx.)



in neural and muscle tissue, and form bradyzoites (tissue cysts). Cats become reinfected by consumption of this infected tissue or sporulated oocysts in the environment. Humans become infected by consuming infected tissue or food or water contaminated with sporulated oocysts. In humans, the tissue cysts are formed in such tissue as skeletal muscle, myocardium, brain, and eyes and may persist for the life.[67]

Toxoplasmosis is the illness associated with *T. gondii* infection and may be one of the most common human infections. Serologic surveys indicate that upward of 22.5% of the US population has been infected with *T. gondii* at some time.[67] The illness is usually asymptomatic in immunocompetent individuals, though some with acute illness may develop a flulike illness or cervical lymphadenopathy. Ocular infections such as retinochoroiditis may develop. Those with HIV or immunocompromised by other means (e.g., suppressive drugs) may develop a variety of complications. Pregnant women may also pass the infection to the fetus, with results varying by the trimester of acquisition. Individuals who acquire infection congenitally are at greater risk for developing eye lesions by adulthood. Treatment is available, but is generally reserved for pregnant women and those who are immunocompromised.

There are of course a large number of other parasites we could discuss, but these five do well to illustrate the techniques and challenges associated with their

analysis. Other organisms that may be of interest include *Entamoeba histolytica* and select helminths.

Sample Collection and Analysis

Blood Specimen Collection

The collection of these samples is specified by the CDC's Division of Parasitic Diseases. Venous blood is suitable and of sufficient volume for most tests. Anticoagulants may interfere with some analyses (e.g., malaria), so capillary samples are accepted for some uses. *Thick smears* use lysed red blood cells in a thick layer on the slide. This allows for greater concentration of any parasites, but may also obscure distinctive morphology and make differential identification difficult. *Thin smears* use less blood and spread the layer such that the thickness decreases to a monolayer toward one side of the slide. Though less sensitive that a thick smear (fewer potential parasites present), it allows for much better viewing.[68]

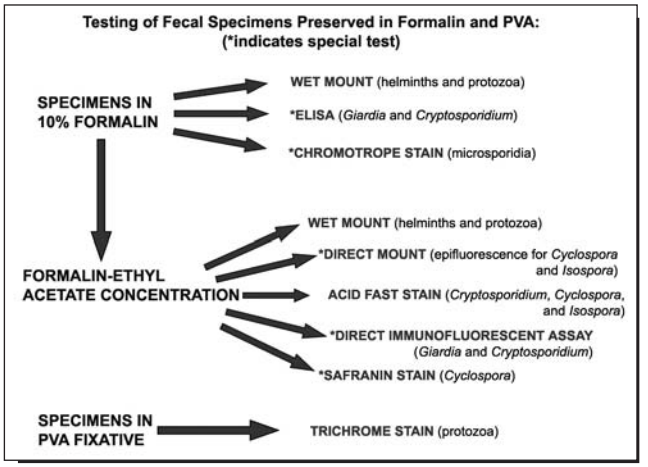
Stool Specimen Collection

The collection of these samples is also specified by the CDC's Division of Parasitic Diseases. Stools are collected in dry, leakproof, clean containers and examined immediately (within 30–60 minutes). Alternatively, they may be chemically preserved with variety of solutions, including 10% formalin, merthiolate iodine formaldehyde (MIH), and Schaudinn's fixative. Each offers different advantages and disadvantages that vary by protozoan. Formalin and polyvinyl alcohol are the most commonly used but may interfere with molecular analysis. Samples preserved in formalin may be directly tested via wet mount, immunoassay, chromatin stain, and UV fluorescence. Sample concentration removes fecal material, concentrates parasitic organisms, and allows further testing such as direct mount, acid-fast, or safranin staining and direct immunofluorescent assays.[68] Figure 5-31 shows how the CDC processes stool samples preserved in formalin.

Sample Analysis

There are multiple methods available for the analysis of clinical (stool) samples for *Cryptosporidium*, *Cyclospora*, and *Giardia*. As a general rule, at least three stool specimens should be examined before a negative result is determined.[69] This rule holds for other sample types as well (e.g., food). Also worth noting is that three samples indicates three separately collected samples, not one sample divided into three sample vials. Finally, a negative result does not guarantee the absence of parasites because of the likely highly heterogeneous distribution of pathogens in the sample.

Figure 5-31 Flowchart showing how the CDC processes and tests samples preserved in formalin and polyvinyl alcohol. (Courtesy of CDC/DPDx.)



The *ova and parasite* (O&P) *examination* is commonly employed for the analysis of stool samples and consists of three steps. First a wet mount is done of the sample to look for motile trophozoites. This cannot be performed on preserved samples, however. Second, any oocysts, cysts, and spores are concentrated from the sample with the use of a flotation or sedimentation procedure (formalin-ethyl acetate solution may be used for sedimentation). This is also examined as a wet mount with/without iodine stain. The third step is the permanent stained smear (e.g., trichome) for the identification of protozoa. Further analyses may be done as required for the particular organism or for more definitive identification.

Wet mounts are moderately useful for screening samples that may have a relatively high number of *Cryptosporidium* and *Cyclospora* oocysts and *Giardia* cysts. This should be done in combination with a more sensitive stain technique or assay. *Cryptosporidium* and *Cyclospora* oocysts are typically round and 4–6 μm and 7–10 μm in diameter, respectively. *Giardia* cysts are typically oval/ellipsoid in shape and are generally 10–14 μm long. An example of these three observed via wet mount is seen in Figures 5-32 to 5-34. A very useful characteristic of *C. cayetanensis* in particular may be used here as it autofluoresces with a bright blue color under UV illumination. An example is seen in Figure 5-35.

Figure 5-32 Wet mount of *Cryptosporidium parvum* oocysts. (Courtesy of CDC/DPDx.)

Figure 5-33 Wet mount of *Cyclospora cayetanensis* oocyst. (Courtesy of CDC/DPDx.)

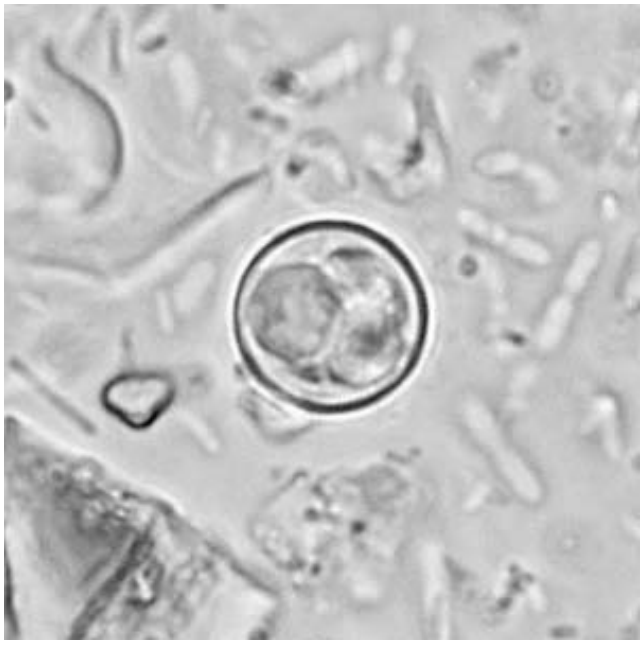


Figure 5-34 Wet mount of *Giardia lamblia* cyst visualized via differential interference contrast. (Courtesy of CDC/DPDx.)

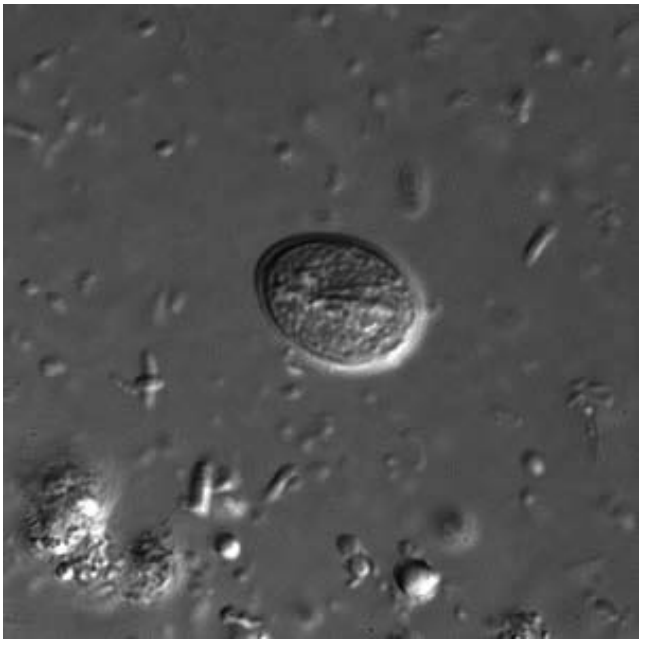


Figure 5-35 *Cyclospora cayetanensis* oocysts fluorescing under UV microscopy. An image bank of full-color photos is available online at http://www.jbpub.com/catalog/9780763771027/. (Courtesy of CDC/DPDx.)

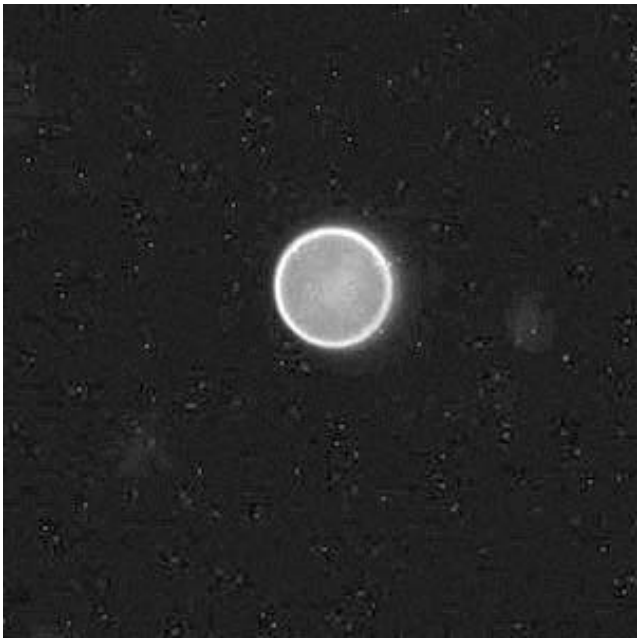


Figure 5-36 Example of an unstained *Toxoplasma gondii* cyst. (Courtesy of CDC/DPDx.)

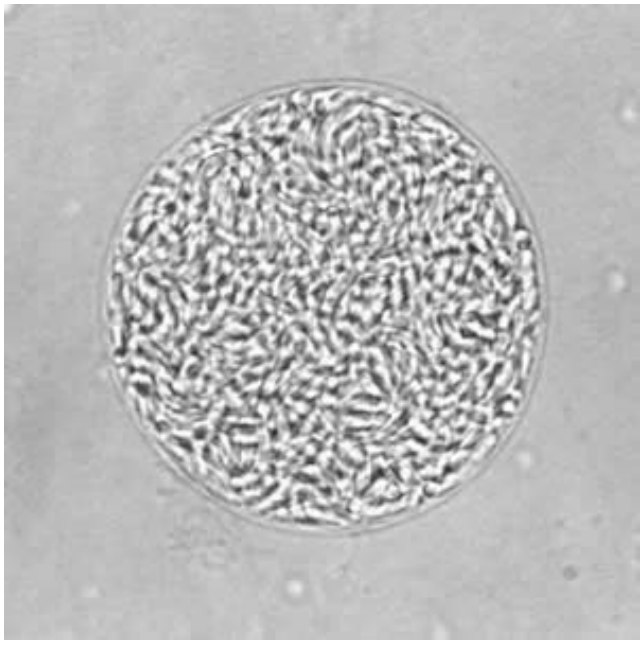
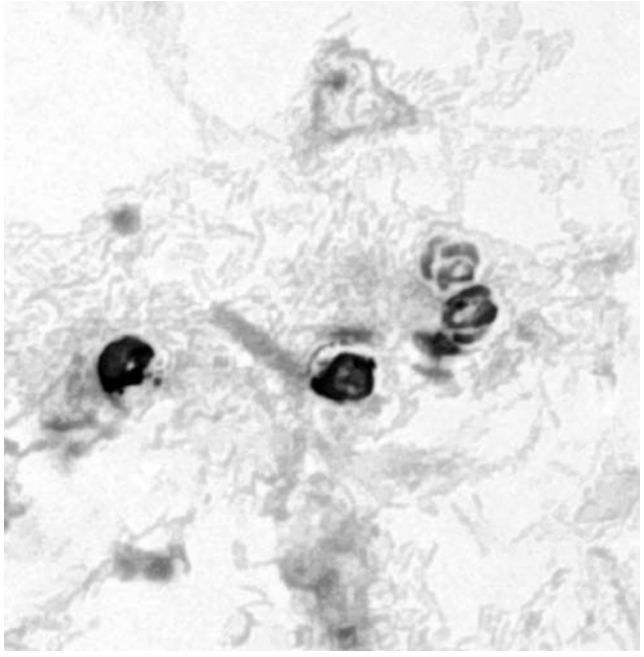


Figure 5-37 *Cryptosporidium parvum* oocysts visible by modified acid-fast stain (sporozoites visible within the two on the right). An image bank of full-color photos is available online at http://www.jbpub.com/catalog/9780763771027/. (Courtesy of CDC/DPDx.)



There are no methods available for the analysis of stool samples for *T. gondii* as humans do not shed oocysts. Serologic testing is the routine method for diagnosis. Tissue biopsies may also reveal infection, but these are not usually performed in PHLs.[69] For the purposes of comparison to other protozoa cysts/oocysts, an example of a *T. gondii* cyst is seen in Figure 5-36. The cysts are typically round in shape and may be anywhere from 5 to 50 μm in diameter.

Staining is often useful to identify parasites and resolve their structure. *Acid-fast staining* is superior to other parasitology stains, such as Giemsa, for the identification of *Cryptosporidium* as it differentiates between oocysts and similarly sized yeasts. Oocysts stain red against a blue-green background. An example is seen in Figure 5-37. There are modifications to this basic technique. *Modified-safranin staining* is better suited for *Cyclospora* as the modified acid-fast technique used for *Cryptosporidium* is variable for this organism. Oocysts stain red/reddish-orange against a blue-green background. An example is seen in Figure 5-38. *Trichrome staining* is more effective for *G. lamblia* analysis than other protozoa discussed, and the trophozoites in particular become quite visible. They are pear shaped and

Figure 5-38 *Cyclospora cayetanensis* oocysts uniformly stained red with safranin. An image bank of full-color photos is available online at http://www.jbpub.com/catalog/9780763771027/. (Courtesy of CDC/DPDx.)

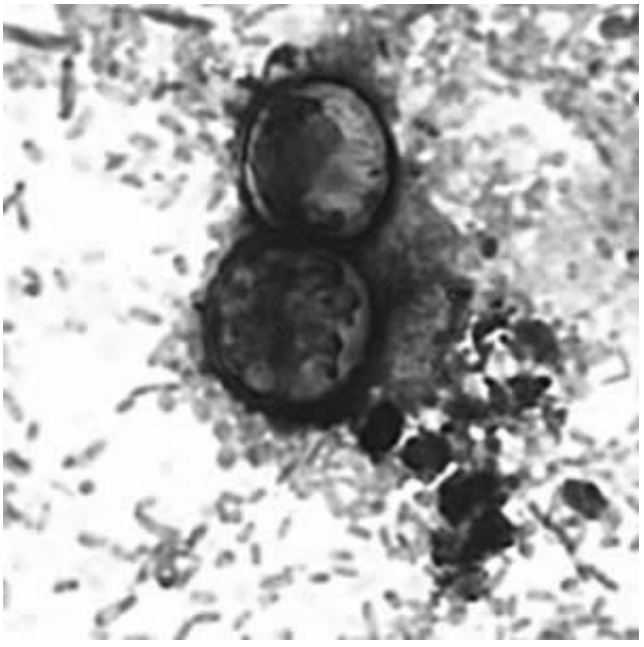
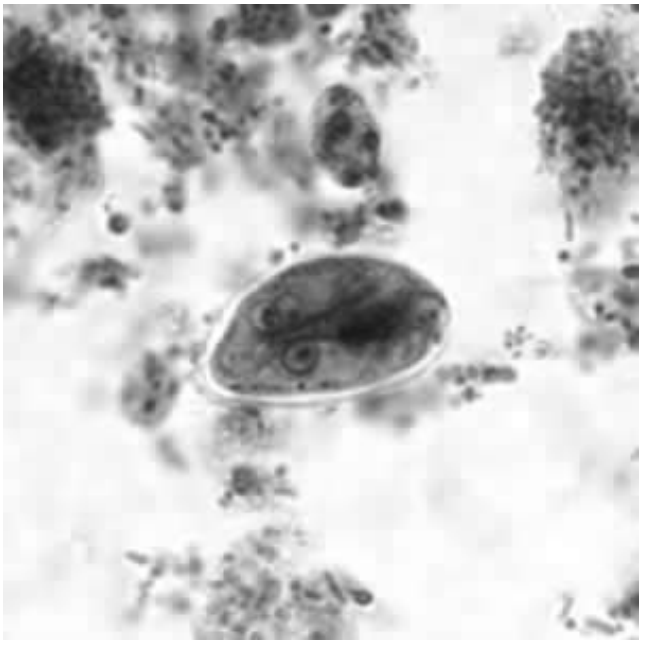


Figure 5-39 *Giardia lamblia* trophozoites visualized with trichrome stain. (Courtesy of CDC/DPDx.)



10–20 μm long. Figure 5-39 shows a trichrome-stained trophozoite with the sucking disk visible.

Staining is also useful for the identification of *Plasmodium*. Here the sample is blood and the goal is to detect the parasites within blood cells. The morphological characteristics of the four *Plasmodium* species known to infect humans are distinguishable through staining and observation. Techniques involve the use of thin and thick blood smears for slides and Giemsa staining. Detailed information concerning the differentiation of *Plasmodium* species can be found on the malaria bench aids Web site at http://www.dpd.cdc.gov/dpdx/HTML/Malaria.htm.[70] Examples of thick and thin smear stained slides are seen in Figures 5-40 and 5-41.

Immunofluorescence antibody analysis has generally superior sensitivity and specificity to staining techniques. However, some fixatives may interfere with the reactions. Fluorescently labeled antibodies are broadly reactive to *Cryptosporidium* spp. and available through multiple commercial sources. An example of fluorescently labeled *Cryptosporidium* oocysts and *Giardia* cysts are seen in Figure 5-42. *Indirect fluorescent antibody* detection is useful for screening blood for *Plasmodium*, but cannot differentiate between newly acquired (acute) infection and the persist presence of immune response to past infection.

Figure 5-40 Thick blood smear showing ring-form trophozoites of *Plasmodium falciparum.* (Courtesy of CDC/DPDx.)

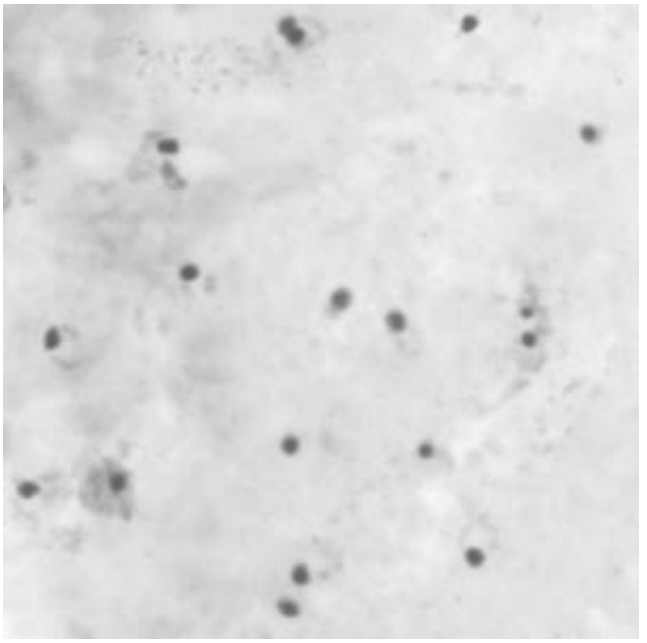


Figure 5-41 Thin blood smear showing ring-form trophozoites of *Plasmodium malariae.* (Courtesy of CDC/DPDx.)

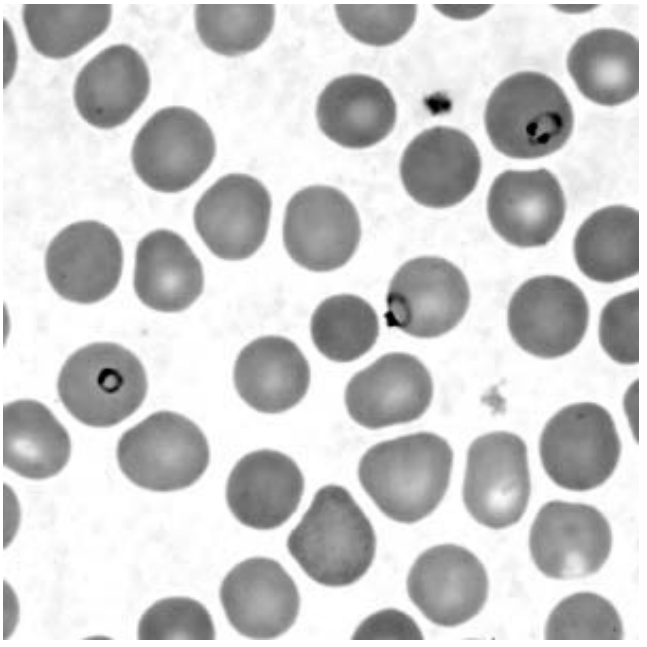
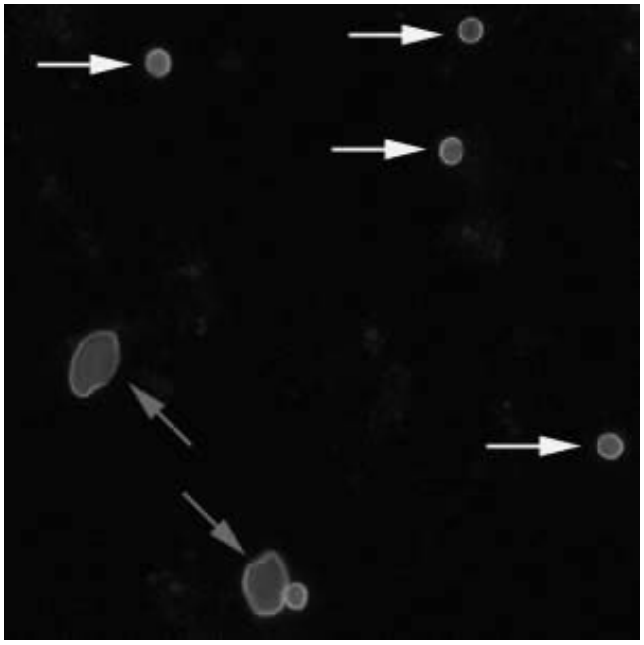


Figure 5-42 Example of immunofluorescent labeling of *C. parvum* oocysts and *G. lamblia* cysts. (Courtesy of CDC/DPDx.)



Species-specific test kits are available, but may cross-react with *Babesia* spp. In the test, blood stage schizonts are exposed to patient serum and homologous antibodies bind to form a complex (if present). Fluorescein-labeled antihuman antibody is added, binds to the complex, and fluoresces a bright green color (Figure 5-43).

Antibody detection in patient sera is the primary method used to diagnose infection with *Toxoplasma gondii*. There are multiple commercial kits available, though their sensitivities and specificities vary widely. (This is of considerable concern as treatment, especially for pregnant women, may be determined on test results.). The tests use two different assays. The IgG assay tests for the presence of *Toxoplasma*-specific antibodies. This indicates whether a person was ever infected. Positives are followed up with an IgM antibody immunoassay to determine recent infection. This is relatively insensitive, as IgM antibodies may remain detectable 18+ months after acute infection. Because of these complexities and the variability of test results, the CDC has developed a testing algorithm to interpret test results. This is available at the Toxoplasmosis diagnostic findings Web site at http://www.dpd.cdc.gov/dpdx/HTML/Toxoplasmosis.htm. Figures 5-44 and 5-45 show positive and negative reactions to immunofluorescent antibodies.

Figure 5-43 Positive immunofluorescence antibody test for *P. malariae* schizonts. An image bank of full-color photos is available online at http://www.jbpub.com/catalog/9780763771027/. (Courtesy of CDC/DPDx.)

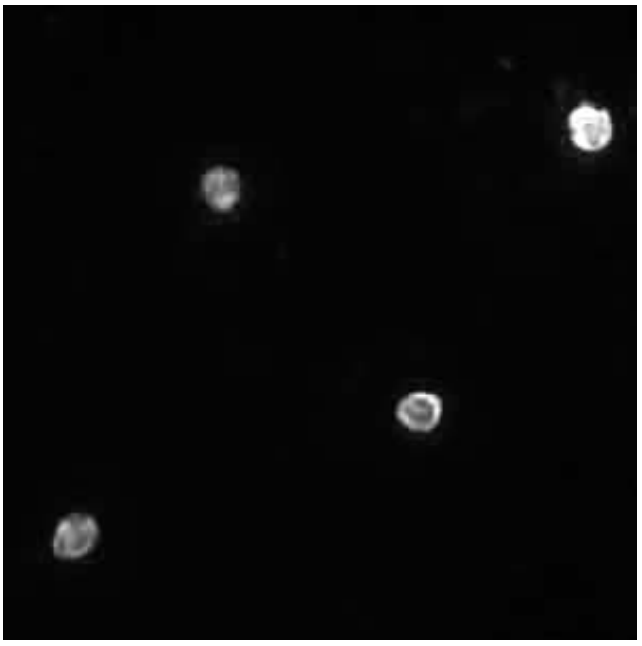


Figure 5-44 Example of a specimen positive for *Toxoplasma gondii* by IgG immunofluorescent. (Courtesy of CDC/DPDx.)

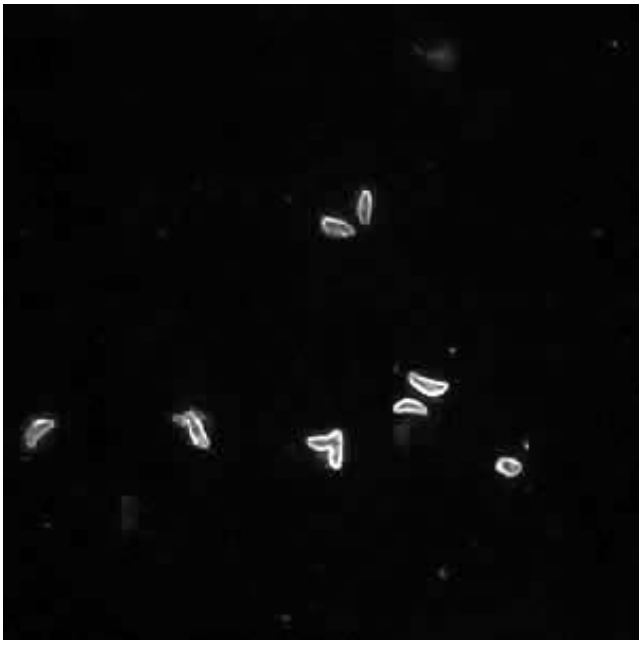


Figure 5-45 Example of a specimen negative for *Toxoplasma gondii* by IgG immunofluorescent assay. (Courtesy of CDC/DPDx.)

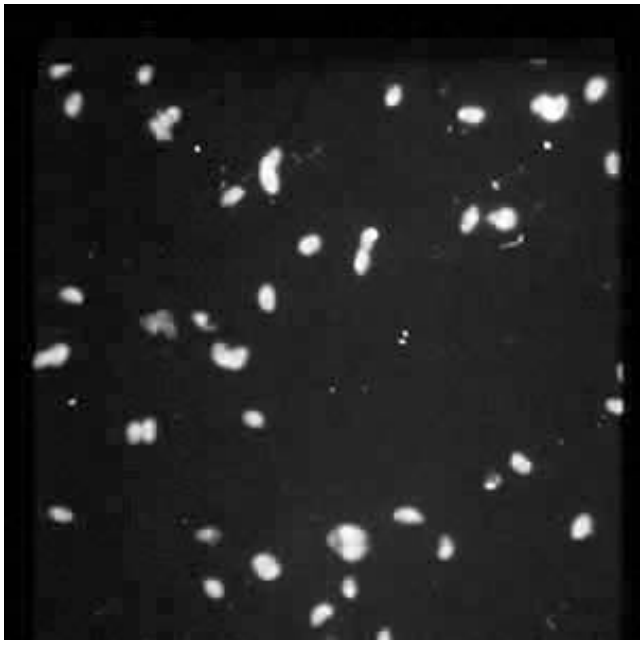
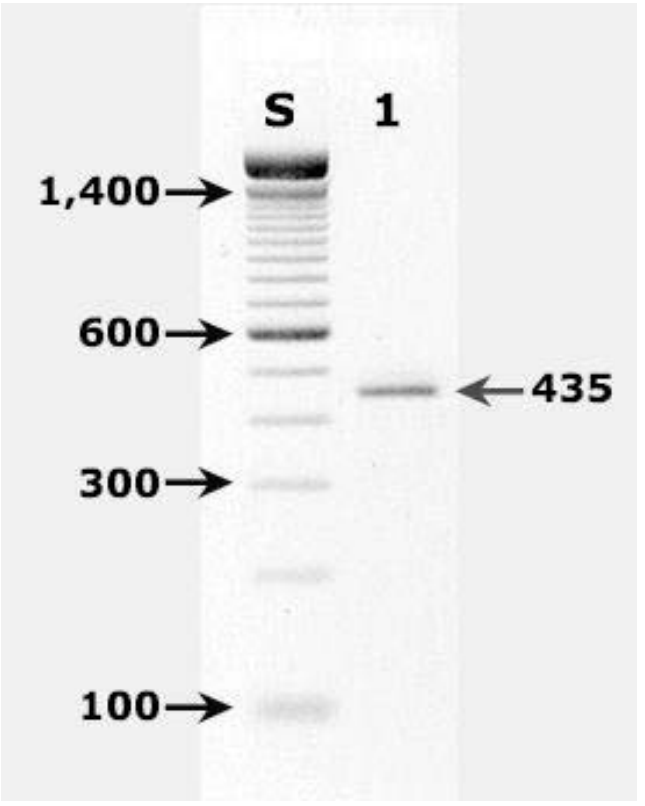


Figure 5-46 Molecular diagnosis of *Cryptosporidium parvum* by PCR with diagnostic band at 435 base pairs. (Courtesy of CDC/DPDx.)



RT-PCR techniques have been developed and validated by the CDC. Figure 5-46 shows an agarose gel analysis with the *C. parvum* diagnostic band at 435 base pairs. *Nested PCR* techniques have been developed, but they may have low sensitivity (62%).[60] The nested technique uses two primers for the first amplification round followed by a different pair for the second. Figure 5-47 shows an agarose gel analysis with the *C. cayetanensis* diagnostic band at 308 base pairs.[63] Straight *PCR* technique has been developed to identify *G. lamblia*. Figure 5-48 shows an agarose gel analysis with the *G. lamblia* diagnostic band at 183 base pairs.[64] *Nested PCR* techniques have been developed and can identify and distinguish the four *Plasmodium* species known to infect humans. Figure 5-49 shows an agarose gel analysis with the diagnostic bands for *P. vivax*, *P. malariae*, *P. falciparum*, and *P. ovale* at 120, 144, 205, and 800 base pairs, respectively.[65]

Other tests include e*nzyme immunoassay kits*, which are available from at least four commercial sources. The sensitivity and specificity of the kits is reportedly superior to that for microscopic examination with sensitivities ranging from 66.3 to 100% and specificity from 93 to 100%.[60] *Other immunologic and biochemical tests* are available that specialize in the detection of *Plasmodium* species-specific antigens and biochemical reactions. These include the detection of a specific antigen, such as histidine-rich protein-2, or a specific enzyme, such as lactate dehydrogenase.

It is worth noting that the absence of stages other than rings and trophozoites suggests a diagnosis of *P. falciparum*, some care must be taken to rule out *Babesia* infection, which causes symptoms similar to malaria. The patient's travel history; the presence of tiny, pleomorphic, and pyriform rings; and an unusual number of erythrocytes infected with multiple parasites can indicate the presence of *Babesia* species rather than *P. falciparum*. For a definitive identification in doubtful specimens, PCR tests are available. The CDC's A–Z Index of Parasitic Diseases (at http://www.cdc.gov/ncidod/dpd/parasites/index.htm) lists over 100 different parasites of all three types (protozoa, helminths, and ectoparasites). This underscores the breadth of potential infection both worldwide and in the United States and

Figure 5-47 **Molecular diagnosis of *Cyclospora cayetanensis* by PCR with diagnostic band at 308 base pairs. (Courtesy of CDC/DPDx.)**

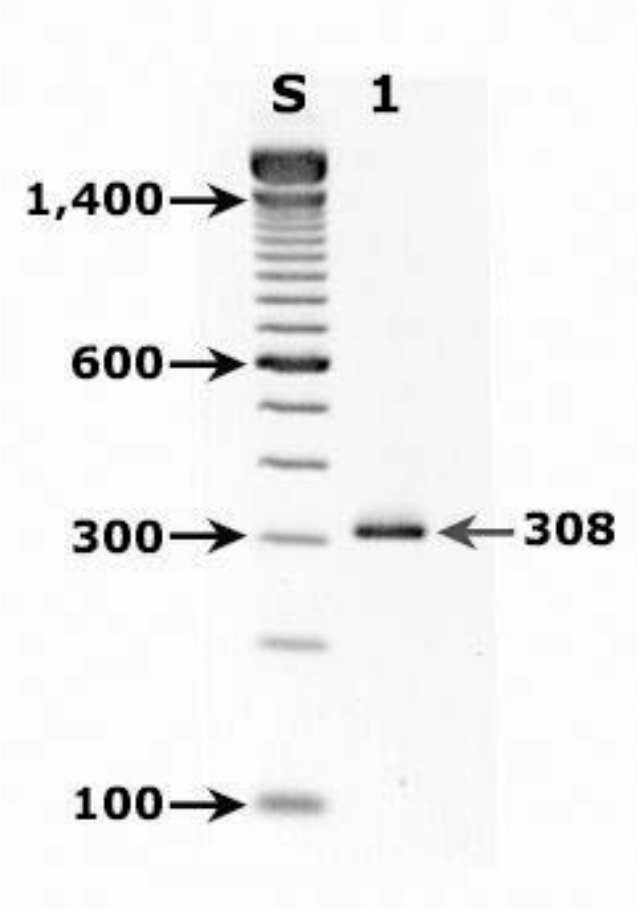


Figure 5-48 **Molecular diagnosis of *Giardia lamblia* by PCR with diagnostic band at 183 base pairs. (Courtesy of CDC/DPDx.)**

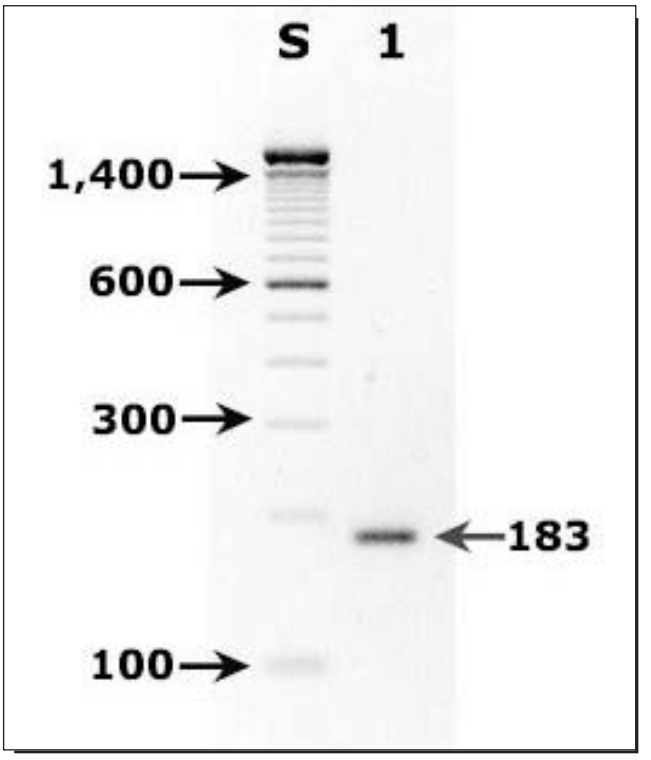
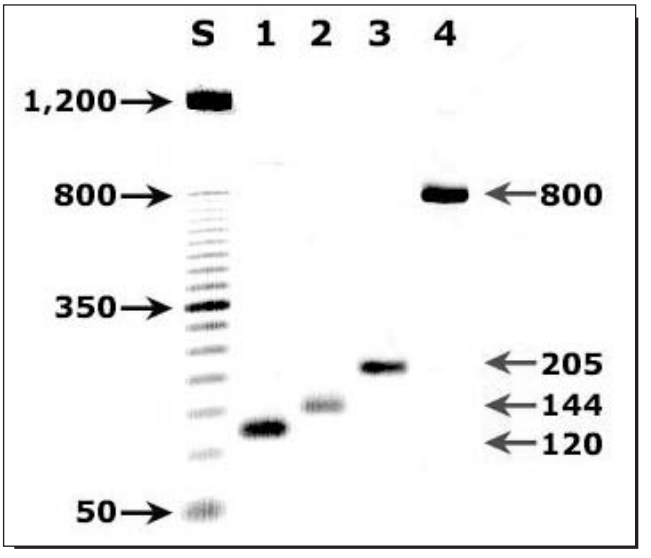


Figure 5-49 **Molecular diagnosis of *Plasmodium* spp. by PCR with diagnostic bands for *P. vivax, P. malariae, P. falciparum,* and *P. ovale* at 120, 144, 205, and 800 base pairs, respectively. (Courtesy of CDC/DPDx.)**



the importance of laboratory capability for swift analysis. The IDPH Division of Laboratories offers routine analysis for 20 individual parasites and intestinal flagellates as a group.

The IDPH laboratories received 2988 samples for parasite analysis (including 45 blood samples) from July 2007 through June 2008 (unpublished data). The number is interesting in that it is so small. If more people and clinicians recognized the importance of stool collection and analysis in outbreak investigations, the number submitted could easily be increased by an order of magnitude. As it is, PHL analysis of stool samples for protozoan parasites is an important component in ensuring the safety of drinking and recreational waters. It is also an important part of surveillance and diagnosis of malaria, which has the potential to become endemic in the United States once again.

Sexually Transmitted Infections

A sexually transmitted infection (STI), also known as a sexually transmitted disease (STD), is one that has a significant chance of transmission through vaginal, anal, or oral contact. The term STI is preferred over STD as one may be infected with and/or pass an infectious agent without any symptoms of disease. STIs run the full gamut from relatively benign (e.g., herpes) to potentially life-threatening (HIV), and from the curable (chlamydia) to those with symptom-only treatments (herpes). In 2008, it was reported 26% of US adolescent females were infected with at least one STI and intervening in their transmission and identifying those infected for treatment remains a large public health challenge.[71]

Chlamydia trachomatis, *Lymphogranuloma venereum*, and *Neisseria gonorrhoeae*

Chlamydia

Chlamydia infection is caused by the bacterium *Chlamydia trachomatis* (CT). It is the most commonly reported bacterial infection worldwide and the most commonly reported STI in the United States. In 2006, the number of reported cases exceeded 1 million for the first time.[72] It is estimated that the actual prevalence is in excess of 2.2 million. It is difficult to get an accurate count of those who are infected as up to three quarters of women and half of men show no symptoms (are asymptomatic). Chlamydia is spread during oral, vaginal, and anal sex and may be spread from mother to child during childbirth. Studies have shown that that those infected with chlamydia are at increased risk for coinfection with other STIs such as gonorrhea, and up to five times more likely to acquire HIV (if exposed).

Such symptoms as do present are often mild and usually resolve (disappear) within a matter of weeks. For women, these may include abnormal vaginal discharge, a burning sensation when urinating, lower abdominal pain, low back pain, nausea, fever, pain during intercourse, and/or bleeding between menstrual periods. It is estimated that the infection will spread to the uterus and/or fallopian tubes and cause pelvic inflammatory disease (PID) in up to 40% of those untreated.[73] Further morbidity includes chronic pelvic pain, infertility, and ectopic pregnancy. In men, symptoms present more frequently and may include penile discharge, a burning sensation when urinating, and/or burning and itching around the opening of the penis. Further morbidity is rare. All who have receptive anal intercourse may acquire infection in the rectum that may present as rectal pain, discharge, or bleeding. Those engaged in oral sex may acquire infection in the throat.

The infection itself is readily treated once detected. The two most used medications are single doses of azithromycin and a twice-daily week-long regimen of doxycycline.[74] However, for a treated individual to become and remain free of infection, their infected partner must also be treated. It has been quite difficult for this to happen to a high degree for a number of reasons (e.g., refusal to divulge/inform partners; partner unknown). Many individuals are therefore at high risk for reinfection and return on multiple occasions for testing and the CDC recommends that those infected return for retesting after 3 months. Unfortunately, multiple infections increase the risk of serious morbidity.

Chlamydia infection interventions therefore remain a high priority for public health. The annual cost of treating morbidity associated with untreated infection is estimated to be in excess of $2.4 billion in the United States and $10 billion worldwide.[75,76] Because of the relative mildness of symptoms presented by the minority of cases, many people not only remain infected but also go on to infect others. Untreated chlamydial infection is one of the leading causes of infertility in the United States (as reflected in the name of the CDC grant program, Infertility Prevention Program, which funds chlamydia intervention efforts by state health departments).

Lymphogranuloma venereum

Lymphogranuloma venereum is an ulcerative STI caused by three strains of CT (L_{1-3}). The frequency of infection is thought to be quite low but, because the symptoms may be mistaken for ulcerative colitis, it may often be misdiagnosed. Symptoms may include genital papule, ulcers in the genital area or rectum, bleeding, pain, and discharge. Currently recommended treatment is a 3-week regimen of doxycycline (compared to 1 week for common CT infection). Diagnosis is based on clinical examination, and there is no currently validated laboratory test.[77]

Gonorrhea

Gonorrhea infection is caused by the bacterium *Neisseria gonorrhoeae* (GC). It is the second most commonly reported bacterial disease in the United States, with 355,991 reported in 2007 and infecting 118.9/100,000 of the general population.[78,79] This is a $>$ 4% increase from 2005 and shows the somewhat intractable nature of the infection in the population. Unlike chlamydial

infection, GC is more equitably distributed by gender with men and women becoming infected at approximately equal rates (113.7 versus 123.5/100.000). Unfortunately, the disparity of infection by race is even greater for GC than CT, with rates among Blacks approximately 19 times greater than among Whites (662.9 versus 34.7/100,000).[80]

Similar to CT, the majority of women are asymptomatic, and untreated infection may lead to the same morbidities (PID, chronic pelvic pain, ectopic pregnancy, and infertility). Symptoms of infection in females include a burning sensation when urinating, vaginal discharge, and vaginal bleeding. These symptoms are often mistaken for bladder or vaginal infections. Infection usually causes symptomatic urethritis in males, occasionally progressing to epididymitis. The most common symptoms include a burning sensation when urinating, a discharge from the penis, and painful/swollen testicles.

The common treatments include single doses of oral cefixime or intramuscular injection of ceftriaxone. It should be noted that the recommendation is to also treat presumptively for chlamydia infection as well unless it has been ruled out. The treatment varies by dose and antibiotic regimen(s) depending on the location of the infection and its degree of resistance. Gonorrhea has become increasingly resistant to antibiotics in recent years. These data are gathered through the Gonococcal Isolate Surveillance Project (GISP), which was established in 1986 to monitor antimicrobial trends in selected STI clinics nationwide. In 2007, 27% of isolates collected from these sites were resistant to the following antibiotics: penicillin, tetracycline, and ciprofloxacin.[80] The CDC has more recently determined that fluoroquinolones as a class are no longer recommended because of widespread resistance, advised that only cephalosporins are still recommended and available for treatment.[81]

It is important to identify and treat those infected with GC. Gonorrhea is spread during oral, vaginal, and anal sex and may be spread from mother to child during childbirth. Studies have shown that those infected with gonorrhea are often coinfected with chlamydia and are more likely to acquire HIV (if exposed).[82] Left untreated, gonorrhea may cause PID in women, which may be followed by chronic pelvic pain, ectopic pregnancy, and infertility. Gonorrhea may also be passed to infants during birth, leading to potential blindness and blood and joint infections if untreated. In men, the most common morbidity is epididymitis. For both sexes, gonorrhea has the potential to disseminate to the joints and blood where it can become life-threatening.

Organism Characteristics

Chlamydia trachomatis is an obligate intracellular parasite and is thus Gram-indeterminate (though the bacterium contains elements that are associated with Gram-negativity). There are multiple strains (A–L) causing various diseases. *Neisseria gonorrhoeae* is a fastidious, Gram-negative, nonspore forming, nonmotile diplococcus (appearing in pairs) with a distinctive kidney-shaped appearance. It requires chocolate agar (or other specialized media) and supplemental carbon dioxide (CO_2) for optimal growth and is oxidase positive.[83]

Sample Collection

The collection of clinical samples for the analysis of CT/GC is fairly standard. There are slight variations by test manufacturer in terms of preservation and time requirements. In general, the following describes how different samples are collected:

- Endocervical swabs: Excess mucous is removed from the cervical os, a sample collection swab is inserted into the cervical canal and rotated 10–30 seconds and removed, and the swab is placed in a transport tube (with or without preservative) for delivery to the lab.
- Male urethral swabs: The patient should refrain from urination for 1+ hours prior to collection, the sample collection swab is inserted 2–4 cm into the urethra and rotated 2–5 seconds and removed, and the swab is placed in a transport tube (with or without preservative) for delivery to the lab.
- Urine: The patient should refrain from urination for 1+ hours prior to collection, 15–60 ml of first-catch urine is collected in a specimen cup. This may be mailed directly, or an aliquot taken and added to preservative first.
- Vaginal swabs: The sample collection swab is inserted into the vagina approximately 2 in. past the introitus and rotated 10–30 seconds, removed, and placed in a transport tube.

The delivery of collected samples is a significant issue for PHLs, and conditions vary by test and manufacturer. Some sample types must be maintained between 2° and 8°C and be delivered within 6 days of collection. Others may be maintained at up to 30°C and may be stored for up to 30 days. Ensuring that samples sent from all over the state arrive at the laboratory within the required time

frame, and at the proper temperature, is a significant issue for the laboratory to address.

Sample Analysis

Microscopy

Microscopic examination of a genital swab smear for gonorrhea infection has limited utility and is usually done onsite rather than in the laboratory. Gram staining of urethral smears from symptomatic men may have sensitivities in excess of 90% and can be considered diagnostic. However, the sensitivities fall off rapidly with asymptomatic men and other sample types (e.g., sensitivity of endocervical swabs is estimated at 50–70%) because of the potential colonization of the swabbed area with other Gram-negative coccobacillary organisms.[84] Thus, the testing of other sample types (e.g., endocervical or rectal) is not recommended.[74] Though not recommended, Figure 5-50 shows GC in a stained rectal smear as an example.

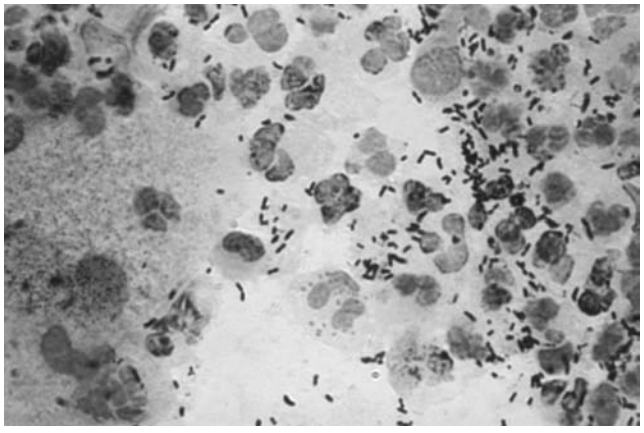
Nucleic Acid Amplification Test

The methodology of nucleic acid amplification testing (NAAT) has the benefits of being quite sensitive, quite specific, and remarkably swift. These tests (and the nonamplified tests described later) have been widely adopted and have replaced most other methods for routine testing. In fact, they have recently been officially recommended as the primary means of analysis of samples for both CT and GC.[85] The increased sensitivity is because of the ability (in theory) to find, amplify, and detect a single strand of target genetic material. Tests that at one point took days/weeks to complete may now be performed in a matter of hours. In addition, the analyses are able to accommodate a wider variety of samples, which may make sample collection and screening compliance easier. For a more detailed description of the basis of nucleic acid amplification, see Chapter 2. There are several popular commercial test systems using different variations of DNA/RNA analysis:

- Becton, Dickinson and Company's ProbeTec (Franklin Lakes, NJ) uses strand displacement on the cryptic plasmid DNA
- Gen-Probe's Aptima (San Diego, CA) uses transcription-mediated amplification on ribosomal RNA
- Roche's Amplicor (Basel, Switzerland) use PCR on cellular DNA

All these tests offer methods/reagents for the analysis of both CT and GC. Two of the more popular tests are the BD and Gen-Probe kits, with vendor-specific variation that may allow samples to be tested for both CT and GC, either individually or simultaneously. Sample analyses are tightly controlled by the test kit manufacturers and unique to each test method. They are automated to varying extents, based on both proprietary instrumentation and test methodology. Table 5-1 shows the reported sensitivities and specificities of these tests in test populations in comparison to culture. While individual studies of test method sensitivities vary in actual reported values, the conclusion is that CT NAATs are more sensitive than non-NAATs. Test sensitivity is not as clear cut with GC, as the superiority of NAATs versus hybridization (discussed later) and culture vary depending on sample type, with the best NAAT performance on endocervical swabs and lower on urine.[86] The positive predictive value (proportion of all test positives that are truly positive) for both tests are greatly dependent on the local prevalence, and low prevalence populations may need confirmatory analysis if the initial test is positive. For a discussion of the definitions and calculations of sensitivity, specificity, and positive and negative predictive value, see Chapter 12.

Figure 5-50 ***Neisseria gonorrhoeae*** **revealed by Gram stain in a rectal smear. (Courtesy of CDC/Joe Miller.)**



Nonamplified Nucleic Acid Probe Tests

There are two FDA-cleared hybridization assays for the analysis of CT and GC. The Gen-Probe Pace 2C and Digene Hybrid Capture II (Digene Corporation, Valencia, CA) are able to analyze for the presence of both organisms

Table 5-1 Comparison of Chlamydia and Gonorrhea Method Performances

Test	Sample type(s):*	Chlamydia		Gonorrhea	
		Sensitivity	Specificity	Sensitivity	Specificity
Culture[84,87]	Genital swabs	70–80	> 99	> 95	> 99
Aptima Combo 2[88]	Endocervical swabs	94.2	97.6	99.2	98.7
	Male urethral swab	95.9	97.5	99.1	97.8
	Female urine	94.7	98.9	91.3	99.3
	Male urine	97.9	98.5	98.5	99.6
	Vaginal swab	96.6	96.8	96	99.2
ProbeTec[89]	Swabs, urine (Fuller)	97	100	99	100
	Urine (Gaydos)	96	100	—	—
	Urine (Chan)	95.3	99.3	100	99.7

*In addition to these sample types, others are in development and the process of validation. These include the use of self-collected swabs from the vagina, urethra, and rectum.

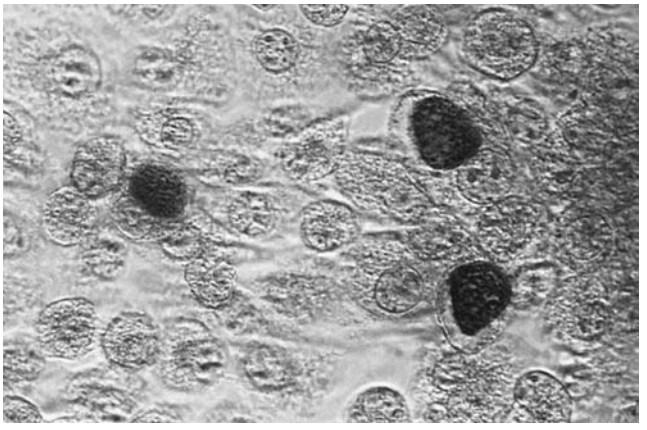
within a single specimen. The dual tests do not differentiate between organisms, but Gen-Probe offers the Pace 2CT and Pace 2GC variants that do differentiate. The Gen-Probe assay uses a DNA probe that is complementary to specific ribosomal RNA. The Digene assay uses probes specific for genomic and cryptic plasmid CT/GC DNA sequences. Hybridization assays have a general advantage of not requiring sample refrigeration and allowing longer time periods between collection and analysis.[86]

Culture

Culturing is still considered the gold standard for the identification of many microorganisms, though the emergence and validation of the nucleic acid tests described previously for CT and GC are beginning to supplant culture. Few laboratories still perform this test for chlamydia, and the actual testing methodologies are not standardized among labs. Chlamydia culture relies on the inoculation of a confluent monolayer of host cells (e.g., cycloheximide-treated McCoy cells) with a suspension of the specimen. The cells are then examined after 48–72 hours for the presence of inclusion bodies (CT bacterium) that are observable via immunofluorescent staining. Figure 5-51 shows a McCoy cell monolayer with three observable CT inclusions.

The culture methodology for GC is not as varied or subject to individual lab characteristics as CT methods. Specimens are streaked on appropriate medium, incubated at a higher temperature (35° to 36.5°C) and 5% CO_2, and checked at 24-hour intervals. Presumptive identification of the resultant growth can be made via Gram staining and oxidase testing. Despite the time requirements and technical difficulty, culturing is sometimes the method of choice when the sample may be part of a legal case as an actual isolate is retained. Culturing is also needed for microbial susceptibility studies. Unfortunately, there are significant time delays compared to other methods. While culture's specificity may be quite high, the sensitivity of newer testing methods may be significantly higher.

Figure 5-51 *Chlamydia trachomatis* inclusion bodies in a McCoy cell monolayer. (Courtesy of CDC/Dr. E. Arum; Dr. N. Jacobs.)



Enzyme-linked Immunoassay Tests

There have been several immunoassay tests marketed for the detection of CT. Similar tests for GC have not been shown to be cost-effective and are not in general use. The immunoassays for CT make use of a monoclonal antibody linked to an enzyme. The antibody is directed to genus-specific lipopolysaccharide proteins (LPS). When bound, the enzymes turn a colorless substrate into a colored form that can be detected with a spectrometer. There is the potential for false-positive results as the antibody is not species specific and may cross-react with the LPS of other organisms or *Chlamydia* spp. Positives should be followed up with a species-specific EIA. Examples of current commercial tests include the Clearview Chlamydia MF (Inverness, Orlando, FL) and BioStar Chlamydia OIA (Biostar, Inc., Boulder, CO).

Direct Fluorescent Antibody Tests

Direct fluorescent antibody (DFA) tests are much the same as EIA in that the primary method of detection uses specific antibodies. Whereas the EIA test kits mix the sample with the antibodies and use linked enzymes to affect a color change, DFA uses a microscope slide smear of the specimen. The smear is then stained with fluorescein-labeled antibodies directed toward either LPS (described previously in EIA) or chlamydia major outer-membrane protein (MOMP). The latter is considered highly specific to CT, while the former retains the potential for cross-reactivity with other organisms. While the identification of GC by DFA is possible, and has been done for specialized purposes, there are no current tests available. Current CT tests include the Pathfinder Chlamydia DFA (Bio-Rad Laboratories, Hercules, CA). Figures 5-52 and 5-53 are examples of each organism identified through the use of fluorescent antibodies.

Figure 5-52 ***Chlamydia trachomatis*** **in HeLa cells observed with fluorescent antibody stain. (Courtesy of CDC/Joe Miller.)**

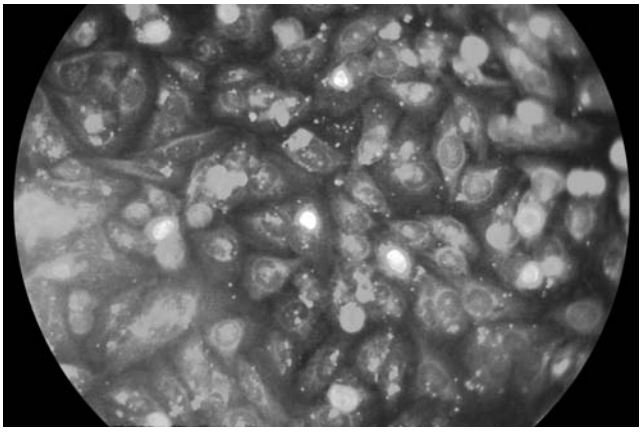
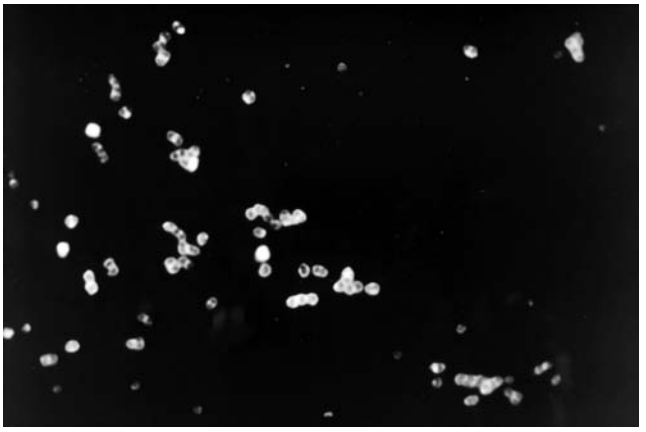


Figure 5-53 Fluorescent antibody stain used to reveal *Neisseria gonorrhoeae.* (Courtesy of CDC/Dr. M. S. Ferguson.)



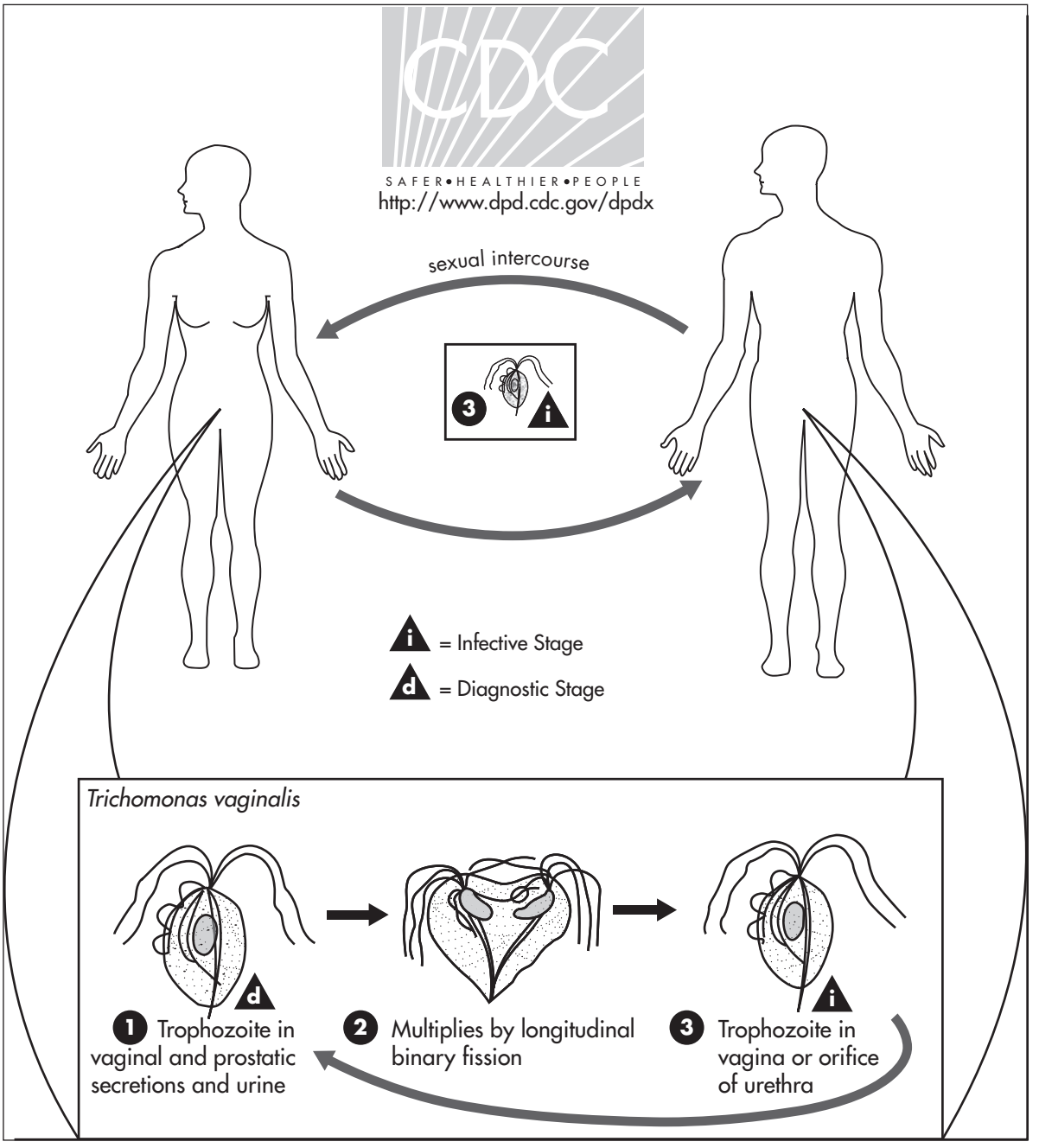
Serology Tests

Serological tests for CT infection are not considered of value for finding new/current infections. The antibody response to CT infection is frequently long-lasting and the presence of antibodies may not correlate with current infection. There are no comparable serologic tests for GC.

Trichomonas vaginalis

Trichomoniasis (Trich) is caused by the parasite *Trichomonas vaginalis*. It is the most common pathogenic protozoan of humans in industrialized countries and is the most common curable STI in sexually active younger women. Unfortunately, there are currently no national surveillance data, and accurate estimates of rates and prevalence are subject to more variability than they are for other reportable STIs. That said, the CDC estimates that there are approximately 7.4 million new cases annually.[90,91] Overall US prevalence is 3.1%, with Blacks being disproportionately infected at 13.3/100,000 (from 2001 to 2004 National Health and Nutrition Examination Survey [NHANES] data).[92] The life cycle of the protozoa is seen in Figure 5-54.

Figure 5-54 Life cycle of *Trichomonas vaginalis.* (Courtesy of CDC/DPDx.)



The vagina in women and urethra in men are the most common points of infection. Transmission occurs through penis–vagina or vulva–vulva contact with an infected partner. While women can acquire it from partners of either gender, men usually only contract it from infected women partners. The great majority of men do not present with any symptoms. Those that do present are usually mild and include mild penile irritation, slight discharge, or slight burning after urination or ejaculation. Women are more likely to become symptomatic within 5–28 days after exposure. Common signs include a frothy, yellow-green discharge, odor, discomfort during urination or intercourse, and/or irritation and itching in the genital area. Lower abdominal pain may occur, but is rare. Once detected, recommended treatment is metronidazole or tinidazole and it is recommended that the patient's partner

be treated concurrently.[74] It is possible for an individual who has either never had symptoms, or had mild symptoms that resolved on their own, to still infect others.

Organism Characteristics

Trichomonas vaginalis is a flagellated protozoan. The trophozoite form is oval and approximately 7 by 15 μm in size with 5 flagella. It displays characteristic jerky and nondirectional movements under wet mount. Unlike most other protozoa discussed in this text, trichomonas may be cultured under anaerobic conditions and decreased pH.[93]

Sample Collection

Swabs from the vaginal cavity may be used for all analyses and swabs from the male urethra may be used for culture.

Sample Analysis

Analysis of samples for trichomonas is infrequently performed at the state laboratory because of the availability of relatively good, and swift, onsite tests. Three of the more commonly used methods include:

- Direct microscopy: This is a direct examination of a wet mounted slide preparation of the vaginal secretion for the parasite. Sensitivity is only on the order of 60–70% and results are immediately available. This method is not sensitive for samples from males.[74] Figure 5-55 shows the presence of trichomonas in a vaginal smear.
- Immunoassay: Genzyme Diagnostics has a rapid test (OSOS Trichomonas Rapid Test; Cambridge, MA) using color-conjugated antibodies targeted to trichomonas proteins. Presence of the proteins causes conjugation with the antibodies and a subsequent observable color change as the sample travels along a dipstick. Sensitivity compared to a composite reference is $> 83\%$.[94] Results are available in ~10 minutes.
- Nucleic acid probe: Becton Dickinson has a test (Affirm VP III) using a probe for the detection of DNA from *T. vaginalis* (and *Candida* spp. and *Gardnerella vaginalis*).[95] Sensitivity is $> 97\%$. Results are available in ~45 minutes.

Culture

The false-positive rate in low prevalence populations can be high, and culturing is still the gold standard for sensitivity and specificity. For men, the best test is culturing of a urethral swab, urine, or semen. Culture consists of the inoculation of selected medium (e.g., trichomonas medium or modified Columbia agar [MCA]), incubation for up to 6 days, and analysis by wet mount microscopy. The use of MCA yielded a sensitivity of 98.5%, and is reported to be reliable and easy to perform.[96] An example of cultured and Giemsa-stained *Trichomonas vaginalis* is seen in Figure 5-56.

Bacterial Vaginosis

Unlike the previous infections, bacterial vaginosis (BV) is not associated with infection of a single organism. The

Figure 5-55 ***Trichomonas vaginalis*** **as seen via phase contrast wet mount microscopy. (Courtesy of CDC.)**

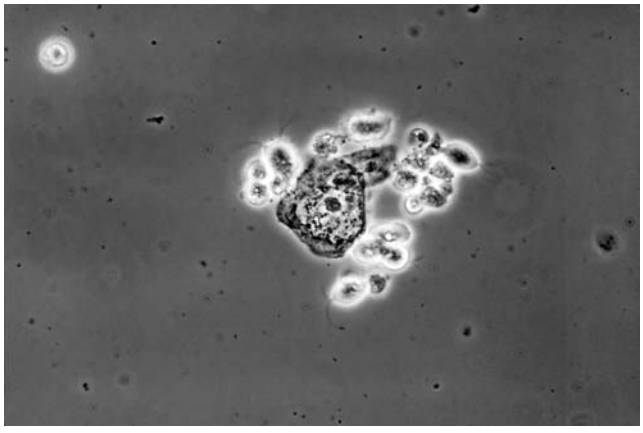
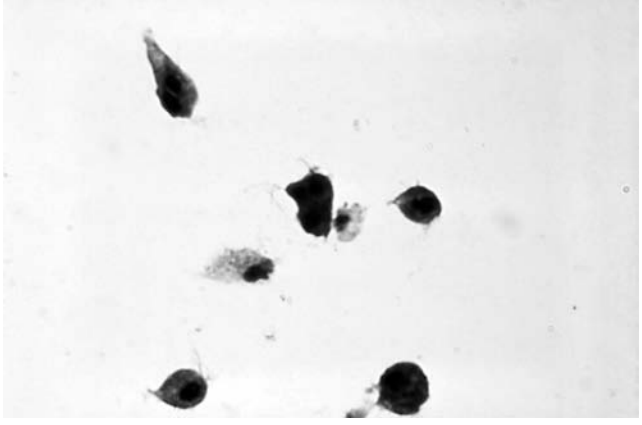


Figure 5-56 ***Trichomonas vaginalis*** **as revealed by Giemsa-stained culture. (Courtesy of CDC.)**



condition arises when the normal distribution of naturally occurring flora in the vagina becomes disrupted, and is the most common cause of vaginal discharge. Specifically, levels of *Lactobacillus* spp. decrease while levels of *G. vaginalis*, *Mycoplasma hominis*, and anaerobic species, such as *Prevotella* and *Mobiluncus*, increase.[74,97] While having a new or multiple sex partners is a risk factor for getting BV, it is unknown what role sexual activity plays. In fact, those who have never had intercourse may still be affected.

BV data are not collected in the same manner as STIs, and prevalence and incidence rates are difficult to calculate. Studies do indicate that BV is relatively common among women of reproductive age, with a prevalence as high as 16% in pregnant women. Racial disparities are again evident as the rate among Whites is approximately 9% versus 23% in Blacks.[98] Greater than 50% of women with BV are asymptomatic. For the others, the most common symptoms of BV include a strong/unpleasant odor, vaginal discharge, burning during urination, and itching in the genital area. Recommended treatment is oral metronidazole twice daily for 7 days, metronidazole gel intravaginally once a day for 5 days, or clindamycin intravaginally once a day for 7 days.[74]

Sample Collection

Swabs from the vaginal cavity are used for all analyses.

Sample Analysis

Diagnosis is often done onsite by a pelvic exam using established clinical criteria. The Affirm VP III discussed previously as part of trichomonas analysis may be clinically useful onsite by testing for high concentrations of *G. vaginalis*.

Microscopic examination of a Gram-stained sample is considered the gold standard for diagnosis and may be done onsite or in a laboratory. Specifically, the examination looks for the relative concentrations of long Gram-positive rods (lactobacilli), Gram-negative and Gram-variable rods and cocci, and curved Gram-negative rods. Culture is not recommended because of nonspecificity.

Treponema pallidum

Syphilis is one of the oldest recognized STIs and remains a significant source of disease in the present day. After a steady decline in the 1990s, the number of cases reported annually has increased steadily in the 21st century. There were 40,920 cases of syphilis (all stages) reported in the United States in 2007, an increase of 10.7% from 2006. Syphilis is more geographically distinct than other STIs, with half of all 2007 primary and secondary (P&S) cases being reported from only 23 counties and 2 cities in the United States. Additionally, the south accounted for 48.8% of all P&S cases in 2007. This geographic clustering, coupled with low incidence in the late 1990s, led the CDC to develop the National Plan to Eliminate Syphilis in 1999.[99,100]

Gender disparities in infection are seen as men are more likely than women to be diagnosed with P&S syphilis (6.6 versus 1.1/100,000). This disparity is the opposite of chlamydial infection where women are approximately four times more likely than men to be infected. Also different from CT is the age group at highest risk for infection, with the group aged 25–29 at highest risk. Racial disparities continue to be exhibited, with Blacks more likely to become infected than Whites (14 versus 2/100,000). Syphilis is also different in that the majority of new P&S cases are from men who have sex with men (MSM; 65%, 2007). Syphilis infection also increases the risk of passing or acquiring HIV. Early syphilis detection is of special concern for pregnant women as the infection may pass to the baby, resulting in perinatal deaths in up to 40% of cases. Studies have also shown that untreated infection in the 4 years preceding pregnancy may lead to fetal infection in upward of 80% of cases. Congenital syphilis occurred at 10.5 cases/100,000 live births in 2007.[99,100]

Syphilis is transmitted through direct contact with a chancre (open syphilis sore.) There is usually a single sore, which appears on the external genitalia, anus, or lips or within the vagina, rectum, or mouth. Internal sores are more likely to go unnoticed, increasing the risk of transmission if the individual is not aware of his or her status and engages in unprotected sex. Transmission may occur during vaginal, anal, or oral sex. Treatment of P&S syphilis for those infected for over 1 year is a single intramuscular injection of benzathine penicillin. Longer infections may require additional doses, though there is no consensus on this.[74]

Sidebar 5-10 Stages of Untreated Syphilis Infection[99,101]

- *Primary:* Characterized by the appearance of an open sore (chancre) at the site of entry into the body within 10 to 90 days. There may be multiple sores. Though open, they are not usually painful, resolve on their own within 3 to 6 weeks, and may go unnoticed (especially if in

the vagina or rectum). Therefore, there is good opportunity for disease transmission as this occurs when in contact with a sore.

- *Secondary:* Characterized by the appearance of one or more nonitching rashes. Rashes on the palms of the hands and bottoms of the feet are especially characteristic, though do not always appear. Rashes may also resemble those caused by other diseases and may be too faint to notice. Other characteristics of secondary syphilis may include lesions in different mucous membranes, fever, sore throat, headaches, patchy hair loss, fatigue, and swollen lymph glands. These will all resolve without treatment.
- *Latent (early and late) and Late:* During the latent stage, the individual is still infected but the bacterium is not active. If detected within 1 year of infection, it is termed early latent. All other cases are considered late latent. The latent period may last 10 to 20 years. Approximately 15% progress to late stage disease. This is potentially life threatening and may include damage to internal organs, heart and blood vessels, nerves and eyes, liver, bones, and joints. Symptoms include uncoordinated muscle control, numbness, gradual blindness, dementia, and possibly death.
- *Neurosyphilis:* This is characterized by infection of the brain and spinal column and symptoms generally occur 10 to 20 years after the initial infection. The four types are asymptomatic, general paresis, meningovascular, and tabes dorsalis. Symptoms are related to nervous effects and may include blindness, depression, headache, inability to walk, paralysis, seizures, and tremors.

Organism Characteristics

Treponema pallidum is a Gram-negative, motile spirochete (characterized by long, helically shaped cells). The flagella are located lengthwise along the bacterium rather than at either end. As a practical matter, syphilis is not readily identified by Gram staining because it is too thin for the color to be observed. *T. pallidum* displays a characteristic corkscrew motion under wet mount. Figures 5-57 and 5-58 show the corkscrew nature of the spirochette and a closeup of one end.

Sample Collection

There are two types of samples that may be used for the analysis of syphilis. The first is a swab of the chancre that is then used for microscopy. The darkfield microscopic examination for the syphilis spirochete (*corkscrew shaped*) may be done onsite. The other sample type, a blood draw, is used for serological analyses.

Sample Analysis

Syphilis cannot be cultured on artificial media, so diagnosis is dependent on corroborative clinical and diagnostic tests. The spirochete itself is quite distinctive and can be viewed using both normal and darkfield microscopic techniques (Figures 5-59 and 5-60). The bacterium wiggles vigorously and this may be observed in newly collected samples. Microscopy may be done onsite from swab smears, and are not often done after shipment to a state laboratory. Once there, laboratories rely on other serologic techniques for analysis.

Syphilis infection causes the production of two types of antibodies by the host: nontreponemal-specific antilipoidal antibodies (reagin) that react with lipid antigens (cardiolipin, cholesterol, and lecithin) and treponemal-specific antibodies. Consequently, there are two basic types of tests: nontreponemal specific and treponemal specific. There are good reasons for using both types of tests for the accurate determination of current disease status:

- Nontreponemal tests detect proteins that are not solely specific to syphilis, so general prevalence in the population may be relatively high and result in a number of false positives. A positive nontreponemal test result is also not a lifelong result, and therefore indicates recent infection.
- Treponemal tests detect antibodies specific to *T. pallidum*, but are also reactive with some other

Figure 5-57 Illustration of the *Treponema pallidum* bacterium. (Courtesy of CDC.)

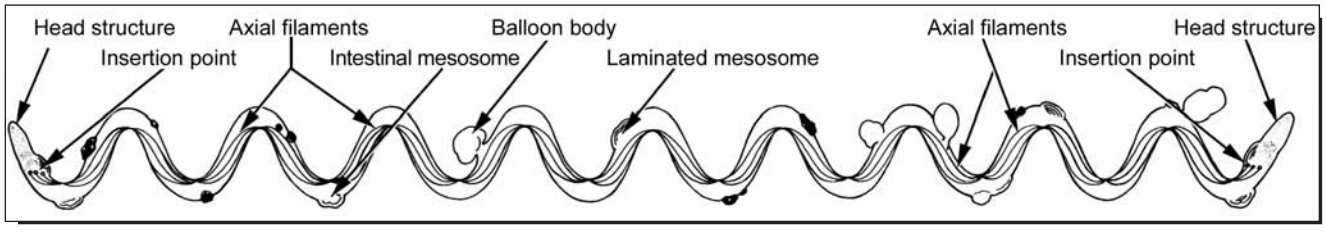


Figure 5-58 Closer illustration of one end of the *Treponema pallidum* bacterium. (Courtesy of CDC.)

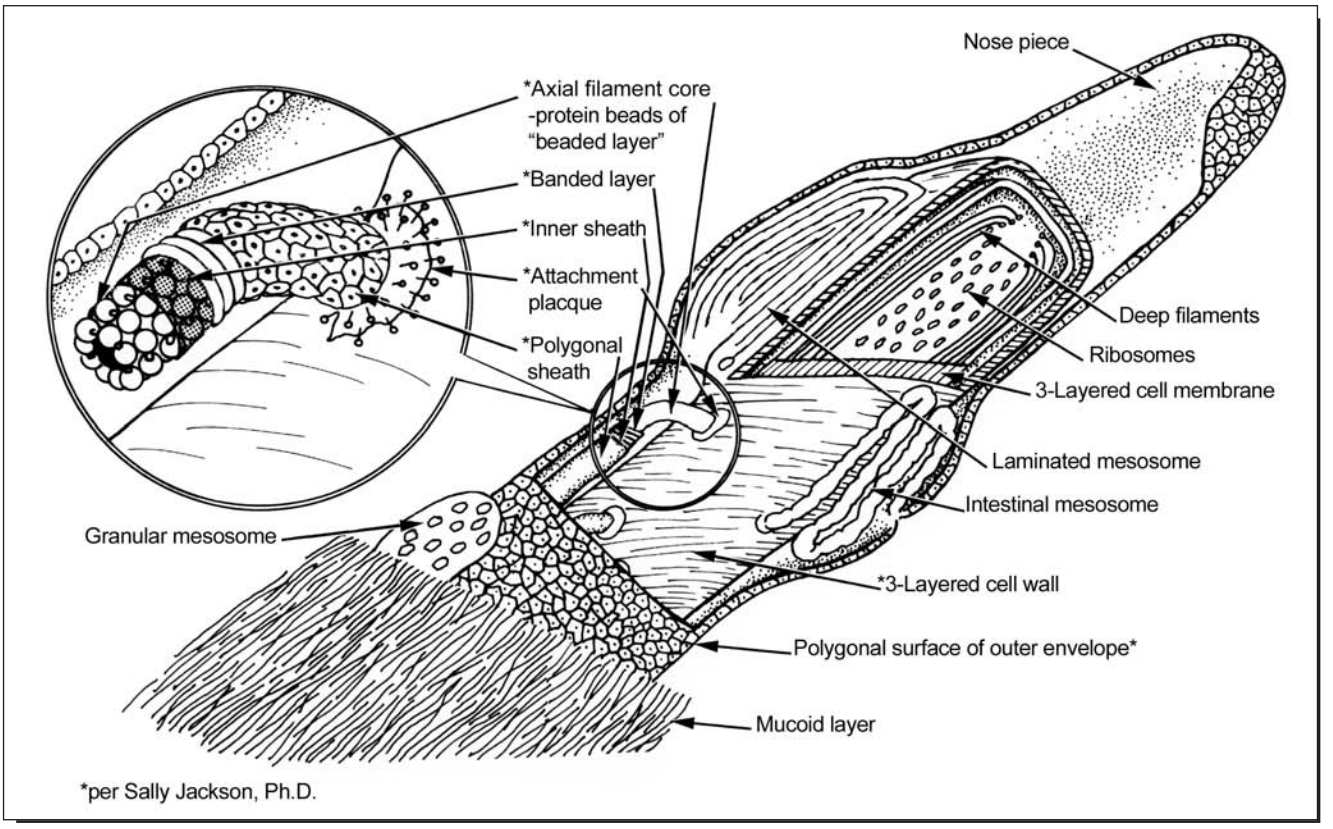


Figure 5-59 A whole mount photomicrograph of a *Treponema pallidum* bacterium. (Courtesy of CDC, VDRL Department.)

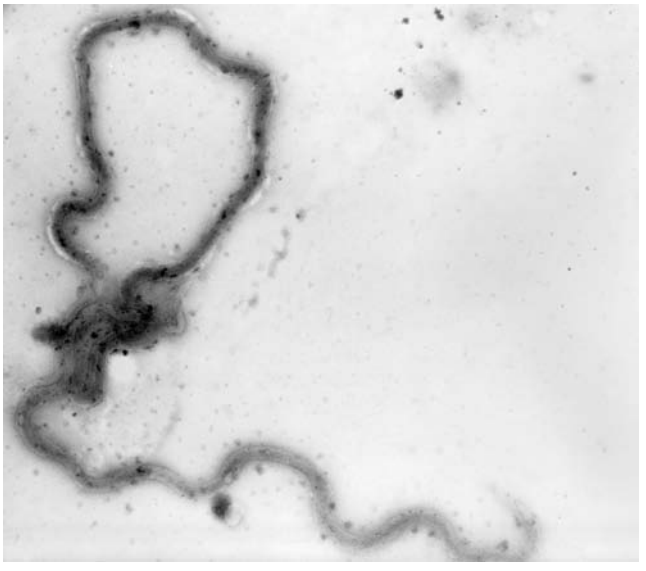
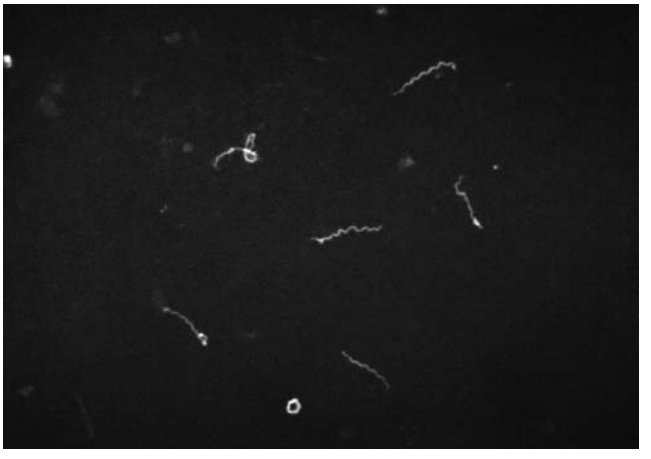


Figure 5-60 *Treponema pallidum* observed via darkfield microscopy. (Courtesy of CDC/W. F. Schwartz.)



treponemal subspecies (e.g., *T. pertenue* and *T.carateum*). While these species are rare in North America, the potential exists for a false-positive result. In addition, infection with syphilis often causes a lifelong antibody response, even after successfully treated.

As a result, the two testing methodologies complement each other. Treponemal tests are sensitive for *T. pallidum*, but lack a time dimension, whereas nontreponemal tests are not specific for *T. pallidum*, but may differentiate between past or present infection. Nontreponemal tests are less expensive and easier to perform and are often chosen for screening and the identification of preliminary positives. A treponemal test would then be done on preliminary positives for confirmation.

It should be noted that there is a lack of consensus about the best methodology for the detection and confirmation of syphilis. Treponemal and nontreponemal tests offer different information and have different strengths and weaknesses. Is it better to perform a nontreponemal test first as a screen (with a fairly high false-positivity rate) followed by a treponemal test for confirmation? Or would it be better to perform a treponemal test first (which is more specific, but would also detect those who were successfully treated and are no longer infected) followed by the nontreponemal test? There is no easy answer to this question, nor to which of each type of test is the best. Choices are usually made based on local prevalences, test and instrumentation costs, and expertise of the laboratory.

PHLs often develop testing algorithms that define which tests are used in which order, and how the results are interpreted. The IDPH laboratory in Chicago has adopted an algorithm published by the CDC.[102] A sample is initially screened with an immunoassay (treponemal specific). Those that are negative are reported as such. Those that are repeatedly positive are then confirmed by rapid plasma reagin (nontreponemal specific). Should that test be nonreactive, the sample is retested with fluorescent treponemal antibody absorbance (double stain). While this is also treponemal specific like the screening test, it is an indirect versus direct immunoassay method. These tests are discussed later and their performance characteristics compared in Table 5-2. Other tests that are not used as frequently are also briefly described after these three.

Table 5-2 Comparison of Syphilis Method Performances

Test	Sensitivity*	Specificity
EIA – Phoenix[103]	100	99.8
RPR – Remel[104]	97	99
FTA-ABS[105]	100	97.8

*Test sensitivity may vary by the stage of syphilis in the tested individual.

Enzyme Immunoassays

Enzyme immunoassays (EIAs) are treponemal-specific tests that use serum or plasma as samples. Like other well-based immunoassays, these often utilize recombinant treponemal antigens bound to microtiter plate wells. The sample is added and antitreponemal antibodies (if present) bind to the antigen forming a complex. After rinsing, a second antitreponemal antigen is added that binds to the first antigen/antibody complex, essentially forming a sandwich of antigens with the antibody in the middle. This second antigen is conjugated to a colorizing agent (e.g., HRP) that causes an added substrate to change color. The color intensity is measured by a spectrophotometer and is proportional to the amount of antibody present in the patient's serum/plasma. An example is the TREP-SURE screen by Phoenix Bio-Tech Corp (Mississauga, Ontario, Canada).

Rapid Plasma Reagin

The rapid plasma reagin (RPR) test is a nontreponemal test using either serum or plasma as sample types. The basic principle of the RPR is the use of microparticulate carbon-bound cardiolipin antigens targeted to reagin. When in solution with sample serum containing reagin, these particles clump (flocculate) together. These clumps are visible to the naked eye. An example is the RPR Card Test produced by Remel (Lenexa, KS). The RPR may be used as a qualitative assay (for screening) as well as a quantitative assay by performing serial sample dilutions (for monitoring treatment).

Fluorescent Treponemal Antibody Absorbance

The fluorescent treponemal antibody absorbance (FTA-ABS) test is a treponemal-specific indirect fluorescent antibody test using serum as a sample. The sample serum is mixed with a sorbent (extract from Reiter's treponeme) to remove nonspecific antibodies and then added to a prepared slide coated with treponemal antigen. Treponemal antibodies, if present in the sample, bind to the antigens to form an antigen/antibody complex. Antihuman

Figure 5-61 ***Treponema pallidum*** **revealed by FTA stain. (Courtesy of CDC.)**

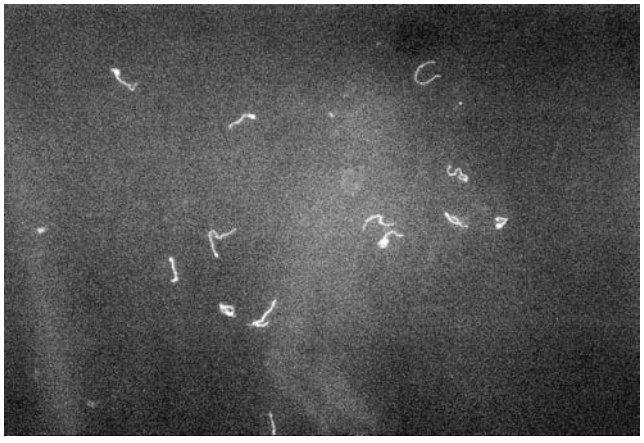
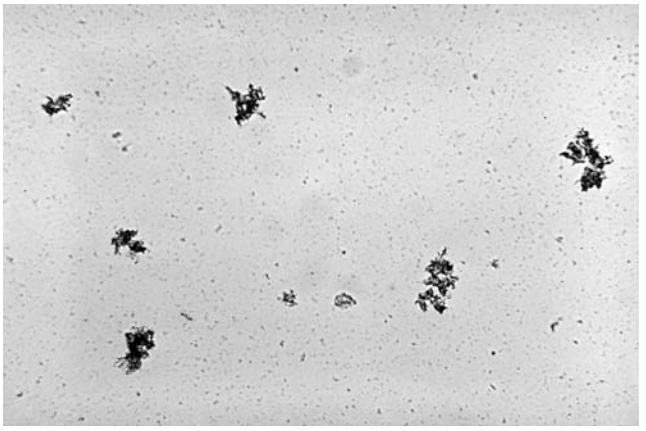


Figure 5-62 Example of a VDRL test positive control showing clumping (100× magnification). (Courtesy of CDC/ Renelle Woodall.)



immunoglobulin conjugated with fluorescent agent such as FITC is added that binds to the previously formed complexes. This is much the same type of "sandwich" described previously for EIA, but instead fluoresces yellow-green under UV illumination rather than enzymatically producing a color change. Figure 5-61 shows syphilis spirochetes stained using FTA. An example is the FTA-ABS Test System by Diagnostic Automation, Inc (Calabasas, CA).

Fluorescent Treponemal Antibody Absorbance Double Stain

The FTA-ABS double stain (DS) test is similar to the FTA-ABS but has an additional conjugate, tetramethylrhodamine isothiocyanate (TMRITC) antihuman IgG. The slides are viewed on an incident light fluorescent microscope, first using a FTIC filter to locate the treponemes, and then a rhodamine filter to read the specific red fluorescence. An example is the FTA-ABS DS IFA Test System by Zeus Scientific, Inc (Raritan, NJ).

Venereal Disease Research Laboratory

The Venereal Disease Research Laboratory (VDRL) method is a nontreponemal, microflocculation test using either serum (qualitative and quantitative) or CSF (qualitative only) as sample types. The basic principle of the VDRL is the use of cardiolipin and lecithin bound to microparticulate carbon. When in solution and mixed with a sample containing antibodies targeting these proteins, the antibodies/antigens are bound and clump together (flocculate) to a size that may be visible to the naked eye, but are routinely viewed using a light microscope. An example is the VDRL Antigen with Buffered Saline kit produced by Becton Dickinson. Figure 5-62 shows a reactive VDRL control slide.

Treponema Pallidum Particle Agglutination

The treponema pallidum particle agglutination (TPPA) test is treponemal specific and uses serum as a sample. The basic principle of the test is the mixing of gel particles sensitized with *T. pallidum* antigens and sample serum. *T. pallidum* antibodies in the serum react with the antigen-covered gel particles to form clumps (agglutination) that are visually distinctive. An example is the Serodia™ Treponema Pallidum Particle Agglutination Test (Fujirebio, Tokyo, Japan).

Other Tests

There are other tests in existence that are not often used or have been replaced with more sensitive and specific methods. Such tests include the *T. pallidum* hemagglutination assay and the microhemagglutination assay (TPHA and MHA-TP, respectively). These both use sensitized erythrocytes (avian or ovine) coated with *T. pallidum* antigens. When mixed with serum containing syphilis antibodies, the cells aggregate in distinctive patterns. There is also the DiaSorin chemiluminescent assay that is specific for IgG and IgM antibodies to *T. pallidum* and the Bio-Rad multiplex system that is based on the capture of treponemal antibodies on magnetic beads and identification by laser.

Human Immunodeficiency Virus

HIV is the causative agent for acquired immunodeficiency syndrome (AIDS). There are two known variants in humans, HIV-1 and HIV-2. The majority of infections in the United States are from HIV-1, though HIV-2 is predominant in West Africa. The virus acts by invading parts of the immune system sent to remove it, and the persistent infection eventually weakens the immune system to the point where the body can no longer effectively fight off infections. This is the point where AIDS is established, and the actual diagnosis is dependent on the presence of one or more specific infections, certain specific cancers, and/or a low number of certain white blood cells (CD4-presenting T cells).[74,106]

HIV data for 2007 from all 50 states show trends along the lines of the more comprehensive HIV/AIDS data previously. The overall rate of new infections was 22.8/100,000. Males accounted for 73% of new cases (estimated at 41,400) at a rate of 34.3/100,000 compared to the female rate of 11.9 (estimated 15,000 cases). Blacks accounted for 45% of new cases (estimated at 24,900) at a rate of 83.7/100,000 compared to Whites at 35% of new cases (estimated at 19,600). Those aged 13 to 29 years accounted for the largest percentage of new cases at 34% (19,200 cases), with the highest rate in those aged 30 to 39 at 42.6/100,000. The highest risk categories for HIV acquisition was MSM at 31% (16,800 cases) and high-risk heterosexual contact at 31% (16,800 cases).[107]

AIDS case data for 2007 from all 50 states again shows similar trends. The overall estimated rate of new AIDS cases was 11.9/100,000. Males accounted for 73% of new cases at a rate of 21.6/100,000 compared to the female rate of 7.5. Blacks had a rate of 47.3/100,000 compared to Whites at 5.2. Those aged 40 to 44 years had the largest number of new cases, accounting for 19%. Through 2007, there have been a total of 1,030,832 persons reported as having AIDS in the United States and dependent areas.[107]

Sidebar 5-11 Complexities Associated with HIV and AIDS Statistics[107]

The extent of HIV in the United States and rates of new infection are more difficult to determine than some other STIs, largely because of the potentially long latent period between infection and symptom presentation. All states have been reporting HIV case data to the CDC since 2004. One difficulty is attempting to determine if a newly diagnosed case represents a newly acquired infection. The serologic testing algorithm for recent HIV seroconversion (STARHS) can determine the difference between new (within approximately 5 months) and ongoing infections. The CDC funds 34 areas to include STARHS in their HIV surveillance. The cost of HIV/AIDS, in terms of morbidity, mortality, and their associated resources, has resulted in the establishment of HIV and AIDS monitoring and surveillance activities unlike any other STI. Multiple federally funded programs collect information in trends, demographics, and behaviors related to HIV acquisition and AIDS treatment. Examples include the Morbidity Monitoring Project (MMP), the National HIV Behavioral Surveillance (NHBS) System, and the collection of data from all AIDS cases in the United States through surveillance. Surveillance is not at 100% as only 34 states participate in name-based reporting of new cases.

HIV is transmitted through sex with an infected partner, sharing needles with an infected person, before/during birth, and through breastfeeding. The virus is fragile and does not survive long outside a host. Infection with other STIs such as chlamydia, gonorrhea, and syphilis increase the risk of both transmitting and acquiring HIV infection. The pace of progression from HIV infection to AIDS varies. In untreated patients, progression ranges from several months to 17 years with a median time of approximately 10 years. Viral replication is active during this entire time and increases as the immune system deteriorates. Early detection is important as infection may make the individual more susceptible to other infections (e.g., TB) and may alter the effectiveness of other disease treatment, and increasing viral load increases the risk of transmission to another person. Current CDC guidelines state that anyone seeking STI testing or treatment should be screened for HIV as well.

HIV infection progresses to AIDS through two distinct phases. The acute phase occurs immediately after infection and is characterized by high viral loads but low antibody response. During the acute phase there are some symptoms that may be observed. Such signs include fever, malaise, lymphadenopathy, and skin rash. After 3 to 5 weeks, the individual enters the latent phase where body's immune system is able to resist the virus and the viral load drops and antibody levels rise. This time is essentially a power struggle between the immune system, which seeks to eliminate the virus, and the virus, which

is infecting portions of the immune system. Over time, the immune system is weakened to the point where viral replication outpaces the body's ability to remove it, viral load is increased and immune system is depressed, and the individual becomes more acutely ill and susceptible to opportunistic infections and specific cancers. There is no cure for HIV infection. Treatment includes a lifetime regimen of highly active antiretroviral treatment (HAART).[74]

Organism Characteristics

HIV is a lentivirus in the family *Retroviridae* and is single-stranded, positive sense, and enveloped. The virion is ~100 nm in diameter and contains two copies of the genomic RNA, which are coated with a nucleocapsid protein and further capsulated. Once the host has been invaded, the viral RNA is transcribed into double-stranded DNA, which is then incorporated into the host's DNA and available for expression.[108] In addition to the two HIV types (1 and 2), there are four known strains of HIV-1: M, O, N, and S. M may be further subtyped into 10 distinct "clades" (A to J) and circulating recombinant forms (CRF). There are also at least five strains of HIV-2 (A to E).[109] Electron micrographs of HIV virions are seen in Figures 5-63 and 5-64. A view of an HIV virus interior is seen in Figure 5-65.

Sample Collection

Samples commonly used for HIV antibody testing include blood, serum, plasma, urine, and oral fluid. Blood samples may be from a finger stick or venipuncture. Blood from venipuncture is centrifuged to separate the serum. Oral fluid samples are collected with an OraSure collection device by rubbing the absorbent material between the cheek and gums. Alternative testing media (dried blood spots, vaginal fluid, CSF, and cadaveric fluids) have also been used for HIV antibody testing.

Figure 5-63 Transmission electron micrograph of stained HIV showing surface glycoproteins. (Courtesy of CDC/ Dr. Edwin P. Ewing, Jr.)

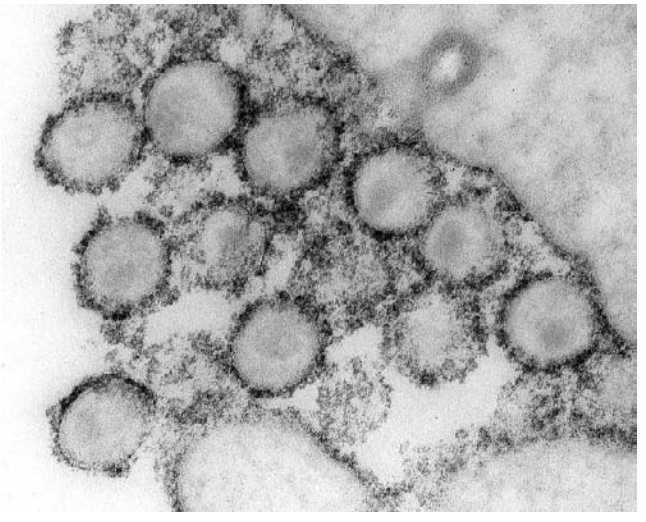
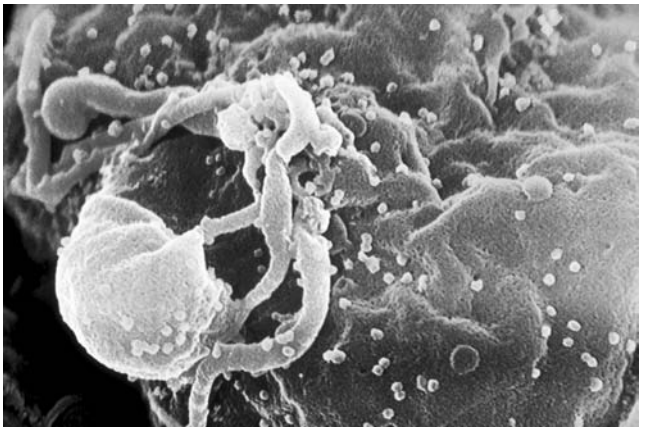


Figure 5-64 Scanning electron micrograph of HIV-1 budding from a lymphocyte. (Courtesy of CDC/C. Goldsmith, P. Feorino, E. L. Palmer, W. R. McManus.)



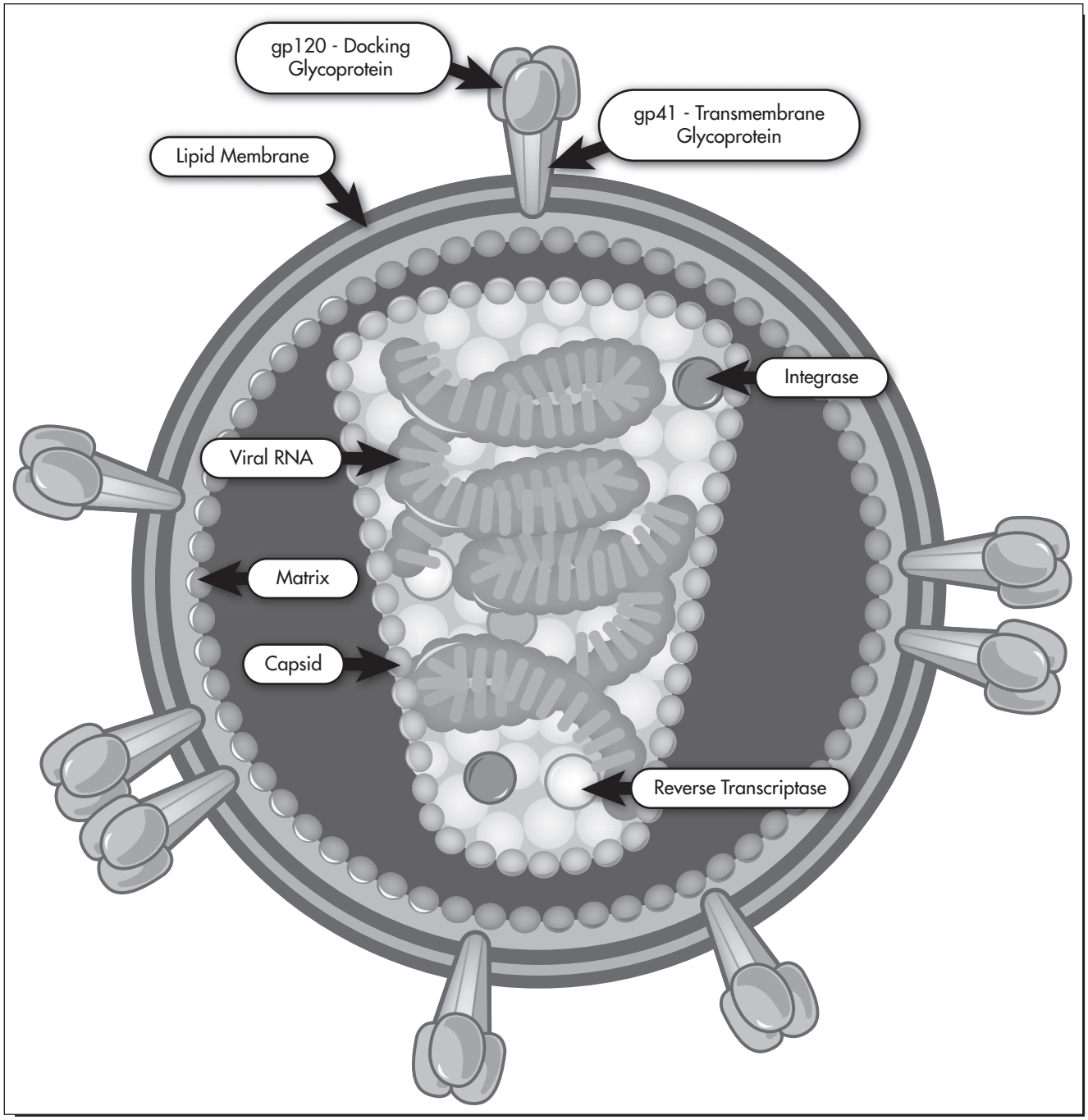
Home Tests

Immunoassay screening tests for HIV are available, with some available commercially, and can be done in an individual's home with results within minutes. The FDA has not approved these test kits and there have been reports that some give erroneous results. Also troubling is the potential for someone to test positive, but not seek treatment and counseling. Other tests require an individual collect their own sample and send it for analysis. While the test quality may be better for this type, there is a concern about contamination through the self-collection of blood samples.

Sample Analysis

There are currently six FDA-approved rapid tests available in the United States. While these are used extensively onsite in clinical settings, they are also frequently used in state laboratories as well. All are immunoassays that detect antibodies against HIV. All utilize blood samples (serum, plasma, or whole blood) except the OraQuick ADVANCE, which utilizes oral fluid. The

Figure 5-65 Organization of the HIV-1 virion. (Courtesy of National Institute of Allergy and Infectious Diseases.)



specificities and sensitivities of these tests range from 99.3 to 100% sensitive and 98.6 to 100% specific[110]:

- OraQuick ADVANCE Rapid HIV-1/2 Antibody Test (OraSure Technologies, Inc., Bethlehem, PA): This test can be performed with whole blood, plasma, or oral fluid. The test is sensitive to both HIV-1 and HIV-2, but does not distinguish between the two.
- Reveal G3 Rapid HIV-1 Antibody Test (MedMira Laboratories, Inc., Halifax, Nova Scotia, Canada): This test can be performed with serum or plasma. The test is sensitive to HIV-1 only.
- Uni-Gold Recombigen HIV Test (Trinity Biotech, Bray, Ireland): This test can be performed with whole blood, serum, or plasma. The test detects HIV-1 only.

- Multispot HIV-1/HIV-2 Rapid Test (Bio-Rad Laboratories, Hercules, CA): This test can be performed with serum or plasma. The test is sensitive to both HIV-1 and HIV-2 and can distinguish between the two.
- Clearview HIV 1/2 Stat Pak (Inverness Medical, Princeton, NJ): This test can be performed with whole blood, serum, or plasma. The test detects to both HIV-1 and HIV-2, but does not distinguish between the two.
- Clearview COMPLETE HIV 1/2 (Inverness Medical, Princeton, NJ): This test can be performed with whole blood, serum, or plasma. The test detects both HIV-1 and HIV-2, but does not distinguish between the two.

The basis for these tests rests on the specific affinity of reagent antigens to HIV-specific antibodies found in the sample. The kits all have these antigens conjugated to a color-producing compound and bound on a solid matrix. When the sample is analyzed, any HIV-specific antibodies present in the sample bind to the conjugated and bound antigens, producing a color change (very similar to home pregnancy tests). The absence of a color change in the test region of the kit indicates no detectable levels of HIV antibodies. If the test returns a positive result, it is considered presumptive until a confirmatory test may be done at the state PHL.

HIV analysis presents a bit of a conundrum when multiple tests and technologies are used. Different tests are used to limit the potential for false positives, but how does one interpret discordant results such as a positive first test but negative second? Does the laboratory perform repeated tests, by other methodologies? In a fashion similar for syphilis analysis, laboratories often develop testing algorithms to create a mechanism for result interpretation. Also similar to syphilis analysis is the lack of agreement on which algorithm is best. The Association of Public Health Laboratories (APHL) has published a status report of different proposed algorithms and laboratories may be advised to adopt one of them. As an example, Algorithm 1 uses a standalone HIV-1 immunoassay as the screening test (A1) and a Western blot (WB) or indirect immunofluorescence assay (IFA) as a supplemental test (B1). Samples repeatedly positive by A1 are also tested by B1. If A1 and B1 are positive, HIV-1 is reported as present. NAAT testing (B) may also be done for confirmation. NAAT testing may also be used to resolve indeterminate B1 tests. Finally, if the sample is repeatedly positive by A1, but negative by B2, then supplemental testing by B1 is called for. There are five different proposed algorithms and some are useful for both HIV-1 and -2.[111]

IFAs are very similar to other indirect immunoassays discussed previously. Typically, wells in a microtiter plate are coated with HIV-1- and HIV-2-specific proteins (antigen) obtained from infected cell lines. Prepared sample is added to the well and antibodies to HIV-1/2 (if present) bind to these antigens. After rinsing, peroxidase-conjugated antigens are added, which bind to the previously established antigen–antibody complex. Following another rinse, a colorizing solution is added that is detected by a spectrometer. These reagents are available as kits from multiple venders (e.g., Bio-Rad Laboratories and OraSure Technologies).

WB tests for HIV-1 are considered the gold standard and may confirm a positive immunoassay. The WB tests are more specific to the HIV-1 viral proteins than the rapid tests. The principle of the WB test rests on the combination of electrophoretic separation followed by an immunoassay. In brief, the kit manufacturer propagates HIV in a cell line. The cells are harvested and subjected to reagents that disrupt the virus, freeing multiple proteins. These are then separated by size during passage through a polyacrylamide gel (PAG). The now-separated proteins in the gel are then electrotransferred onto a nitrocellulose membrane. This produces a "ladder" pattern of proteins based on increasing molecular weight (size).

Analysis involves preparing a sample is adding it to this membrane. Antibodies specific to HIV-1 (if present) will bind to their corresponding target protein. They are visualized after rinsing by the addition of a phosphatase-labeled conjugate and color development reagent. The HIV-1 WB test results are determined by first locating band reactions and then grading the level of intensity to the HIV-1 specific proteins (p) and/or glycoproteins (gp); for example, p17, p24, p31, gp41, p51, p55, p66, gp120, and gp160. According to the CDC/Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) criteria, the bands of diagnostic significance are any two of the following: p24, gp41, and gp120/gp160.[112] An example is the Abbott HIVAB HIV-1/HIV-2 (rDNA) EIA (Abbott Laboratories, Abbott Park, IL).

RT-PCR tests are extremely sensitive to the RNA that is specific to HIV-1. They also have the advantage over immunoassays in being able to detect the virus much earlier, theoretically within days of infection when the viral load is increasing but there is still an undetectable immune response. There are currently 10 FDA-approved tests. Not all are suitable for screening individuals, with some designed for screening donated blood and others for sample pool-

ing (where multiple samples negative by immunoassay are pooled and reanalyzed by RT-PCR). An example is the APTIMA HIV-1 RNA Qualitative Assay (Gen-Probe).

Clinics and other venues often perform rapid immunoassay tests to screen clients. These are quite sensitive and specific and are useful for preliminary diagnoses. Those clients testing positive have confirmatory testing performed at the state laboratory, usually by WB or other test in accordance with their adopted algorithm. While RT-PCR techniques are approved, they are generally more expensive. Because of the potential of RT-PCR to detect infection in the early acute phase, where an immunoassay would be negative, some states are performing pooled negative sample testing. Samples that are negative by immunoassay are pooled together (e.g., 48 samples into one tube) and analyzed by PCR. The idea is that they might identify individuals still in the acute phase where they are most infectious. By altering their behavior, more people may avoid exposure. This is not instituted in many places yet, and the jury is still out as to whether this is a cost-effective method. That is, is the substantial cost associated with reanalyzing thousands of sample worth the effect of identifying a small number of individuals? This will likely be answered in the next couple of years, and be dependent on such things as test and treatment costs and local prevalence.

Human Papillomavirus

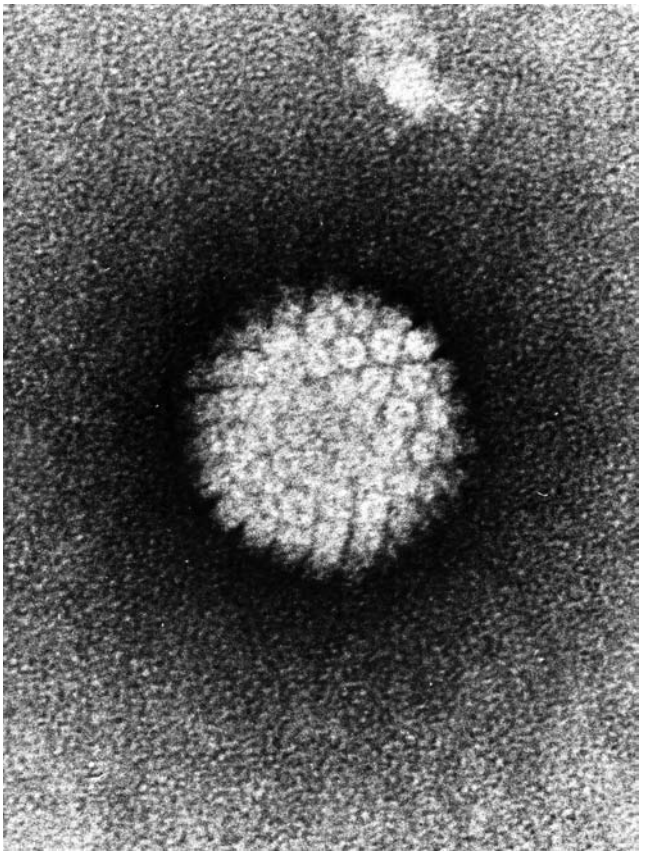
Human papillomavirus (HPV) is the most common STI worldwide. It is estimated that there are 20 million American currently infected, with 6.2 million new infections annually. The virus is spread through genital contact and approximately 50% of sexually active people acquire HPV at some point. Limited prevalence testing showed that those aged 14 to 19 were at greatest risk with 35% testing positive. Fortunately, the great majority of people do not have any symptoms or ill effects, and 90% clear the infection without treatment within 2 years.[92,113]

There are more than 100 variants of HPV, and more than 30 of them infect the genital area. Infection with some may progress to significant morbidity. "Low-risk" variants are those types that cause genital warts (usually types 6 and 11) and "high risk" are those that may cause cervical and other cancers (e.g., types 16, 18, 31, 33, and 35). Less common cancers include those of the vulva and vagina, anus, and penis. The most common visible sign of infection is the presence of genital warts. These may or may not resolve without treatment, and approximately 1% of sexually active adults have genital warts at any given time. NHANES data for years 1999 to 2004 showed that 5.6% of sexually active individuals aged 18 to 59 reported a history of genital warts. The cancers associated with HPV infection are usually not noticed until advanced, which underlies the importance of screening through the use of Papanicolaou (Pap) tests and regular checkups. There is no treatment for infection. A newly introduced vaccine protects females from the four variants (6, 11, 16, and 18) that cause the majority of cervical cancers and genital warts. There is no currently approved vaccine for males, though some are in development. Visible warts may be removed by the individual with over-the-counter products (e.g., podofilox), or this may be done by a clinician (e.g., acid or cryotherapy).[74,92,113]

Organism Characteristics

HPV belongs to the family *Papillomaviridae* and is spherical with a diameter of ~55 nm. They contain double-stranded DNA that is approximately 7900 base pairs long and contained in a nucleocapsid. An example is shown in Figure 5-66 as seen via electron microscopy.

Figure 5-66 Electron micrograph of negatively stained human papillomavirus. (Courtesy of NCI/Laboratory of Tumor Virus Biology.)



Currently, HPV cannot be cultured in vitro and immunoassays are not adequate to determine infection within cervical cells. There are FDA-approved DNA tests (e.g., Hybrid Capture 2 High-Risk HPV DNA Test, Digene Corporation), but it is not recommended to test individuals for HPV except as part of cervical cancer screening. There are newer cell suspension methods used with Pap tests that allow for greater sensitivity and also allow for nucleic acid testing if warranted.

Herpes Simplex Virus

There are two types of the herpes simplex virus, HSV-1 and HSV-2. Approximately 50 million adolescents and adults in the United States are infected with HSV-2. Data on HSV infections are not collected nationally, and data from the National Disease and Therapeutic Index shows an increasing number of clinician visits for genital herpes. Meanwhile, the NHANES data shows a decrease in seroprevalence of HSV infection in 14 to 49 year olds from 1988–1994 to 1999–2004 (from 21 to 17%). This discrepancy between increasing office visits and decreasing reported prevalence highlights how most people are unaware they are infected.[92,114]

The majority of genital herpes cases are caused by HSV-2, and twice as many women as men are infected (approximately 25% versus 12.5%). HSV-1 is more often associated with "fever blisters" on the mouth and lips. The virus can be passed through contact with viral sores, and also from skin not appearing to have a sore. HSV-2 is generally only acquired through sexual contact with someone who is infected. HSV-1 can be acquired through both genital–genital and oral–genital contact. Though recurrent HSV-1 outbreaks are less frequent than HSV-2, HSV-1 may cause up to 50% of first episode cases. [114] The identification of the specific viral infection thus has important implications for counseling.

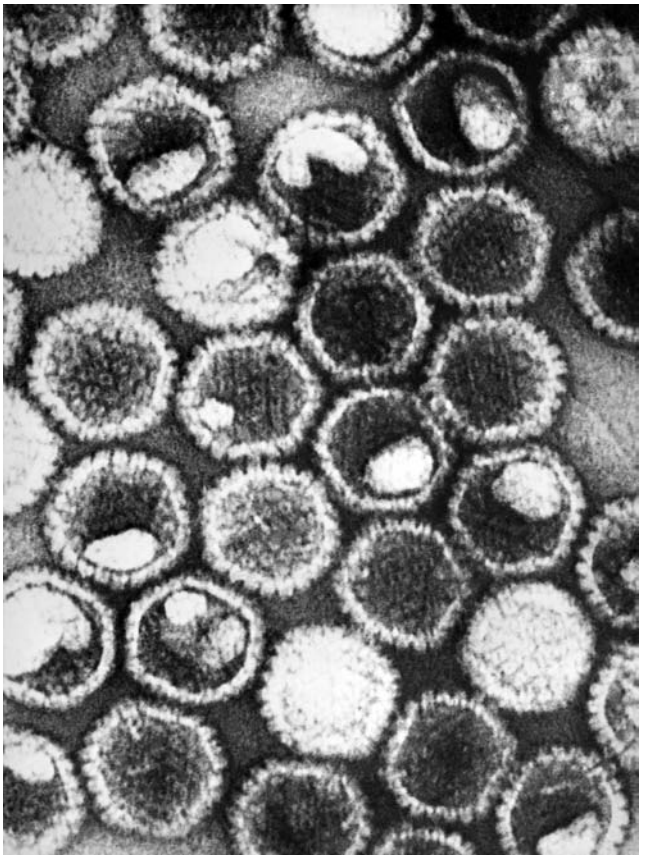
While most individuals with HSV-2 never have sores or noticeable symptoms, these do occasionally appear during the first outbreak and may be significant. Symptoms include the appearance of sores, which heal within a couple weeks. This may be followed by additional sores, fever, swollen glands, and general flu-like symptoms. The infection may progress to cause recurrent sores that may be painful. Infection in pregnant women may lead to potentially fatal infections in newborns, but this is quite rare. HSV infection is also associated with increased risk of HIV acquisition and spread, neonatal infection, and HSV encephalitis.

There is no cure for infection. Treatment of first episodes with antiviral medications such as acyclovir, famciclovir, and valacyclovir, taken for a 7- to 10-day regimen, can shorten outbreaks. Episodic treatment for recurrent outbreaks uses the same medications in different amounts. Suppressive therapy using these same medications may reduce the number of recurrences by 70 to 80% for those who have them frequently, and also reduce the potential for transmission to uninfected partners.[74]

Organism Characteristics

Both HSVs belong to the family *Herpesviridae* and are large, enveloped, and approximately 180–200 nm in length. They contain double-stranded DNA that is approximately 160,000 base pairs long, encodes for ~75 proteins, and is contained in an icosahedral nucleocapsid. HSV-1 and HSV-2 share most surface antigens, but are differentiated by glycoprotein gB (HSV-1 has gB1 and HSV-2 has gB2).[115] An example of HSV virions observed by electron microscopy is shown in Figure 5-67.

Figure 5-67 Negatively stained transmission electron micrograph of herpes simplex virions. (Courtesy of CDC/Dr. Fred Murphy; Sylvia Whitfield.)



Sample Collection

Viral culture and PCR methods use samples of skin and fluid from an active sore. The base of the lesion may be lightly scraped as the virus resides in the skin cells and these must be collected in addition to vesicular fluid. Immunoassays (both onsite and laboratory performed) use blood or serum samples.

Sample Analysis

Culture

Viral culture is a preferred analysis for patients who seek treatment for genital ulcers and mucocutaneous lesions, but is plagued with low/variable sensitivity. Cell lines (e.g., mink lung, MRC-5) are infected and some cytopathic effects, such as cytoplasmic granulation, may be observed. HSV typing is done by testing an isolate by fluorescent antibody (FA) analysis using commercially obtained reagents (e.g., Trinity Biotech and Diagnostic Hybrids, Inc.). The method has generally low sensitivity, due in part to intermittent and/or low viral shedding, and a negative result does not rule out infection.

Polymerase Chain Reaction

These tests are not currently FDA approved for genital specimens, though they are more sensitive than culture methods. They are used for the detection of HSV in spinal fluid.

Immunoassays

There are two kinds of HSV immunoassays. The older tests are non–type-specific and cannot distinguish between HSV-1 and HSV-2. More recently developed immunoassays are type-specific and can distinguish between HSV-1 and HSV-2. These newer tests are specific to HSV-specific glycoproteins. Examples of type-specific tests include HerpeSelect 1 and 2 Immunoblot IgG (Focus Technology, Inc., Mooresville, NC) and HSV-2 ELISA (Trinity Biotech). Both tests use immobilized G-specific antigens bound to a solid matrix. G-specific antibodies, if present in the sample, bind to the antigens and are retained after a wash step. The matrix is then incubated with conjugated goat antihuman IgG. This is followed by the addition of a substrate that produces color in the presence of the conjugated proteins. The reaction is then detected by a spectrometer. This type of test is often preferred because of its ease of use, relatively high sensitivities and specificities, and the long-term presence of HSV antibodies in serum after initial infection.

The testing of samples for STIs may definitely be considered one of the purposes for a state PHL. The IDPH laboratories received 60,905 HIV samples (oral and serum), 168,806 CT/GC (swab and urine), 4141 GC culture, 70,161 syphilis (EIA, RPR, and FTA), and 1023 herpes samples from July 2007 through June 2008 (unpublished data). The total number, 305,036, represents 45% of all samples received and eclipses those received for any other type of analysis. Given the nature of the different organisms (bacteria, viruses, and protozoa), these samples are frequently analyzed in different test sections based on the request. One test section may perform CT/GC analyses and another the syphilis and HIV. In Illinois, the tests are also distributed among all three laboratories. This is done to both distribute the burden of testing but to also facilitate sample delivery. We discussed at the beginning of this section the importance of mail delivery and holding times, and having laboratories able to perform these analyses in different locations greatly facilitates sample delivery within the proper holding time.

Enterics

Enterics is often an identifiable testing section of PHLs, in the same manner as environmental chemistry and blood lead are readily identifiable. The purpose of the section is to analyze clinical samples (usually stools) for the presence of microorganisms that cause primarily gastrointestinal illness. Most of the cases from which the samples are obtained were caused by some sort of microorganism contamination, whether of food or water. Thus, a positive result may trigger an outbreak investigation. This in turn might result in further enteric samples from those who may have been exposed, and food or water samples that are identified as potential sources of exposure.

Serotyping the enterobacteria is based in part on the identification of known antigenic factors. The "O" antigen is an outer membrane lipopolysaccharide, the "K" antigen is the presence of a surface capsule or amorphous slime layer, and the "H" antigen is the presence of flagella resulting in motility. Thus, *E. coli* O157:H7 is a motile serotype of *Escherichia coli* containing the #157 O antigen and #7 H antigen. This is different than the O157:NM serotype that shares the same O antigen but is not motile (lacking flagella). This classification system applies for other enterobacteria where needed, such as *Salmonella*.

Possible Agents of Enterics Illness

Escherichia coli

Contrary to what some may think from news reports, the vast majority of the hundreds of *E. coli* serotypes are harmless to humans. One exception is *E. coli* O157:H7, which has received much media attention associated with food-borne poisonings. Indeed most illnesses caused by O157:H7 are associated with undercooked beef, but it does occasionally happen that people become ill from contaminated water (as in 1999 in Washington County, NY).[116] *E. coli* as a whole are present in great quantities in the intestines and feces of warm-blooded animals.

Human illness is associated with infection by specific strains of *E. coli*, and it is worthwhile to describe how they are classified. This system described here is somewhat arbitrary and some serotypes may be legitimately assigned to multiple classes[117]:

- STEC: These are the *S*higa-*t*oxin producing *E. coli* and they produce one or two toxins (coded by the genes *stx1* and *stx2*) that are quite closely related to those produced by *Shigella dysenteriae*. There are more than 400 serotypes known. Examples include O157 (the most common serotype identified in North America), and non-O157 serotypes O26, O111, and O103.[118] Much of PHL analyses are concerned with identifying a particular pathogen as STEC or non-STEC.
- EHEC: These are the *e*ntero*h*emorrhagic *E. coli* and are essentially a subset of STEC that cause hemorrhagic colitis/hemolytic uremic syndrome (HUS) in humans. *E. coli* O157:H7 is an example of a STEC serotype, which is also enterohemorrhagic.
- ETEC: These are the *e*ntero*t*oxigenic *E. coli* and they produce enterotoxins that are heat stabile and/or heat labile. They are a leading cause of diarrheal illness.
- Other classes include *e*ntero*p*athogenic *E. coli* (EPEC), *a*ttaching and *e*ffacing *E. coli* (A/EEC), *e*ntero*agg*regative *E. coli* (EAggEC), *d*iffuse *a*dherent *E. coli* (DAEC), and *e*ntero*i*nvasive *E. coli* (EIEC).

STEC toxin may cause severe damage to the intestinal lining and infection symptoms usually develop 3 to 4 days after exposure. These vary by individual but often include severe stomach cramps, bloody diarrhea, vomiting, and mild fever (if present). Illness usually lasts 5 to 7 days and resolves without treatment. However, 5 to 10% of those diagnosed with STEC infection go on to develop HUS, a potentially life-threatening illness.[118]

E. coli are Gram-negative, facultative rods that ferment lactose and glucose and are oxidase negative. Most also ferment sorbitol (except O157 and a few others). Some serotypes are flagellated and thus motile. There are in excess of 150 known O antigens and many K and H antigens as well.[119] The infective dose may be as low as 10 organisms. Almost all documented outbreaks, and many individual cases, have been associated with the consumption of raw or undercooked hamburger.[120] Figures 5-68 and 5-69 show different strains of *E. coli.*

Figure 5-68 Transmission electron micrograph of *E. coli* O157:H7. (Courtesy of CDC/ Peggy S. Hayes.)

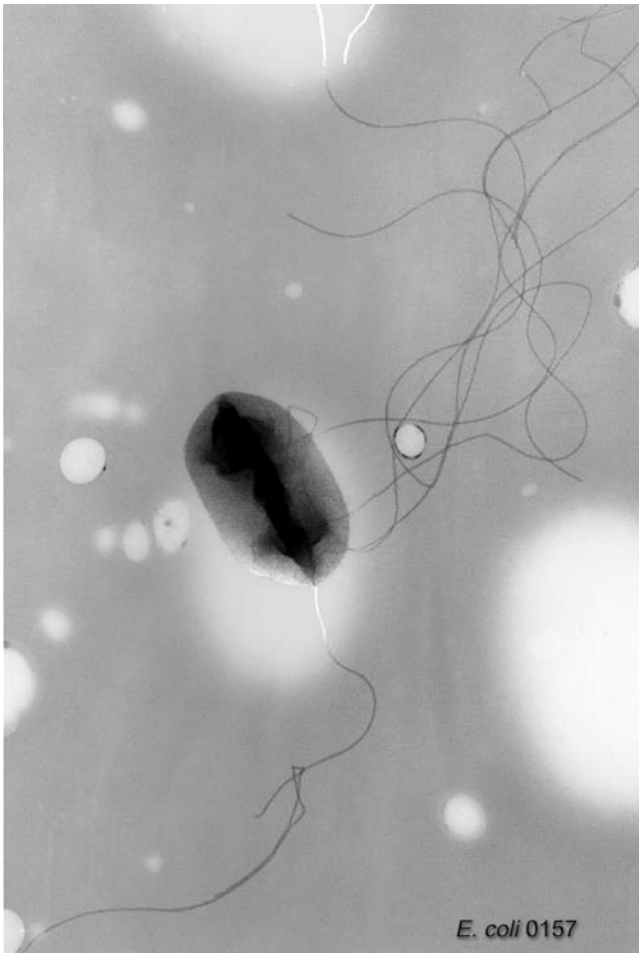


Figure 5-69 Scanning electron micrograph of *E. coli* O169:H41. (Courtesy of CDC/Janice Haney Carr.)

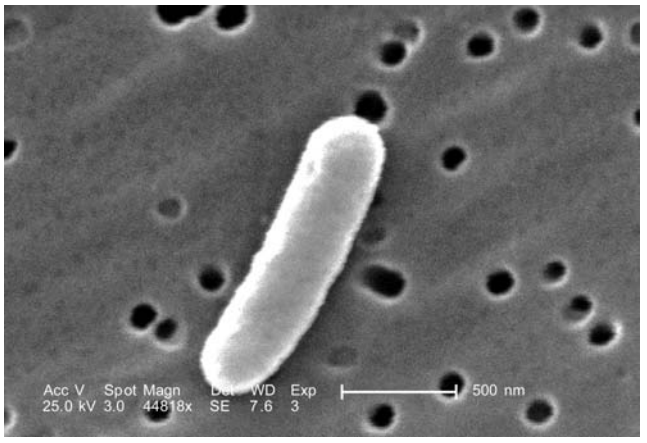
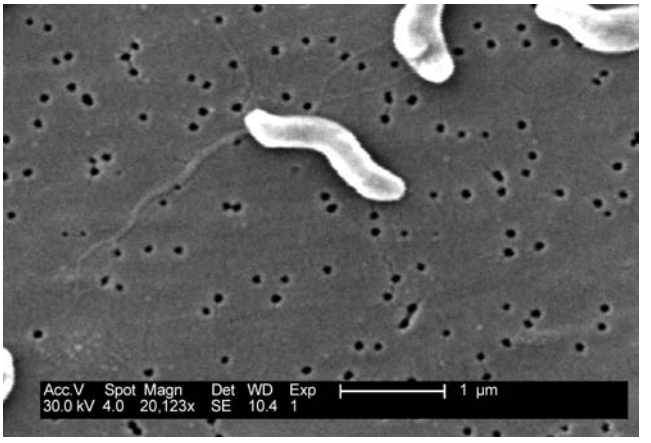


Figure 5-70 Scanning electron micrograph of *Campylobacter jejuni* (20,123x). (Courtesy of CDC/Dr. Patricia Fields, Dr. Collette Fitzgerald, Photographer: Janice Carr.)



Campylobacter Species

Campylobacter spp. were the confirmed etiology in 20 outbreaks in the United States in 2007 (16 were *C. jejuni* and the other 4 were unknown species).[121] Campylobacteriosis is the illness caused by infection with *C. jejuni* and is one of the more common sources of diarrheal illness, causing an average of 13 cases per 100,000 people annually, affecting over 2.4 million people each year in the United States.[122] Almost every case is an isolated event and not part of a larger outbreak. Illness usually occurs within 2 to 5 days of exposure and symptoms may include diarrhea, abdominal pain, nausea, fever, and vomiting. The disease usually lasts 1 week and most people recover without specific treatment, though antibiotics may be effectively prescribed to shorten the disease course.

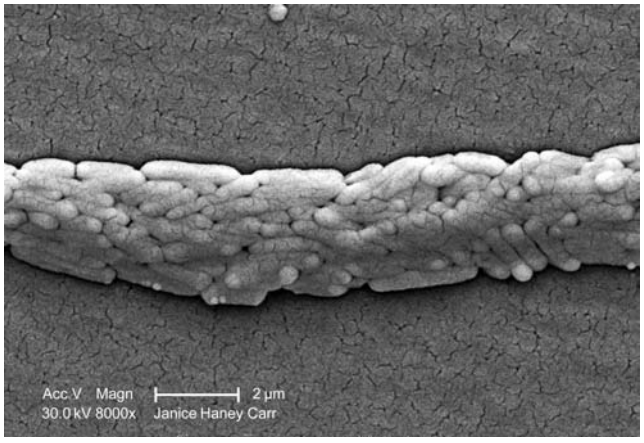
Campylobacter is a Gram-negative, slender, curved, motile rod requiring reduced oxygen levels for growth (microaerophilic). It is oxidase positive. Because of these growth requirements it is frequently not a part of standard testing algorithms and its analysis may be specifically requested. It is estimated that 400 to 500 organisms are necessary to induce disease, and it is a frequent contaminant of poultry where studies have shown that 20 to 100% of retail chickens are contaminated.[120] An example shown in Figure 5-70.

Salmonella Species

Salmonella spp. are the causative agents of salmonellosis and were the confirmed etiology in 135 outbreaks in the United States in 2007 (including 28 *S. enteritidis*, 20 *S. typhimurium*, and 17 *S. newport*).[121] More information concerning the impact of *Salmonella* spp. in food-borne illness may be found in Chapter 8. Illness usually occurs within 12 to 72 hours of exposure and symptoms presented by most people include diarrhea, abdominal pain, and fever. The disease usually lasts 4 to 7 days and most people recover without specific treatment, though antibiotics may be effective if the infection spreads into the bloodstream. A small number of infected individuals develop Reiter syndrome. This is characterized by pain in the joints and eyes and painful urination. Symptoms may persist for months or years, and potentially lead to chronic arthritis.[123]

Salmonella is a Gram-negative, usually motile rod (with some nonmotile exceptions) with widespread occurrence in swine, poultry, water, and soil. Most ferment glucose, mannitol, and maltose. *S. typhi* is the only pathogenic *Salmonella* that does not produce gas. There are only two known species of *Salmonella* (*S. enterica* and *S. bongori*) with six known subspecies of *S. enterica* (subsp. *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *indica*) and at least 2500 serotypes. Serotyping is based on the identification of the O and H antigens that are used to identify more commonly known strains such as *S. typhi*. The flagellar antigens of most *Salmonella* undergo phase variation, resulting in the potential for two H designations.[119] *Salmonella* is one of the more complex organisms to properly name. It is estimated that only 15 to 20 organisms are necessary to induce disease, and it is a frequent contaminant of poultry products and reptiles.[120] An example is shown in Figure 5-71.

Figure 5-71 Scanning electron micrograph of a *Salmonella typhimurium* grouping (8000×). (Courtesy CDC/ Bette Jensen; Photographer: Janice Haney Carr.)

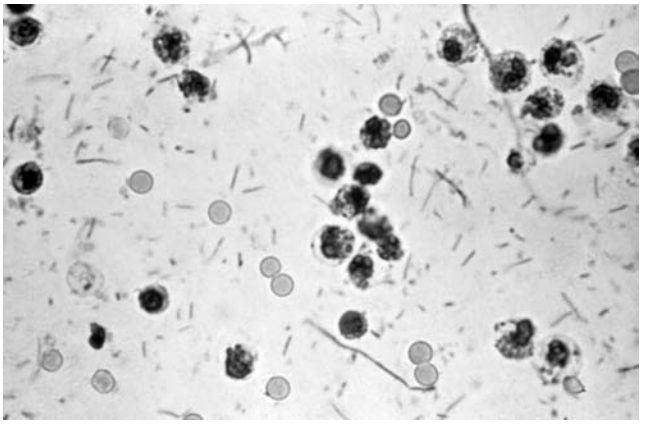


***Shigella* Species**

Shigella spp. are the causative agent for shigellosis and were the confirmed etiology in 10 outbreaks in the United States in 2007 (9 were *S. sonnei* and the other was unknown species).[121] Annually, there are ~14,000 cases reported in the United States (> 300,000 estimated), and children aged 2 to 4 are the most likely to get shigellosis.[120,124] *Shigella* is present in the feces of those infected and fecal contamination is the primary method of exposure (e.g., unwashed hands of contaminated sewage on fruits and vegetables). Illness usually occurs within 1 to 2 days of exposure and symptoms may include diarrhea (often bloody), abdominal pain, and fever. The disease usually lasts 1 week and most people recover without specific treatment. Antibiotics such as ampicillin and ceftriaxone may be prescribed to shorten the disease course, though some bacterium have become resistant.

Shigella spp. are Gram-negative, nonmotile, nonsporeforming rods that do not ferment lactose and do not produce gas when fermenting glucose. There are four species *S. dysenteriae* (serogroup A), *S. flexneri* (serogroup B), *S. boydii* (serogroup C), and *S. sonnei* (serogroup D) consisting of at least 38 serotypes. Assignment to species is based on biochemical reactions and O antigen groups, with species serotype specific to the O antigen.[119] It is estimated that only 10 organisms are necessary to induce disease.[120] An example is shown in Figure 5-72.

Figure 5-72 Photomicrograph of stool exudates from an individual with shigellosis. (Courtesy of CDC.)



Sidebar 5-12 Multidrug-resistant *Shigella sonnei*[124]

Antibiotics are not normally needed for recovery from shigellosis, but may be prescribed to shorten the disease course. This may be the case when those infected attend child care facilities and authorities wish to limit further spread. However, some strains have become resistant to some of the antibiotics commonly prescribed to children. In 2005, Kansas, Kentucky, and Missouri all had outbreaks of *Shigella sonnei* associated with child care centers. There were 201 laboratory-confirmed cases in Kansas City, KS, from May 1 to December 31, 2005. Antimicrobial susceptibility testing on 60 isolates revealed that 53 were resistant to ampicillin and TMP/SMX and 8 were resistant to ampicillin/sulbactam. There were 645 confirmed cases during that same period in Kansas City, MO, and 25 of 28 isolates were resistant to ampicillin and TMP/SMX. Finally, there were 148 confirmed cases in Fayette County, KY, from May 1 to August 31, 2005. All 12 obtained isolates were resistant to ampicillin and TMP/SMX. Antimicrobial resistance testing done by the National Antimicrobial Resistance Monitoring System Laboratory on isolates obtained nationwide during 1999 to 2003 showed that 80% of all isolates were resistant to ampicillin, 47% to TMP/SMX, and 38% to both. This case serves to highlight the importance of antimicrobial susceptibility surveillance testing to ensure that proposed plans of intervention are indeed effective and instigate the development of alternatives if not.

Norwalk-like Virus

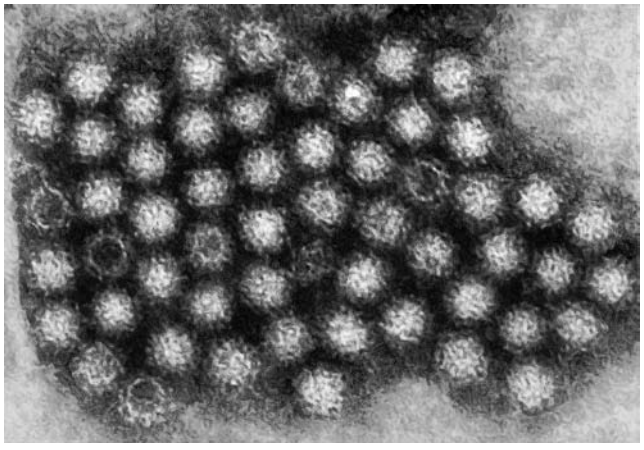
Norovirus is the official genus name for the group temporarily termed Norwalk-like virus, and was the confirmed etiology in 199 outbreaks in the United States in 2007.[121] The CDC estimates that 50% of food-borne outbreaks of gastroenteritis can be attributed to this group, causing 23 million cases.[125] Norovirus is present in the feces of those infected and fecal contamination is the primary method of exposure (e.g., unwashed hands or contaminated sewage on fruits and vegetables). Illness usually occurs within 1 to 2 days of exposure and symptoms usually include water diarrhea, abdominal pain, nausea, and vomiting. The disease usually lasts 2 to 5 days and dehydration is the most common complication.

Noroviruses are nonenveloped round viruses with a diameter of 27 to 32 nm, a single strand of RNA 7500 bases long, and one structural protein. They belong to the family *Caliciviridae* and there are five main groups (GI to GV), further divided into at least 31 genetic clusters. They are also highly contagious with as few as 10 viral particles thought required to induce infection.[120,125] An example is shown in Figure 5-73.

Other Potential Organisms

There are other organisms that are either no longer as big a concern as they once were, are much more regional in occurrence, or are not as widespread in occurrence. Examples include *Klebsiella* spp., *Enterobacter* spp., *Yersinia enterocolitica*, *Vibrio parahaemolyticus*, and *V. cholerae*.

Figure 5-73 Transmission electron micrograph of norovirus virions. (Courtesy of CDC/Charles D. Humphrey.)



Sample Collection and Analyses

Stool Sample Collection and Preparation

Fresh samples are collected in dry, leakproof, clean containers and should be examined within 2 hours for maximum bacterial viability. Alternatively, they may be thermally preserved in a refrigerator or in a Cary-Blair medium and delivered to the laboratory as soon as possible.

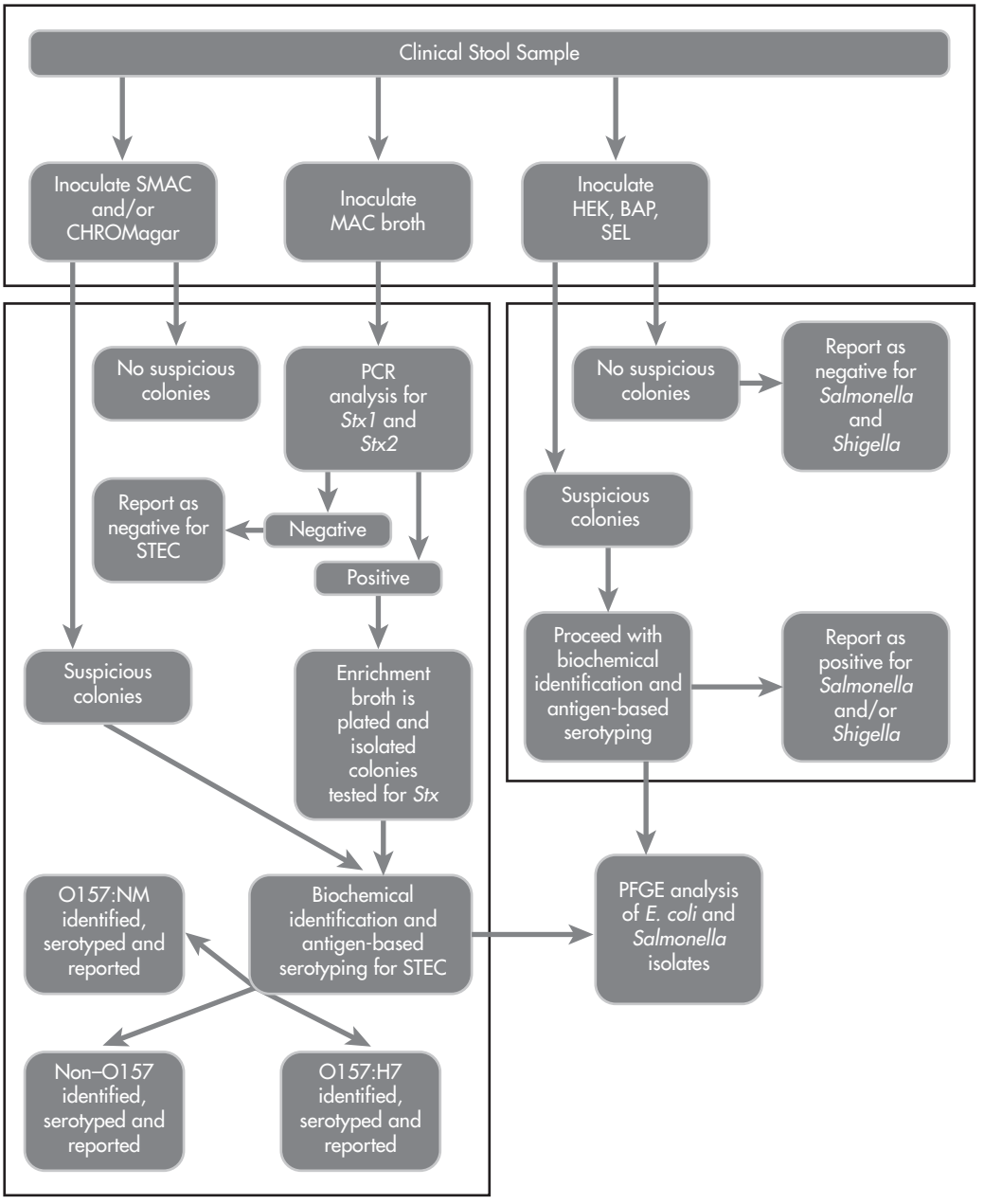
Sample Analysis

The types of sample analysis to which the sample(s) are subjected are based in large part by the instructions of the investigator or clinician from which they come. Based on the clinical presentations of the patient, the clinician or investigator will make the initial determination that the sample should be analyzed for enteropathogenic bacteria, protozoa, or viruses. For example, presentation with bloody diarrhea or hemolytic-uremic syndrome (HUS) would indicate to the clinician to test for STEC. If a viral cause is suspected, the sample will be prepared and analyzed by PCR for norovirus. If a parasitic cause is suspected, the sample will be sent to the parasitology section for microscopic and immunoassay analyses. It is worth noting that CDC recommends that all stool samples be tested for *stx1* and *stx2* because their presence will greatly influence treatment.[126] Studies have shown that 56% of those infected with STEC and treated with antibiotics will develop HUS, but only 8% of those not treated with antibiotics.[127] The assay may be performed with a commercially available immunoassays (e.g., Immuno*Card* STAT!, Meridian Bioscience, Inc., Cincinnati, OH) or PCR. Either method can usually differentiate between *stx1* and *stx2*. The rest of this section will describe the processes of analysis for enteropathogenic bacteria.

Standard enteric bacterial panels usually include *Salmonella*, *Shigella*, and STEC *E. coli*. *Campylobacter* may not be routinely included as it requires low oxygen conditions (versus aerobic or anaerobic conditions). An example of a stool sample testing algorithm (as used by the IDPH laboratory) is shown in Figure 5-74. There are three readily identifiable groups of activities, delineated by color in the figure.

There are three activities performed as a first step (activities in the top box) in sample analysis. Sorbital-MacConkey (SMAC) and/or CHROMagar plates are streaked with the sample to test for the presence of *E. coli* O157 (Figure 5-75). As O157 does not ferment sorbitol, colonies will be colorless/pale (as will some non-O157 strains), while most non-O157 strains, which can ferment sorbitol, will produce pink colonies. CHROMagar

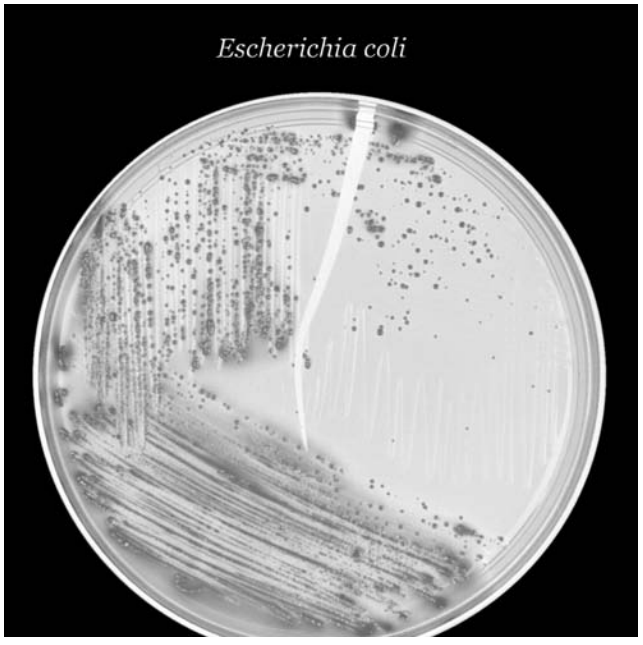
Figure 5-74 Illustration of IDPH enterics sample analysis algorithm.



colonies will be mauve if O157 and pink or blue if non-O157. MacConkey (MAC) broth is also inoculated with a sample portion to grow up *E. coli* spp. Growth is indicated by turbidity after incubation. Lastly, Hektoen and blood agar plates (HEK, BAP) and selenite broth (SEL) are streaked and inoculated with the sample to test for the presence of *Salmonella* and *Shigella*. Hektoen is a differential agar and *Salmonella* colonies will be black or transparent with black centers (Figure 5-76), while *Shigella* colonies will be more greenish in color and appear moist (Figure 5-77). BAP is a nonspecific growth media used to check organism viability. Selenite broth is an enrichment broth media normally used for the recovery of *Salmonella* or *Shigella* specimens from heavily contaminated stool samples.

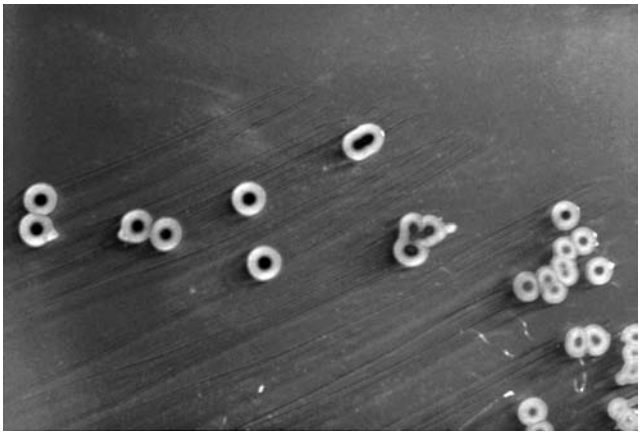
Analyses frequently diverge at this point. We will consider a simpler analysis pathway first, that for the detection and identification of *Salmonella* and *Shigella* (activities in the lower right box). If the HEK plate and SEL broth show no growth (but there is growth

Figure 5-75 ***Escherichia coli*** **grown on a MacConkey agar plate. An image bank of full-color photos is available online at http://www.jbpub.com/catalog/9780763771027/. (Courtesy of CDC.)**



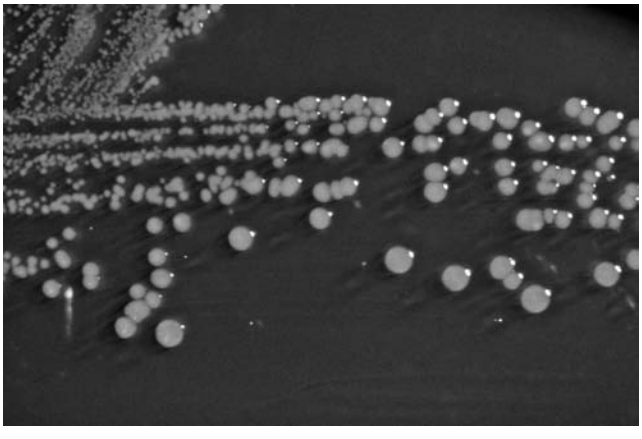
on the BAP), the sample may be considered negative for *Salmonella* and *Shigella*. The appearance of colonies on HEK and/or turbidity in SEL indicates the presence of either organism, presumptively identified based on morphology, and the need for further identification and serotyping. Species identification may be done with the use of miniaturized test kits (e.g., the API 20 E) or automated biochemical analysis system (e.g., MicroScan, Vitek 2). For example, in the API 20 E, aliquots of colony isolates are incubated in individual wells containing various reagents associated with different species-characteristic biochemical reactions. Reactions are observed via color change in the wells. The pattern of reactions is an indication of what genus and species is present. For example, *Salmonella* spp. do not ferment lactose or sucrose but do produce hydrogen sulfide gas (except *S.* Typhi). Positive reactions in these wells, and negative reactions in others, indicates the presence of *Salmonella*. Positives must still be serotyped as biochemical reactions are typically used for the recognition and differentiation of genera and species and not normally used for the identification of the organism serotype. *Salmonella* serotyping is performed by O and H antigen

Figure 5-76 ***Salmonella typhimurium*** **colonies on a Hektoen agar plate. An image bank of full-color photos is available online at http://www.jbpub.com/catalog/9780763771027/. (Courtesy of CDC.)**



slide and/or tube agglutination tests using commercially available antisera. Isolate reactions to the different O and H antisera determine its serotype. *Shigella* serotyping is performed by O antigen agglutination. Isolates identified as *Salmonella* will also be further characterized by PFGE fingerprinting and the data sent to PulseNet (discussed in Chapter 7).

Figure 5-77 ***Shigella boydii*** **colonies on a Hektoen agar plate. An image bank of full-color photos is available online at http://www.jbpub.com/catalog/9780763771027/. (Courtesy of CDC.)**



STEC analyses are somewhat more complicated and require more steps (activities in the lower left box). First, the MAC broth is analyzed by PCR for the presence of the *stx1* and *stx2* genes. If this test is negative, the analysis may be concluded and the sample reported as not containing STEC. Alternatively, a broth positive indicates the presence of STEC species, but not necessarily O157. Colony development on the SMAC plate confirms the presence of O157, but there may be additional STEC species as well (e.g., O26). The presence of Shiga toxin in broth, but no colonies on SMAC, indicates the presence of non-O157 STEC. The analyst needs to be able to identify all STEC stains that are present.

At this point, the broth is streaked on MacConkey to grow colonies of *E. coli* that do ferment sorbitol. After incubation, individual colonies are plucked at random and inoculated into broth tubes for growth. These are then tested by immunoassay (e.g., Immuno*Card* STAT!) or PCR for *stx1* and *stx2*. The idea is to be able to isolate the actual species of STEC (of all *E. coli* species present in the sample). This process of identifying non-O157 serotypes is very time-consuming, complex, and expensive.

Now that isolates of STEC have been obtained, they are subjected to identification as *E. coli* by analysis with the API 20 E (described previously). As is for *Salmonella* and *Shigella*, *E. coli* will engage in specific biochemical reactions that are observed in the individual sample wells. Secondly, colonies are serotested for the differentiation of O157 versus non-O157 and the presence/absence of H7 via latex antibody agglutination tests using commercially available antisera reagents. At this point it may be determined that the colonies are/are not indeed *E. coli* and are/are not O157:H7. If the sample is determined to be STEC O157:H7, it may be reported as such to the submitter at that time. The sample would also be further characterized by PFGE and the data sent to PulseNet. On the other hand, the sample may be determined to be *E. coli,* but non-O157, and is therefore subject to further serotyping testing. This testing would include the antigen agglutination for different *E. coli* O and H antisera. From these results, the other two reports may be made (the first being STEC O157:H7): that the sample contains STEC O157:NM (nonmotile) or STEC non-O157. In either case, the sample is still subjected to further characterization via PFGE and reported to PulseNet.

Campylobacter isolation and identification is done by request as it requires special media and growth conditions. Samples are plated onto charcoal cefoperazone deoxychocolate agar (CCDA) and/or charcoal-based selective medium (CSM). These are both blood free, and a blood-containing medium such as Campy-CVA may also be used. Growth is done under reduced oxygen conditions (O_2 at 5%, CO_2 at 10%, and N_2 at 85%), and specific gas generators can be commercially obtained. Isolates are identified by Gram stain and biochemical reaction. *C. jejuni*, for example, is Gram-negative with a distinctive curve and oxidase positive. Furthermore, it is catalase positive and hydrolyses both hippurate and indoxyl acetate, whereas *C. festus* does not hydrolyze either hippurate or indoxyl acetate.

In some ways the samples received in the enterics section are similar to those received in parasitology; they represent the tip of the iceberg of potential samples. The IDPH laboratories received 5207 enterics samples from July 2007 through June 2008 (unpublished data). Again, many people and clinicians do not realize the importance of sample collection and analysis in identifying and investigating disease outbreaks. The identification of the infectious agent and subtyping and fingerprinting, as occasion warrants, are important to determine the extent of infection on a national level while also providing disease surveillance data to track national trends.

Newborn Screening

Background of Newborn Screening in Public Health Laboratories

Newborn screening (NBS) is perhaps one of the best recognized and most successful health promotions and disease prevention public health programs in the world. Of the many links in the public health chain that contribute to the overwhelming success of this program, the PHL is the first link in the chain, as virtually all newborn blood samples are sent for the initial screening to the state PHL, or to a laboratory contracted by the state public health agency.

NBS is the process of testing newborn babies for treatable metabolic/genetic, endocrine, and hematologic diseases commonly referred to as inborn errors of metabolism (IEMs). Many of these diseases are potentially fatal conditions that are not otherwise apparent at the time of birth. Although the incidence of some conditions are very rare in the newborn population, when one of these conditions is not found and treated, it can affect a newborn's normal physical and mental development. In severe cases, the lack of or delay of treatment can lead to developmental delay, mental retardation, and premature death. PHLs perform the crucial laboratory analyses on NBS samples as soon as the samples are received in the laboratory. These

laboratories perform newborn screening tests for more than 95% of the approximate 4 million babies born in the United States each year. Approximately 3000 babies with severe disorders are identified in the United States annually using state newborn screening programs.[128]

Robert Guthrie, State University of New York at Buffalo in Buffalo, New York, developed and promoted the earliest population based screening for phenylketonuria in the early 1960s by using dried blood spots (DBS) on filter paper obtained by pricking a newborn baby's heel during the first few day of life.[129] Over the next 30 years, Guthrie and his colleagues developed a number of other tests for IEMs.[130–137] In 1973, procedures for hemoglobinopathies and congenital hypothyroidism were published and adopted by many state NBS programs.[138,139] The development of tandem mass spectrometry screening in the early 1990s led to a rapid expansion of potentially detectable IEMs for organic acids, fatty acids oxidation, and amino acids metabolic diseases.[140,141] NBS has been adopted by most countries around the world, though the lists of screened diseases vary widely.

Newborn Screening Programs

In nearly all states, the NBS program is a division of the state public health department. State law mandates collecting a sample onto the filter paper labeled with a unique identifier for the infant and mother and the names of the hospital and primary care physician. Samples for the performance of the NBS tests are collected before the newborn leaves the hospital or birthing center. It is usually specified that the sample be collected between 24 and 72 hours after birth and after protein-containing feedings (i.e., breastmilk or formula) have started, and the postnatal thyroid stimulating hormone (TSH) surge has subsided. States generally require that birthing hospitals as well as independent midwives supervising home deliveries collect the specimens onto the filter paper devices and mail the filter paper cards each day to the public health or contract laboratory.

The state health department agency in charge of screening will either operate a laboratory or contract with a laboratory to analyze the mandated screening tests. The goal is to report the results within a short period of time. If screens are normal, a paper or electronic report is sent to the submitting hospital and parents rarely see reports that are interpreted as "normal." If a potential abnormality is identified, public health followup staff contacts the physician, hospital, and/or nursery by telephone. NBS followup staff conducts a relentless contact string until they can arrange an evaluation of the infant by an appropriate specialist physician (depending on the disease). The specialist will attempt to confirm the diagnosis by performing confirmatory testing. Depending on the likelihood of the diagnosis and the risk of delay, the specialist will initiate treatment or further diagnostic testing and provide genetic counseling and educational information to the family. Performance of the program is reviewed regularly and strenuous efforts are made to maintain a system that identifies and follows every infant with a diagnosis. Guidelines for NBS and followup have been published by the American Academy of Pediatrics (AAP), the American College of Medical Genetics (ACMG), and the National Academy of Clinical Biochemistry (NACB).[142–144]

Sidebar 5-13 Components of a State NBS Program[143]

The public health NBS program in each state is a system generally consisting of six parts: education, screening, followup, diagnosis, management, and evaluation.

- *Education* is given to the parents before the birth of their child by brochure(s) given to the healthcare providers by the state NBS program in order to inform the parents of the benefits of NBS. The brochure explains why NBS is done, what IEMs and other genetic diseases are screened for, and the possible health effects on the child if these diseases are not detected early.
- *Screening* is the process of collecting a blood sample from a newborn (ideally 24–36 hours after birth), for expeditious shipment and subsequent testing at a state PHL, or a laboratory designated by a state public health agency to perform NBS. Timing of specimen collection is dependent on several variables such as birth weight, gestational age, transfusions, and feeding status. Specific requirement are established by each NBS program.
- *Followup* is the process of activating public health department NBS resources to contact parents, doctors, nurses, and other healthcare providers attending the child. This effort is to recall infants who have demonstrated a presumptive positive in the initial screening test to the healthcare setting of their pediatrician, primary care

physician, and/or birthing hospital in order to more thoroughly assess their health status
- *Diagnosis* is performed by direction of highly trained physicians (e.g., pediatric hematologists, biochemical geneticists, pediatric pulmonologists, or pediatric endocrinologists) using additional, more specific tests to confirm that an infant identified by a presumptive positive NBS does indeed have a disease.
- *Management* is the specific treatment plan developed by the attending physicians along with input from the public health genetic program staff to deal with the particular metabolic or genetic anomaly so as to assure normal or near normal development of the child. (For example, an infant diagnosed with phenylketonuria has a plan developed by healthcare providers for use by the child's parents, instructing them on how to serve the child's nutritional needs with a diet low in the amino acid, phenylalanine.)
- *Evaluation* is the ongoing assessment by parents, public health, and primary healthcare providers of the effectiveness of the intervention to assure that normal child development is indeed occurring.

The funding source for this public health activity (including laboratory testing and case management) may be either state general revenue funding, a direct fee for service program, or a combination of the former and private health insurance.

Common Targets of Analysis

As mentioned previously, following Robert Guthrie's implementation of an efficient filter paper blood collection mechanism for analyzing phenylketonuria in the late 1960s, more screening tests followed. The development of tandem mass spectrometry screening in the early 1990s led to an expansion of potentially detectable congenital metabolic diseases that affect blood levels of organic acids, fatty acids, and amino acids. Additional tests have been added to many screening programs over the last 2 decades. Virtually every state NBS program in the United States now includes at least the following tests:

Phenylketonuria (PKU) is an autosomal recessive genetic disorder characterized by a deficiency in the enzyme phenylalanine hydroxylase (PAH). This enzyme is necessary to metabolize the amino acid phenylalanine to the amino acid tyrosine. When PAH is deficient, phenylalanine accumulates and is converted into phenylpyruvate (also known as phenylketone), which can be detected in the urine. In a newborn blood sample, the PHL analyzes for phenylalanine as well as tyrosine. A ratio of phenylalanine to tyrosine may be calculated to assist in identifying cases of PKU or hyperphenylalaninemia (HPA). Left untreated, this condition can cause problems with brain development, leading to progressive mental retardation and seizures. However, PKU is one of the genetic diseases that can be controlled by diet. A diet low in phenylalanine and high in tyrosine can be a very effective treatment. There is no cure and, if untreated, the resultant neurologic damage is irreversible so early detection is crucial. The incidence of PKU is approximately 1 in 15,000 to 20,000 live births in the US population.[128]

Congenital hypothyroidism (CH) is a condition of thyroid hormone deficiency present at birth. Approximately 1 in 4000 newborn infants has a severe deficiency of thyroid function, while even more have mild or partial degrees of impaired thyroid function. If untreated for several months after birth, severe congenital hypothyroidism can lead to growth failure and permanent mental retardation. Treatment consists of a daily dose of thyroid hormone (thyroxine) by mouth. Because the treatment is simple, effective, and inexpensive, nearly all of the developed world practices NBS to detect and treat congenital hypothyroidism in the first weeks of life. In the initial screening test, the PHL analyzes for elevated TSH. Most NBS laboratories use TSH as a primary screening test and if TSH is elevated, a secondary test for T4 is performed.

Congenital adrenal hyperplasia (CAH) refers to any of several autosomal recessive diseases resulting from mutations of genes for enzymes mediating the biochemical steps of production of cortisol from cholesterol by the adrenal glands (steroidogenesis). Most of these conditions involve excessive or deficient production of sex steroids and can alter development of primary or secondary sex characteristics in some affected infants, children, or adults. Some infants will have a life-threatening electrolyte imbalance if this disorder is not recognized early and promptly treated. Approximately 95% of cases of CAH are because of 21-hydroxylase deficiency.

PHL screenings are primarily conducted to detect the lack of the 21-hydroxylase enzyme (e.g., 17-hydroxyprogesterone) but may detect a few other mutated enzymes in the pathway.

Galactosemia (GAL) is a rare genetic metabolic disorder that affects an individual's ability to properly metabolize the sugar galactose. Lactose in food (such as dairy products) is broken down by the body into glucose and galactose. In individuals with galactosemia, the enzymes needed for further metabolism of galactose are severely diminished or missing entirely, leading to toxic levels of galactose to build up in the blood. The PHL analyzes for total blood galactose and/or uridyltransferase enzyme activity. Elevated galactose levels result in hepatomegaly, cirrhosis, renal failure, cataracts, and brain damage. Without treatment, mortality in infants with galactosemia is about 75%.

Sickle cell disease and other hemoglobinopathies are screened for using methods such as HPLC or isoelectric focusing (IEF). As an example, HPLC is an effective method for screening for abnormal hemoglobins such as S (Sickling), C, D, and E as well as the normal hemoglobins found in the neonate, namely fetal (F) and adult (A). Carriers of certain traits (such as sickle cell trait) are identified as well as homozygous cases of disease, such as sickle cell disease. Early identification of sickle cell disease allows timely intervention such as prophylactic antibiotic administration as well as treatment with chemotherapy agents. Crises may be avoided and complications minimized by this early identification. Other hemoglobinopathies detected by these methods are alpha and beta thalassemias. The thalassemias are quite common in some populations (e.g., Mediterranean and South East Asian) and can cause serious anemia.

Cystic fibrosis (CF) is now universally screened for in the United States. CF is an autosomal recessive disorder that results in production of a defective form of CF transmembrane conductance regulator (CFTR) protein. CFTR protein is a component of the chloride channel within the epithelial cells of multiple organs, and regulates movement of salt and water into and out of the cells. More than 1600 individual mutations have been identified, of which the mutation identified as dF508 is the most common. Infants identified early in life benefit from timely intervention and treatment. Advances in CF treatment have improved health and quality of life, along with the median age of survival, which is now approximately 37 years of age. Screening for CF typically uses a two-tiered protocol of an initial screen for elevated immunoreactive trypsinogen (IRT), followed by DNA analysis for specific mutations when the initial IRT is elevated. Neonates identified with a mutation are referred to a CF center for sweat chloride testing (pilocarpine iontophoresis) and clinical evaluation. It is worthy of note that when using the IRT/DNA algorithm, heterozygotic carriers are identified as well as homozygotic cases of CF. Alternate testing algorithms may be employed to best suit the needs of the target population.[145]

NBS results are available within turnaround times published by each NBS screening laboratory. Presumptive positive results are communicated immediately via telephone by laboratory staff to the public health followup staff. Healthcare providers are contacted by the followup staff using telephone and/or fax. Public health followup staff may enlist the assistance of local public health nurses or law enforcement agencies when difficulties are encountered making contact with healthcare providers of families/guardians of newborns.

Tandem Mass Spectrometry

With the development of tandem mass spectrometry, the potential for the analysis of a number of detectable diseases quickly grew, especially in the categories of fatty acid oxidation disorders and organic acid disorders. Advocates from various interests groups and groups of parents with affected children have pressured state health departments to add mass spectrometry (MS/MS) screening and all the disorders recommended by ACMG. The diseases listed in Table 5-3 include most of the disorders capable of being detected by NBS using MS/MS or other methodologies. This list of disorders is not yet universally mandated by all states, but may be privately obtained by parents or hospitals. Perhaps 1 in 4000 to 5000 infants will be positive for one of the metabolic disorders discussed here (unpublished data from IDPH laboratory). The ACMG reviewed many of these disorders, and recommended inclusion in NBS programs as appropriate.[143]

The main clinical features of the amino acid disorders (e.g., hyperphenylalaninemias, maple syrup urine disease, and homocystinuria) are mental retardation,

Table 5-3 List of Disorders Detected by Tandem Mass Spectrometry

Inborn Errors of Amino Acid Metabolism	
Phenylketonuria (PKU)	Argininemia
Hyperphenylalaninemia (HPA)	Argininosuccinic aciduria (ASA)
Tetrahydrobiopterin deficient	Citrullinemia (CIT)
Tyrosinemia I	Maple syrup urine disease (MSUD)
Tyrosinemia II	Homocystinuria (HCY)
Tyrosinemia III	HHH syndrome (Hyperammonemia, hyperornithinemia, homocitrullinuria syndrome)
Nonketotic hyperglycinemia (NKH)	
Inborn Errors of Fatty Acid Metabolism	
Carnitine palmityl transferase deficiency type 2 (CPT II)	Medium/short-chain hydroxy Acyl-CoA dehydrogenase deficiency (SCHAD)
Carnitine palmityl transferase deficiency type 1 (CPT I)	Medium-chain acyl-CoA dehydrogenase deficiency (MCAD
Long-chain acyl-CoA dehydrogenase deficiency(LCAD)	Very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD)
Long-chain hydroxyacyl-CoA dehydrogenase deficiency (LCHAD)	Carnitine/acylcarnitine translocase deficiency (CACT)
Short-chain acyl-CoA dehydrogenase deficiency (SCAD)	Carnitine uptake defect (CUD)
Isobutyryl-CoA dehydrogenase deficiency	Trifunctional protein deficiency (TFP)
Multiple acyl-CoA dehydrogenase deficiency (MADD) or Glutaric acidemia type II	
Inborn Errors of Organic Acid Metabolism	
Glutaric acidemia type I (GA I)	Methylmalonyl-CoA mutase deficiency (MUT)
3-Hydroxy-3-methylglutaryl lyase deficiency (HMG)	Methylmalonic aciduria, CblA, CblB, CblC, and CblD forms (MMA, Cbl A, B, C, D)
Isovaleric acidemia (IVA)	Beta-ketothiolase deficiency (BKT)
2-Methylbutyryl-CoA dehydrogenase deficiency	Propionic acidemia (PROP)
3-Methylcrotonyl-CoA carboxylase deficiency(3MCC)	Multiple-CoA carboxylase deficiency (MCD)
3-Methylglutaconyl-CoA hydratase deficiency	

developmental delay, and some present with seizures and death. The urea cycle amino acid disorders (e.g., argininemia, argininosuccinic aciduria, and citrullinemia), which are in the catabolic pathway of various amino acids, present with hyperammonemia, lethargy, coma, and death. The organic acid disorders present with hypoglycemia, metabolic acidosis, and some with ketoacidosis and hyperammonemia. The fatty acid oxidation disorders present with symptoms ranging from hypoglycemia, hypoketonemia, dilated cardiomyopathy, cardiac and skeletal myopathies, and rhabdomyolysis. If not found and treated, most of the disorders will lead to irreversible brain damage and possible death. Treatment for many forms of these diseases is very effective, but for others the natural history is not well characterized and treatment has only limited effect or is not effective.

Mass Spectrometry Screening and Controversies

Instituting MS/MS screening requires a sizable initial expenditure. When states choose to run their own programs the initial costs for equipment, training, and new staff can be significant. To avoid at least a portion of the

initial laboratory setup costs, some states have chosen to contract with private labs for this screening. Others have chosen to form regional partnerships sharing both costs and resources. But for many states, screening is an integrated part of the department of public health that cannot or will not be easily replaced. Thus, the initial expenditures can be difficult for states with tight budgets to justify. Articles published in *Pediatrics* have suggested that expanded screening maybe cost-effective.[146,147] Advocates are quick to point out studies such as these when trying to convince state legislatures to mandate expanded screening. On the other hand, expanding NBS to include many of the disorders on the list is opposed by some who are concerned that effective followup and treatment may not be available, or that false-positive screening tests may cause more harm than good. Some of these disorders are extremely rare and have later onset forms that may not be detected during the newborn period. Also the natural history for many of these diseases has not been well characterized and effective treatment may not be available.[148,149] A number of conditions may not follow the classic screening protocol, and as such, require discussion among stakeholder groups and the public health community prior to implementation.[150] Pilot testing, including specific informed consent, are helpful for developing and evaluating NBS test algorithms.

It is important to keep in mind that false-negative screening tests are always possible. While false-positives and false negatives are not entirely unavoidable, they can be minimized by employing effective quality assurance programs in the laboratory.

Expansion of NBS for conditions for new genetic diseases will require clinically sound and ethically conducted protocols. Each PHL must establish a protocol for post-analysis storage and disposition of the NBS specimen collection cards. NBS specimen collection cards are unique in that the test requisition containing identification and personal medical information for both baby and mother are part of the actual specimen collection instrument. In other words, filter paper collection cards contain personal medical information and the neonate's blood sample (i.e., DNA). The PHL and its associated NBS program are ethically and legally bound to protect this information from unauthorized access. Prior to disposal after the holding period, the NBS specimens should be rendered biologically inactive (e.g., steam autoclaving) and the included personal information neutralized (i.e., rendered unrecognizable using cross-cut shredding). Public health programs will retain public trust by implementing and maintaining personal information safeguards.

Sample Collection and Analysis

Sample Collection

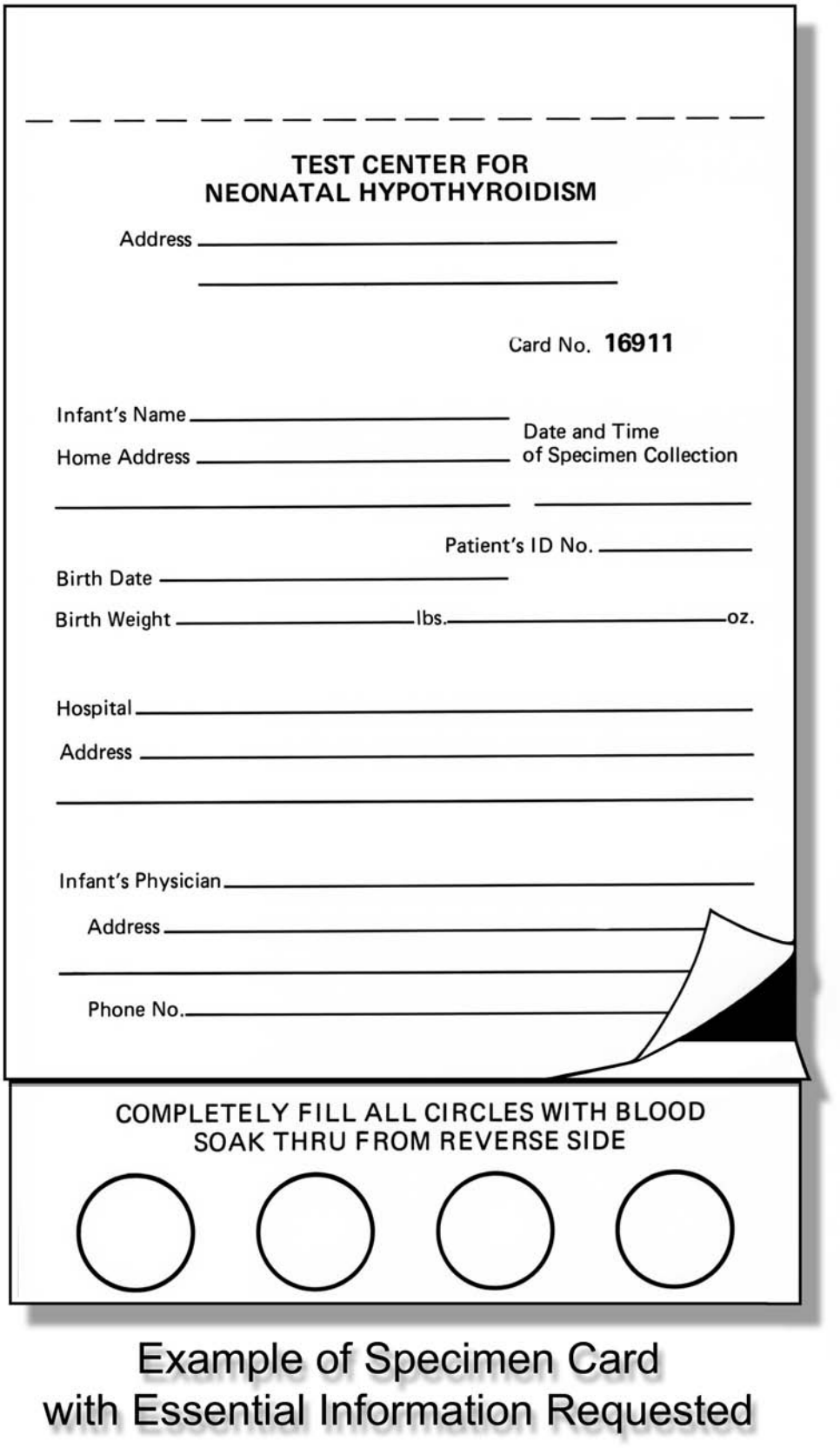
Specimen collection in the hospital nursery is greatly facilitated as the neonate's blood sample is derived from a small lancet stick made in the heel at 24 to 36 hours of age. The few blood drops that are actively expressed from this puncture wound are collected onto a specially manufactured filter paper, having circumscribed circles 13 mm in diameter or slightly smaller than the size of a dime. The filter paper material used for this collection procedure is so highly characterized in terms of fiber content, density, etc., that the filter paper is registered as a medical device by the FDA. An example is shown in Figure 5-78.

Following blood collection within the scribed four to five circles on the paper, the blood is allowed to dry completely before the sample(s) are packaged for shipment to the PHL. Dried blood spots on filter paper are considered to be a minimum health hazard and can be shipped by common carriers in a sealed bond envelope or equivalent.[151] Unpackaged dried blood spots cards should be handled using personal protective equipment (e.g., latex gloves or equivalent and lab coat). Because of the nature of several disorders affecting newborns, it is critical that the samples are shipped to the PHL without delay. "Batching" of the samples (i.e., holding of the samples at the hospital or collection site until a group of samples is available) presents a serious risk to infants with rapid-onset disorders such as classic galactosemia or the salt-wasting form of congenital adrenal hyperplasia. Timely transport to the PHL is considered part of the preanalytic process when appraising the overall NBS "system." Many NBS programs find it advantageous to contract with a common carrier for delivery of the NBS samples to the laboratory, preferably by a service such as "next business morning delivery." Tracking and confirmation of delivery of the individual packages is the responsibility of the submitter. In the event of a delayed or "lost" shipment, the submitter should recall those neonates as soon as possible for resampling in order to avoid the risk of a metabolic crisis in the event a disorder might be present in one of the neonates whose samples were shipped within the delayed or lost package.

Sample Analysis

Once received in the laboratory, the filter paper samples are assessed for adequacy of collection and other relevant quality control (QC) parameters. When QC parameters are satisfied, the sample cards are assigned an accession number or bar code number and subjected to a manual or

Figure 5-78 Example of a standard infant blood sample card. (Courtesy of CDC.)



automated punching process, where 3.2-mm circular paper disks are punched from the filter paper cards into sample testing wells (e.g., most laboratories use 98-well microtiter plates). This punching process is similar to that using a handheld paper punch to make circular holes in a piece of stationery. These 3.2-mm circular paper disks are nearly universally referred to as dried blood spots (DBSs). Subsequent hydration of the DBS with deionized water, buffer, or other suitable reconstituting liquid followed by careful elution yield the liquid samples containing the analytes of interest in NBS. Each laboratory is required by CLIA to have a written protocol(s) for preanalytical testing.

The elements of a public health NBS laboratory contain numerous pieces of complex instrumentation with capabilities that include but are not limited to HPLC, rapid flow analysis using fluorescent or colorimetric detection, immunofluorescent assays, polymerase chain reaction, and tandem mass spectrometry. The actual analysis methods used vary considerably from state to state. In fact, in NBS, we find greater variety and a greater reliance on methods derived from scientific literature than any other testing section. This variety leads to the substantial discussion concerning QC discussed here. In general, though, laboratories use either published methods customized to their own needs, commercially obtained kits, or a combination of these. We can see this variation by examining the QC data for 2008. For all laboratories participating in the 17 α-hydroxyprogesterone test (Lot 751) returning 2011 test results, 92% used commercial methods (63.5% used the AutoDelfia, PerkinElmer, Waltham, MA), 2.4% used an LC-MS/MS method, and 2.0% used an "inhouse" method. We see a high degree of reliance on a commercial product for this analyte. Alternatively, the total galactose test (Lot 727) returning 769 test results had 16.4% performed by either manual fluorometric or colorimetric (generic) with 70.9% performed by identified commercial methods.[152]

NBS by the PHL is the first step in the overall assessment of the newborn. Once the screening is confirmed by diagnostic testing, PHL scientists collaborate with other public health and medical practitioners to make certain that the affected child receives prompt and appropriate followup care. At this point, it is important to pause and reiterate that NBS is just that, screening, not diagnostic testing.

Quality Assurance

To strive for NBS testing that is consistent from one laboratory to another, the state PHLs work very closely with the CDC on all aspects of laboratory newborn testing. CDC's Newborn Screening Quality Assurance Program (NSQAP) works with NBS laboratories on QC and proficiency testing issues relevant to NBS laboratories across the globe. The NSQAP program impacts state NBS laboratories in implementing national recommendations on NBS and genetics testing by participating on national and federal committees.

The QA services of CDC primarily support NBS tests performed by state PHLs; however, the CDC also accept other laboratories and international participants into the QA program. Currently, CDC provides QC materials, proficiency testing (PT) services, and technical support to 73 domestic screening laboratories, 28 manufacturers of diagnostic products, and 300 laboratories in 53 foreign countries. With such an extensive reach, this CDC program is the most comprehensive provider of QA services for NBS worldwide.

The QC program enables screening laboratories to achieve high levels of technical proficiency and continuity that transcends changes in commercial assay reagents while maintaining the high-volume specimen throughput that is required in each of the PHLs performing these important screening tests. The PT program provides laboratories with quarterly panels of blind-coded DBS specimens and gives the laboratory an independent external assessment of its performance. In the event that a laboratory misclassifies a PT specimen, the laboratory must determine whether the error was caused by an analytical failure or a clerical error in reporting the results to CDC. In either case, good laboratory practices require that the laboratory develop a plan of correction in order to avoid or minimize the possibility of a similar occurrence. When laboratories are evaluating their performance on PT challenges, the CDC is available to provide technical assistance.

Regardless of participation in external quality assurance programs, it is the responsibility of the NBS laboratory to implement its own quality assurance program. This program must include periodic reviews of the overall function of the NBS laboratory, not only the analytic component, but also the patient test management activities (i.e., specimen receiving, test result reporting, and followup/case management). Each person, whether laboratory analyst or clerical support, must be aware of the critical nature of their work within the overall context of the NBS program. It is this regard that a logic model for the entire NBS program be developed so that each specialty area (e.g., laboratory, clerical/support, case management) may clearly see their organizational relationships.

At the end of each year, NSQAP prepares and distributes to all participants a summary of all PT and QC data reported for that year. Distributions of PT panels occur in January, April, July, and October. QC materials are distributed in January and July. NSQAP operates the NBS QC and PT programs at no cost to the participants.

Recent advances in technology and genetics have highlighted a growing need to incorporate advanced testing technologies in PHLs and to explore the laboratory's role in genetic testing. As states adopt new tests for additional diseases, APHL and its state and county member laboratories are working with the CDC to broaden services for maintaining the quality of test results associated with the new testing methods. Concerted efforts are also focused on assuring the continuity of testing during emergencies by entering into strategic partnerships among member state PHLs. NBS will continue to effectively serve the need to assess infants at birth for a long list of metabolic/genetic disorders—and the list will get longer. As entities of state government, NBS programs receive attention from stakeholders such as state legislators, advocacy groups (both local and national), hospital associations, and medical specialists.

Public Law and Privacy

Efforts by the US Congress have highlighted the need to assure that NBS program successes continue (Public Law 110-204 Newborn Screening Saves Lives Act of 2008).[153] There is no consensus, however, on the worth of expanding NBS programs when personal privacy issues are considered. There are groups of professional and lay people who oppose expansion, the collection of health-related data, and the storage of residual blood spots. Congress has indicted that the federal government should "continue to carry out, coordinate, and expand research in newborn screening" and "maintain a central clearinghouse of current information on newborn screening ensuring that the clearinghouse is available on the Internet and is updated at least quarterly."[154] A recent Internet-based survey of a nationally representative parent sample has found that a majority of parents are willing or somewhat willing to permit the use of residual NBS dried blood spot samples to be used for research but only with their consent.[155] A majority was also willing to have their children's blood spots stored. Some of the proposed expansions in NBS include actions to:

- Establish a national list of genetic conditions for which newborns and children are to be tested.
- Establish protocols for the linking and sharing of genetic test results nationwide.
- Build surveillance systems for tracking the health status and health outcomes of individuals diagnosed at birth with a genetic defect or trait.
- Use the NBS program as an opportunity for government agencies to identify, list, and study "secondary conditions" of individuals and their families.

As evidenced here, safeguards relating to individual privacy and protection of personal information must be considered and instituted as desired by citizens. But care must be taken not to minimize the documented benefits of the programs carried out so effectively by the nation's public health departments.

NBS may be considered one of the core analysis of PHLs. They certainly constitute a large total share of received samples for any laboratory. The IDPH laboratory in Chicago received 186,419 NBS samples from July 2007 through June 2008 (unpublished data). This is the largest volume received by any testing section and constituted 28% of all samples received that year. When considering the cost of personnel and equipment versus the sheer number of analyses and averted costs associated with undetected medical problems, NBS may be the most cost-effective section in most PHLs.

Blood Lead

Overview of Blood Lead Poisoning

Poisoning with lead remains a preventable health problem in the United States even though the use of lead in gasoline was banned in 1995. This followed the recommendation by the EPA for the phasing out of lead as a gasoline additive in 1973, and the Clean Air Act Amendments of 1990.[156] Remaining sources of lead include paint dust and chips from homes built before 1978 (where lead-based white paint was still used), contaminated soil and water, and industrial sources such as smelters and metal refining. The CDC estimates that 83% of homes built before 1978 still contain lead-based paint and the most important source of exposure for children is associated with paint dust and chips and common hand-to-mouth activity.[157] The New York State Department of Health found in a study of 2006–2007 data that home renovation, repair, and painting activities were the probable source of lead exposure in 14% of elevated child blood lead levels (BLLs).[158]

The addition and widespread use of tetraethyl lead to gasoline was once a major source of environmental

exposure, and its phasing out has been associated with great drops in measured BLLs in children. BLLs in children more than 10 μg/dl are considered "elevated" and have been shown to be associated with significant learning delays, and a level more than 70 μg/dl may have acute symptoms such as seizures, coma, and death. While the mean BLL in children aged 1 to 5 was 15.0 μg/dl in 1976–1980, it dropped to 2.7 μg/dl during 1991–1994.[157] In a similar fashion, data from the NHANES has been shown that the percentage of children with a BLL > 10 μg/dl has dropped from 77.8 to 4.4% between 1976–1980 and 1991–1994. During the 1999–2002 survey, the prevalence of elevated BLL in children aged 1 to 5 was 1.6%, the highest of any age group and associated with ~310,000 children at risk of harmful lead levels. Minorities, the poor, and those living in older homes are at disproportionately greater risk of having elevated BLLs.[159]

Lead is absorbed by the body's soft tissues and bones and can affect almost every system in the body. While exposure to the dust from decaying lead-based paint is the most common exposure source for children, other important sources include the use of certain home remedies (e.g., ayurvedics), improperly glazed pottery and some hobby materials, and contaminated foods and products imported from other countries (e.g., toys and toothpaste). Lead poisoning often does not present with any symptoms, underscoring the need for screening for its detection. The common medical treatment for acute lead poisoning is chelation therapy. Chelators are chemical compounds that bind to lead in the body and are excreted in the urine. This treatment is usually reserved for these with very high BLLs and may be accompanied by diet and lifestyle changes. The most common response to elevated BLLs is a thorough examination and cleaning of the child's living areas. Remediation steps for the removal of lead sources (e.g., old paint) may be suggested and the child/family may have to live elsewhere until the lead source is adequately addressed.

Childhood Lead Poisoning Prevention Program

This program was established by the CDC in response to the Lead Contamination Control Act of 1988.[160] Its primary responsibilities are to prevent childhood lead poisoning through programs, policies, and education; provide grant funding to state and local partners for lead screening and response activities; and support research into the effectiveness of prevention efforts. The program has also instituted the Childhood Blood Lead Surveillance System to which blood lead data from 46 states are reported. Figure 5-79 shows not only the decrease in the proportion of children with elevated BLLs between 1997 and 2006, but also the generally increasing number of all children who are screened.[161] State health department laboratory analysis of blood lead is usually directly funded by this program, and often constitutes a large proportion of all laboratory testing.

Sample Collection and Analysis

Sample Collection

Capillary blood samples are allowed for screening purposes, but are subject to falsely increased values (usually because of poor collection procedures or contaminated materials). Any capillary sample with a high value must be followed by venous blood for confirmation and to institute remediation. It is also recommended that venous samples be preserved with either EDTA or heparin. They are stable for up to 10 weeks if stored at 4°C.[162]

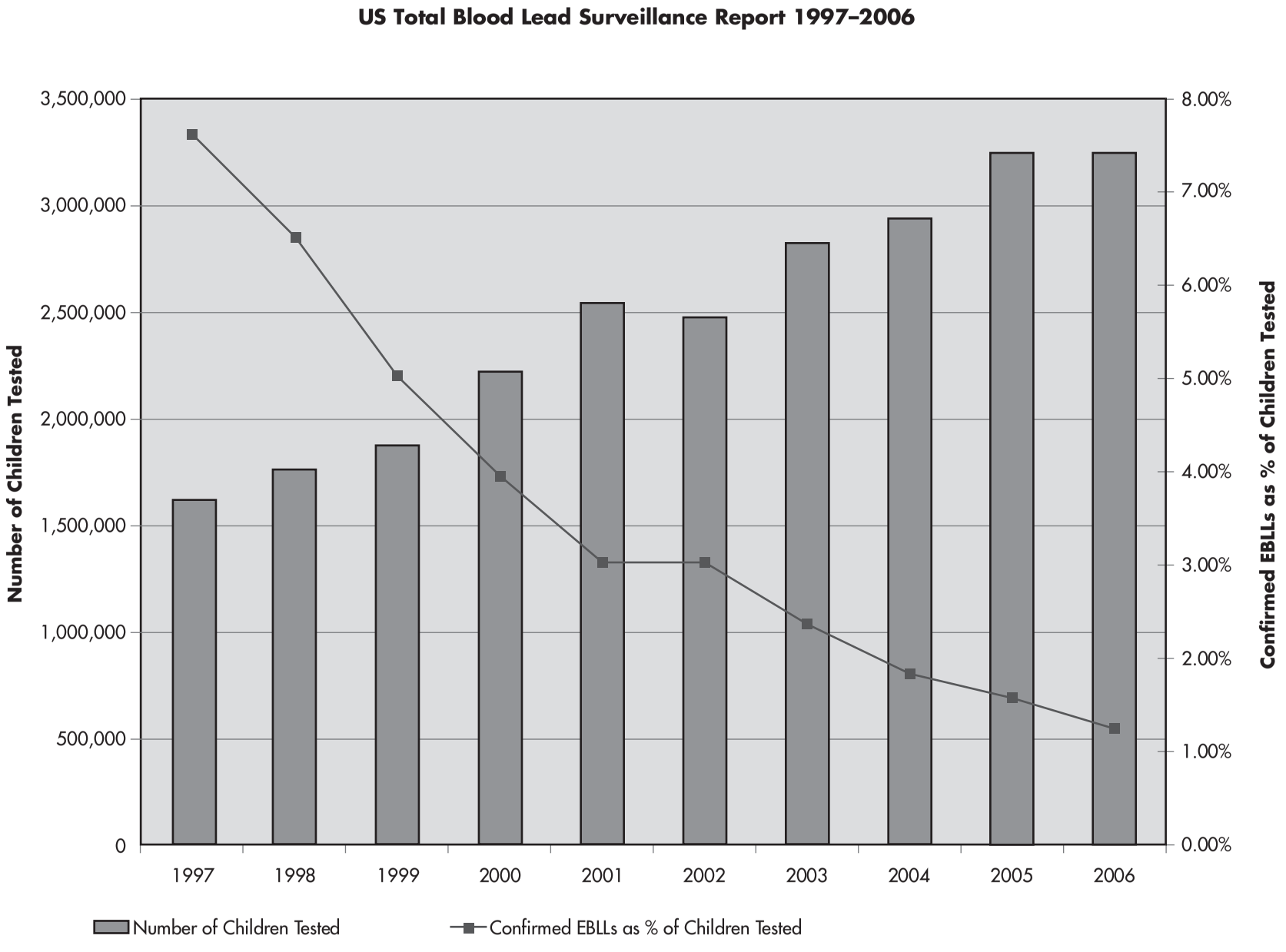
Sample Analysis

Sample analysis is most often performed using graphite furnace atomic adsorption spectroscopy (GFAAS). A small aliquot of sample (e.g., 10 μl) is placed in a graphite tube that is heated via electrical resistance to an excess of 3000°C. The sample is vaporized with every chemical bond broken and passes through a beam of light. The detector measures the absorbance at 283.3 nm, which is characteristic for elemental lead. The amount of absorption is related to the amount of lead present. Methods of analysis are provided by instrument vendor.

Samples may also be analyzed via inductively coupled plasma mass spectroscopy (ICP-MS). Diluted whole blood specimens are converted into an aerosol with a nebulizer and a portion transported into the plasma. The thermal energy of the plasma atomizes and ionizes the sample and a small portion of the ions enter the mass spectrometer that detects ions at specific mass-to-charge ratios and measures their intensity and subsequently their concentration. Lead is determined by measurement of mass 208 (the most abundant isotope for lead). Bismuth (mass 209) is used as an internal standard for the measurement of the lead.

The blood lead testing section (if separate from other sections) may be quite similar to NBS in terms of efficiency. The IDPH laboratory in Springfield received 85,239 blood lead samples from July 2007 through June 2008 (unpublished data). This was the third largest set of samples for a specific group (after NBS and

Figure 5-79 Chart showing the decreasing proportion of EBLLs and increasing numbers of children < 6 years tested annually. (Courtesy of CDC/Lead Program.)



CT/GC) and constituted 12.6% of all sample received. As with NBS and CT/GC, the sheer number of samples received versus the relatively low cost of analysis makes this another of the more cost-effective sections in the PHL.

Discussion Questions

1. This chapter examined the analysis of clinical samples for many different things. Many require specialized (and expensive) training and techniques not readily used elsewhere. Which test or section would you consider the most important and why?
2. If faced with a budget crunch requiring that some testing be discontinued, which test or section would you eliminate first and why?
3. Is TB infection a problem outside of major cities? Find the TB report for your state to see where cases occur and how they are handled.
4. How long does it take to get complete TB analysis results and why is this longer than most other tests?
5. Influenza testing has become more popularly recognized with the emergence of pandemic A H1N1 in 2009. How may laboratory tests for this strain differ from more routine screening?
6. What might be the most important factors when choosing a method for influenza analysis?
7. Read the article "Department of Defense global laboratory-based influenza surveillance 1998-2005" found in *American Journal of Preventive Medicine* 2009, issue 37, number 3. Describe the program and how it complements WHO and state PHL efforts.
8. Many people have a fear of rabies beyond that of other diseases. How might laboratory staff help alleviate a fear of infection?
9. WNV was first identified in the United States in 1999 and has since spread across the country. Find the WNV report for your state and describe how/if it is distributed.
10. How might arbovirus testing algorithms vary from Utah to Georgia?
11. Protozoan infection seems to be widespread, but relatively few cases are laboratory confirmed. Why might this be so?
12. If malaria has not been endemic in the United States since the 1950s, describe at least two reasons for continued testing.
13. Tests for different STIs have become more sensitive, cheaper, and faster and yet the incidence of some diseases continues to either rise or resist significant decline. Why might this be so?
14. The Tuskegee Syphilis Experiment has significantly affected the performance of testing and research in the United States. Using at least two different sources, describe this study.
15. Describe some of the differences between O157: H7, non-O157 STEC, and non-STEC.
16. An enterics algorithm is described in Enterics section. Why is *Campylobacter* not included?
17. Why is there such a pressing need for the swift analysis and reporting of NBS samples?
18. What are some of the issues with implementing tandem mass spectroscopy (MS/MS) screening?
19. What are some of the likely sources of childhood lead exposure?
20. What argument would you present for continuing/implementing a blood lead analysis program?

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6

Water Testing

Introduction

The availability of safe drinking water is a precious resource most people in the United States take for granted. Indeed, the lack of sufficient water supplies is an indirect, and sometimes direct, cause of armed conflict in many other parts of the world. In the United States, we have an abundance of available water (though not always conveniently located). To ensure that water supplied for consumption is safe for use, the US Environmental Protection Agency (EPA) has established rules and guidelines for water quality that must be met by commercial providers of water. Public health laboratories (PHLs) are frequently called on to perform some types of water analyses, though the scope and responsibilities vary greatly by state. Much of the regulatory information concerning water used for consumption and recreation may be found on the EPA's Office of Ground Water and Drinking Water Web site at http://www.epa.gov/safewater/index.html.

Water, both drinking and that used for recreation, continues to be a source of infection and illness in the United States. For the 30-year period of 1971 to 2000, there were 1010 identified outbreaks of water-borne disease. Of these outbreaks, 24% were caused by protozoa, 20% by bacteria, 9% by chemical(s), 7% by viruses, and 40% were of unknown etiology. *Giardia* and *Cryptosporidium* protozoa were the most common identified causes of outbreaks in community water systems, causing 60% of the total. Furthermore, 259 outbreaks were associated with the recreational use of water, such as water parks or lakes.[1] It is for this reason that many PHLs spend significant time testing the quality of water along beaches where swimming is popular.

Water safety in the United States is covered by an act of Congress, the Safe Drinking Water Act of 1974 (revised in 1986 and 1996). Under the Act, the EPA has the responsibility to set national standards for drinking water quality, establish legal limits for specific contaminant levels and required testing schedules, and promulgate adequate methods for analysis. States may also set their own contaminant levels so long as they are at least as rigorous as the federal standards. The original congressionally mandated set of regulated contaminants numbered about 20, and this has since increased to 83. The EPA incorporated six rules in response to the congressional actions.

The reader will quickly discover a significant change in tone from the last chapter to this current one. In Chapter 5, we were concerned with the diagnosis of illness, pathogens, or contaminants within samples obtained from individuals. For these analyses, there were often Food and Drug Administration (FDA)-approved instrumentation and/or kits, such as the GenProbe. For others, it was up to the analyst or laboratory to determine which tests were suitable for their purposes, such as trichrome stain for stool analysis or a particular tandem mass spectroscopic method for a metabolic disorder. This chapter concerns itself with analyzing water samples as part of regulatory compliance in accordance with federal agency rules. Thus, the onus is on the rule-making agency to determine which methods are allowed for analysis and their limits. If a water source is regulated, and may be subject to legal action if the water does not meet

regulated standards, then the regulating agency must ensure that its methods are appropriately robust and sensitive. We will quickly find that the allowed methods are quite specific in their execution (with very specific titles) and have readily determined levels of detection.

Phase I, II, IIb, and V Rules

These rules regulate the majority of chemical contaminants. They set the allowable limit for each compound in drinking water (maximum contaminant level [MCL]), the testing schedule for each compound, and treatment options for their removal. The MCL is legally binding and systems must ensure the levels are met. The maximum contaminant level goal (MCLG) is the estimated level of a parameter at which a person could consume 2 liters per day for 70 years without any ill effects. This level is not legally binding.[2] For known cancer-causing agents, the MCLG is zero. The regulated compounds all have the potential for health risks over the long term, and two (nitrate and nitrite) may have acute effects, especially in infants.

- The Phase I rule became effective in January 1989 and regulated the levels of eight volatile organic compounds (VOCs). These are used in many industries such as plastics, deodorants, and pesticides. A 1982 survey found at least small levels of at least one of these compounds in 28% of those systems serving $>$ 10,000 people, and 17% in those serving $\leq$ 10,000.[2]
- The Phase II and IIb rules became effective in July 1992 and January 1993. The list of regulated compounds has grown to 38 and some of the new ones are widely used in agriculture. MCLs and MCLGs were set for 36 of these compounds. The other two (acrylamide and epichlorohydrin) are used early in the water treatment process. The rules regulate how much of each may be used rather than how much remains in finished water.
- The Phase V rule became effective January 1994 and set standards for an additional 23 contaminants. Some are inorganic compounds and others are pesticides. They have varying health effects when consumed.

Surface Water Treatment Rule

This rule became effective December 1990 and requires that systems filter and disinfect water drawn from surface waters to prevent disease caused by viruses, *Legionella* and *Giardia lamblia*. Viruses and *Giardia* cause different intestinal illness; *Legionella* is dangerous if aerosolized and inhaled. The majority of the nation's large systems draw their water from surface sources defined as those "open to the atmosphere and subject to surface runoff."[2] These include rivers and lakes and they are especially susceptible to contamination via rain, snow melt, and sewage treatment discharge. Many microorganisms are difficult to accurately measure. The EPA therefore does not set an allowable contaminant goal for these but rather specifies a treatment technique (TT), which is considered suitable to adequately lower the level of viable organisms. Treatments consist of disinfection and filtering. Filtering is usually, but not always, required. Filtering effectiveness is measured by the water's turbidity. If the turbidity is low then filtering has worked and removed most of the microorganisms. Disinfection is used to complement the filtering and provide antimicrobial protection throughout the distribution system.

Total Coliform Rule

This rule became effective in December 1990 and set MCLs and MCLGs for total coliform bacteria. These bacteria are part of a large class that is found extensively in the intestines of both humans and animals. While generally harmless, their presence in drinking water implies the presence of other, perhaps pathogenic, organisms as well. Repeated samples positive for total coliform must be followed by analysis for fecal coliform (indicating contamination from feces) and *Escherichia coli* (indicating recent contamination from feces).

Lead and Copper Rule

This rule was published in June 1991 and sets limits on the allowable levels of lead and copper in water. Because these elements entering drinking water come from plumbing sources, they do not have MCLs but rather action levels (lead at 15 g/L and copper at 1.3 μg/L) and associated TTs. Since the contamination may occur anywhere in the distribution system, samples must be taken from the taps of customers and if $>$ 10% exceed any of the two parameters, TTs must be implemented to prevent corrosion and limit the release of these elements from pipes and joints.

Most states retain oversight for water supplies within their borders, and the agency responsible for this monitoring varies by state. Some states have their own EPA (such as the Illinois Environmental Protection Agency [IEPA]) that will perform this regulatory work.

Other states will rely on their health department and/or a combination of other agencies. A water system that operates for 60+ days a year, and serves either 25+ people or has 15+ service connections, is regulated. If a water supply does not meet these conditions, then the service is not regulated and does not need to follow the regulations. Regulated systems include "community public water supplies," which meet the requirement full-time; "transient, noncommunity public water supplies," which meet the requirement for at least 60 days per year; "nontransient, noncommunity public water supplies," which meet the requirement for more than 6 months of the year (up to 1 year or full-time); and private supplies, which include houses that draw water from their own well. There are also other types of systems that escape neat categorization, such as small private schools or rest stops that draw their own water.

It is important to note that not all water samples are tested for all contaminants every time. This would be prohibitively expensive and time-consuming. The largest systems must test for microbiologic contamination at least 480 times per month. Smaller facilities may need only one sample per month, and these may be further reduced if warranted by a sanitary survey. The testing schedule for most chemicals is based in part on their potential for appearance in the system. Thus, areas where there is substantial agriculture and/or rainfall may expect local nitrate and nitrite levels to vary seasonally and therefore test for these parameters on a quarterly basis. On the other hand, a system that does not have asbestos incorporated into portions of its delivery system, or draw water from an area where asbestos is present geologically, may only test once every 9 years.

Water Quality Standards

The EPA has established two sets of standards for public water systems.[3] Primary standards are legally enforceable and all public water systems must meet or exceed the requirements. All these contaminants are known or expected to have adverse health effects when consumed. Secondary standards are encouraged, but not legally enforceable. These contaminants are determined to have merely cosmetic or aesthetic effects. That is, they may cause skin or teeth discoloration or adversely affect the water's taste, but are not considered a health hazard. Each primary contaminant is assigned a TT or MCL, which is the maximum allowed level, and an MCLG. The contaminants are organized into six groups.

Microorganisms include bacteria, protozoa, and viruses. Potential health effects from ingestion may range from mild gastrointestinal illness to specific disease (e.g., Legionnaires'). Some are simply indicators of the presence of other organisms. Sources of contamination by microorganisms are varied and include the natural environment and human and animal fecal waste.

Disinfectants are the chemicals used to treat water. Potential health effects include eye and nose irritation, stomach discomfort, and anemia. They are added to water supplies to control microbes. *Disinfectant byproducts* are the breakdown products of the disinfectant compounds. Potential health effects include anemia, increased cancer risk, and organ problems.

Inorganic chemicals include such things as iron, mercury, and nitrate. Potential health effects are quite varied (by compound) but include increased blood pressure, kidney damage, nervous system problems, and developmental problems. Sources are also quite varied but include discharge from industry (e.g., steel mills), pipe corrosion, and erosion of natural deposits.

Organic chemicals include carbon-containing compounds such as pesticides. As with the inorganic compounds, potential health effects are varied. More common effects include kidney and liver damage, increased cancer risk, and reproductive difficulties. Common sources include factory discharge and pesticide leaching from farmland.

Radionuclides are radioactive elements and their decay products such as uranium and alpha particles. The most likely health effect is increased cancer risk. The most common sources are natural erosion and the decay of manmade deposits.

Why Are Any Contaminants Allowed at Any Level?

Given the toxic nature of many of these contaminants, one may well ask why any of them are allowed in drinking water at any level. After all, the goal of many of these is zero, why not just set the rule at zero now? There are at least three good reasons why the allowable level is above zero, and none directly relate to individual health.

Expense: It is quite expensive to treat water to current standards, and even more so to purify it to the levels seen in commercial and home purification systems. In general, it becomes more difficult (equivalent to expensive) to remove more and more impurities. Consider an example of a handful of sand placed in a liter of water. Simply letting it sit for a few minutes would allow most of it to settle to the bottom and the

water can be poured off. More than 95% of the sand is thus removed with little effort. Next, the water may be poured through some filter paper. A second step, a little more effort, and 99% of the sand is now removed with the fine particulates remaining. Next, the water could be put through a second filter with smaller pores (requiring a pump) or centrifuged (requiring a centrifuge) to remove these fine particles. Expense has gone up considerably, though 99.8% of the sand is now gone. We now find it increasingly difficult and expensive to remove the remaining 0.2%.

Detection: Instrumentation and detectors continue to perform better and achieve lower levels of detection for different compounds and contaminants. This is especially true for chemical compounds where some pesticides can be detected to the sub part-per-billion level (< 1.0 μg/L). However, no matter how sensitive an instrument, there is still a level of contamination below, which it cannot detect the analyte of interest (often referred to as the detection limit). Thus, the pesticide analysis cannot state that there is zero heptachlor in a sample, just that there is less than the instrument can detect (e.g., < 0.08 μg/L). Setting a compound's enforceable contaminant level to zero would not make sense because no analysis could show there were zero compound molecules present. One may also ask if a single pesticide molecule would even have an adverse health effect in the short- or long-term.

Expected health effects: The study of toxicology and the effects of individual components on human health is a continuing process. Current regulation limits are based on studies that show that there is "significant" chance of harm if the controlled contaminants are consumed above the allowable levels. These levels are based on assumed levels of consumption, consumption rate, individual health, and a host of other factors. Also factored is the human body's ability to neutralize or accommodate some contaminants to some level without apparent harm. Consider ethyl alcohol consumption. Consuming 200 oz of 80-proof alcohol in a day would lead to alcohol poisoning and possibly death. Consuming 20 oz in a day would likely lead to a significant level of inebriation/impairment, and daily consumption would lead to greatly increased risk of cirrhosis and other alcohol-related morbidities. However, consuming 2 oz in a day (2 drinks; equivalent to two 12-oz beers or two 4-oz glasses of wine) is actually linked to *improved* health outcomes and a lower risk of coronary heart disease.[4] The point is that not all levels of a compound have a deleterious effect, and the levels in water should be lowered to the point where the risk is minimized.

In the final analysis for what is allowed in water and to what levels, it often comes down to a combination of these three factors. Levels are often set as a compromise between what would decrease risk versus what is technologically achievable. There has been much discussion over the past 5+ years about lowering the allowable level of arsenic from 0.010 to 0.005 mg/L. Epidemiologic studies show that there may be a decreased risk of cancers at the proposed level, but the technological requirements and associated expense mean that most providers would be unable to purify their water to that point or afford the equipment necessary to do so. They would therefore be noncompliant and subject to legal action. In addition, would the effort be cost-effective? Using hypothetical figures as an example, would it be worth spending $100 million to upgrade the water systems in 20 Illinois counties to prevent a single case of bladder cancer in a 10-year time period?

We have so far established that public water systems, and smaller systems of various configurations, are subject to clean water standards set by the EPA. Does this mean that private homeowners utilizing private wells do not need to have their water tested for quality? The answer is both yes and no. The answer is "no" in the sense that the EPA has no legal requirements for testing, though some states and counties have set rules concerning private well water testing. For example, it may be required that a new well be tested bacteria and NO_2/NO_3. In some places, it is mandated that a private well be tested for bacteria and NO_2/NO_3 before the home is sold. The answer is "yes" in that the EPA recommends that private well users have the water tested on a regular basis. Harmful pathogens of sufficient numbers to cause illness may be present in water, yet undetectable by taste, color, or smell. Owners are therefore recommended to have their water tested annually for microorganisms and every 3 years for chemicals. Even well-constructed wells may be subject to contamination from runoff from rain and fields, and metals may leach out of the ground and aquifer from which the water is drawn.[5]

Sidebar 6-1 Widespread Private Well Contamination[6]

There are ~43 million people in the United States who rely on private wells for drinking water. From 1991 through 2004, members of the US Geological

Survey (USGS) sampled > 2100 private wells in 48 states and tested them for a variety of 219 contaminants and properties. Thirteen percent of 1389 wells tested for chemical contaminants contained at least one compound in excess of its MCL, and 23% exceeded the MCL or USGS Health-Based Screening Level. The contaminants most commonly found in excess included radon, some trace elements (e.g., arsenic), nitrate, and fluoride. All but nitrate are from natural sources and many varied regionally. Total coliform bacteria were detected in 34% of the 397 wells tested for microbial contaminants, and *E. coli* found in 7.9%. Finally, 48% of wells had one or more contaminants in excess of the secondary drinking water standards (discussed later). These parameters primarily affect the aesthetic qualities of water such as taste and odor, but may cause damage to pipes and staining to laundry and teeth. It is also worth noting that 73% wells with detectable levels of contaminants were actually co-contaminated with 2+ detectable parameters. The most common co-occurring contaminants included nitrate, arsenic, radon, and uranium. This study underscores the necessity for private homeowners to avoid complacency when it comes to the quality of the water drawn from their private source. As the samples were taken before they entered in-home plumbing, the study also shows the importance of home-based water treatment systems that may remove/reduce most or all of these contaminants to safe levels before consumption. The regional variations in detected contaminants points to the need for different monitoring and remediation efforts in different regions.

Microorganisms

The pursuit and acquisition of water safe for consumption has been a driving force for much of human history. One of the landmark achievements of public health has been the recognition of the association between water consumption and infectious disease, beginning with the investigation of a cholera outbreak in Soho, England, by John Snow in 1854. He found that those individuals consuming water from a specific source were much more likely to become ill with cholera and that simply removing the well pump handle (thereby preventing use of that source) contributed to a reduced incidence of the disease. Since then, researchers have identified hundreds of organisms that have the potential to both cause disease in humans and be spread by contaminated water. While it is not feasible, if even possible, to test drinking water for all potentially pathogenic organisms, the primary standards do specify some parameters that are judged to be good indicators of overall water quality. That is, if the standard is met and selected microorganisms are removed/inactivated, more pathogenic microorganisms are also likely to be removed/inactivated even if they are not specific test organisms. Table 6-1 lists the microorganism parameters and their associated TT or MCL.

Table 6-1 Treatment Techniques/MCLs for Regulated Microorganisms in Water[3]

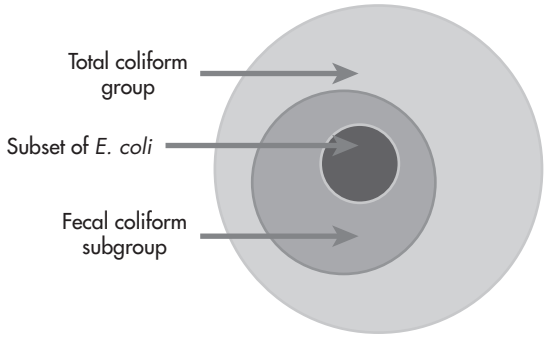
Parameter	MCL
Cryptosporidium	TT — with 99% removal
G. lamblia	TT — with 99.9% removal
Heterotrophic plate count	TT — to < 500 colonies/ml
Legionella	TT — no limit as the effective control of *Giardia* is sufficient for this also
Total coliforms (including fecal coliform and *E. coli*)	5% — or one if less than 40 samples/month
Turbidity	TT — not to exceed 1 NTU ever or 0.3 NTUs for 95% of daily samples
Viruses (enteric)	TT — 99.99% removal/ inactivation

NTU, nephelometric turbidity unit; TT, treatment technique, a required process intended to reduce the level of a contaminant in drinking water.

Total Coliform, Fecal Coliform, and *E. coli*

This section is concerned with the coliform group of bacteria. The total coliform group contains many bacteria found both in nature and the feces of warm-blooded animals. They are defined as being facultative anaerobic, rod-shaped, Gram-negative, non–spore-forming bacteria, all within the family Enterobacteriaceae. Inclusion in the coliform group is largely based on the organism's ability to produce gas and acid by the fermentation of lactose, their primary identifying characteristic. Genera usually listed in the group include *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, and *Serratia*. As a whole, members of

Figure 6-1 Illustration of fecal coliform and *E. coli* subgroups within the total coliform group.



the total coliform group are generally harmless. However, subsets of this group such as fecal coliform or *E. coli* may cause disease or indicate contamination. Their association with the total coliform group is shown in Figure 6-1.

Sidebar 6-2 Water-borne Outbreak of *E. coli* and *Campylobacter jejuni*[7]

The largest known outbreak of water-borne *E. coli* O157:H7 in the United States occurred in August 1999 following a county fair in upstate New York. The source of contamination was found to be a shallow well used to supply water for different vendors to make ice and drinks. Of 775 suspected patients, *E. coli* was isolated by culture from 128, *C. jejuni* was isolated in another 44, and one person was found to be co-infected with both. Samples from the unchlorinated well, its distribution points, a nearby dry well, and a nearby septic tank were screened by polymerase chain reaction (PCR) for the presence of *stx*1 and *stx*2 (genes coding for Shiga toxin production). This was followed by immunomagnetic separation and selective culture for the isolation of *E. coli*. Further analysis of isolates by pulse-field gel electrophoresis (PFGE) helped identify the distribution system as the source of exposure and also show that 117 of 128 individuals were infected with near-identical strains (type 1 or 1a pattern match; three or fewer bands different). This case illustrates the potential for multiple pathogenic agents to be involved in a single outbreak and the importance water treatment.

Many of the organisms that have the potential to contaminate drinking water and cause illness come from feces and are not necessarily included in the coliform group. However, it is very expensive to test for them all. Testing for coliform bacteria, on the other hand, is relatively easy and inexpensive. Their presence in drinking water indicates there may be an opening in the system whereby other, potentially harmful, organisms may enter. Total coliform testing is thus used as an overall indicator of general water quality.[8] EPA-approved methods for the analysis of water for total coliform include the following.

- Multiple tube fermentation: Standard Method 9221 B *Multiple-Tube Fermentation Technique for Members of the Coliform Group* describes the activities associated with this analysis. In brief, a set of five test tubes (or as many as 10) containing lauryl tryptose broth and small inverted vials is inoculated with serial dilutions of the water sample. They are incubated for 24 hours at 35.0° ± 0.2°C and examined for gas formation (seen in the inverted vials) or a color change associated with acid production. If there are no observable results, the tubes are returned to incubation for an additional 24 hours. Samples positive for gas and/or growth must be confirmed.[9]

- Most probable number (MPN): A continuation of Standard Method 9221 (9221 C) and uses a series of sample dilutions to determine the approximate number of coliform units in the sample. The method combines the number of tubes with statistical analysis and tables to give an estimate of bacterial density.[9]

- Membrane filter: Standard Method 9222 B *Standard Total Coliform Membrane Filter Procedure* describes the activities associated with this analysis. In brief, a water sample is collected and poured through a filter with pores fine enough to retain bacteria. The filter is then placed on a growth enrichment media (e.g., M-Endo) saturated pad in a petri dish. The dish is inverted and incubated for 24 hours at 35° ± 0.5°C. Individual bacteria captured on the filter grow into dome-shaped colonies. Coliform colonies are red with a golden/metallic sheen and are counted. The presence of coliform colonies must be confirmed because noncoliform colonies might also form.[9]

- Confirmation: This is done as a continuation of Standard Method 9221 and involves the inoculation of a tube of brilliant green lactose bile broth with a small portion of the presumptive sample test. The tubes contain inverted vials, are incubated at 35° ± 0.5°C, and if any gas forms within 48 ± 3 hours, the sample is confirmed positive.[9]

- Enzyme substrate: Standard Method 9223 B *Enzyme Substrate Coliform Test* allows for the simultaneous detection of both coliform bacteria and *E. coli*. A chromogenic substrate is added to the sample that is acted on by the enzyme β–D-galactosidase (produced by coliform bacteria). This enzyme cleaves the substrate, resulting in a visible color change. A fluorogenic substrate is also added, which is acted on by the enzyme β-glucuronidase (produced by *E. coli*). This enzyme hydrolyzes the substrate, releasing a product that fluoresces under ultraviolet (UV) light. The IDEXX Colilert (IDEXX Laboratories, Inc., Westbrook, ME) is one commercially available test and is very widely used in laboratories because of its ease of use, 18- to 24-hour time frame, and low cost. The initial test mixes the sample with the reagent substrate in a plastic jar. After 24 hours, the sample solution is colored yellow if coliform bacteria are present. The sample will also fluoresce under a black light if there are *E. coli* present. This test also offers a quantitative version that is based on MPN calculations.[9]

- Also approved are Standard Method 9221 D *Presence–Absence Coliform Test*, 9222 C *Delayed-Incubation Total Coliform Procedure*, EPA Method 1604 *Total Coliforms and* Escherichia coli *in Water by Membrane Filtration Using a Simultaneous Detection Technique (MI Medium)*, and five other commercially available tests.

Fecal coliform are a subgroup of the total coliform group and are present in large numbers in the intestines and feces of mammals (including humans). Their presence in drinking water indicates contamination with fecal waste. This represents a greater potential for contamination with harmful pathogens than a high total coliform level alone. Fecal coliform testing is done by some of the same procedures listed previously for total coliform, with two differences. First, the enzyme substrate test is *not* used as it cannot distinguish between fecal coliform and total coliform. Second, an enriched lactose medium is used and the incubation temperatures are increased from 35.0° to 44.5°C. These conditions are selective for fecal coliform, and the temperature is especially important with incubation often done in temperature-controlled water baths. EPA-approved methods for the analysis of water for fecal coliform include the following.

- Membrane filter: Standard Method 9221 E *Fecal Coliform Procedure* describes the activities associated with this analysis. In brief, the broth from a sample positive for total coliform from 9222B is stirred. A small amount is used to inoculate a test tube prepared with EC broth and an inverted vial. The samples are incubated at 44.5° ± 0.2°C for 24 hours. Any gas production with growth is considered positive for fecal coliform.[9]

E. coli are a subset of the fecal coliform group and were discussed previously in Chapter 5. Their presence in drinking water almost always indicates the presence of recent fecal contamination and an elevated risk of contamination with more dangerous pathogens. Figures 6-2 and 6-3 show a close look at *E. coli* and the result of fluorescent antibody staining.

EPA-approved methods for the analysis of water for *E. coli* include the following.

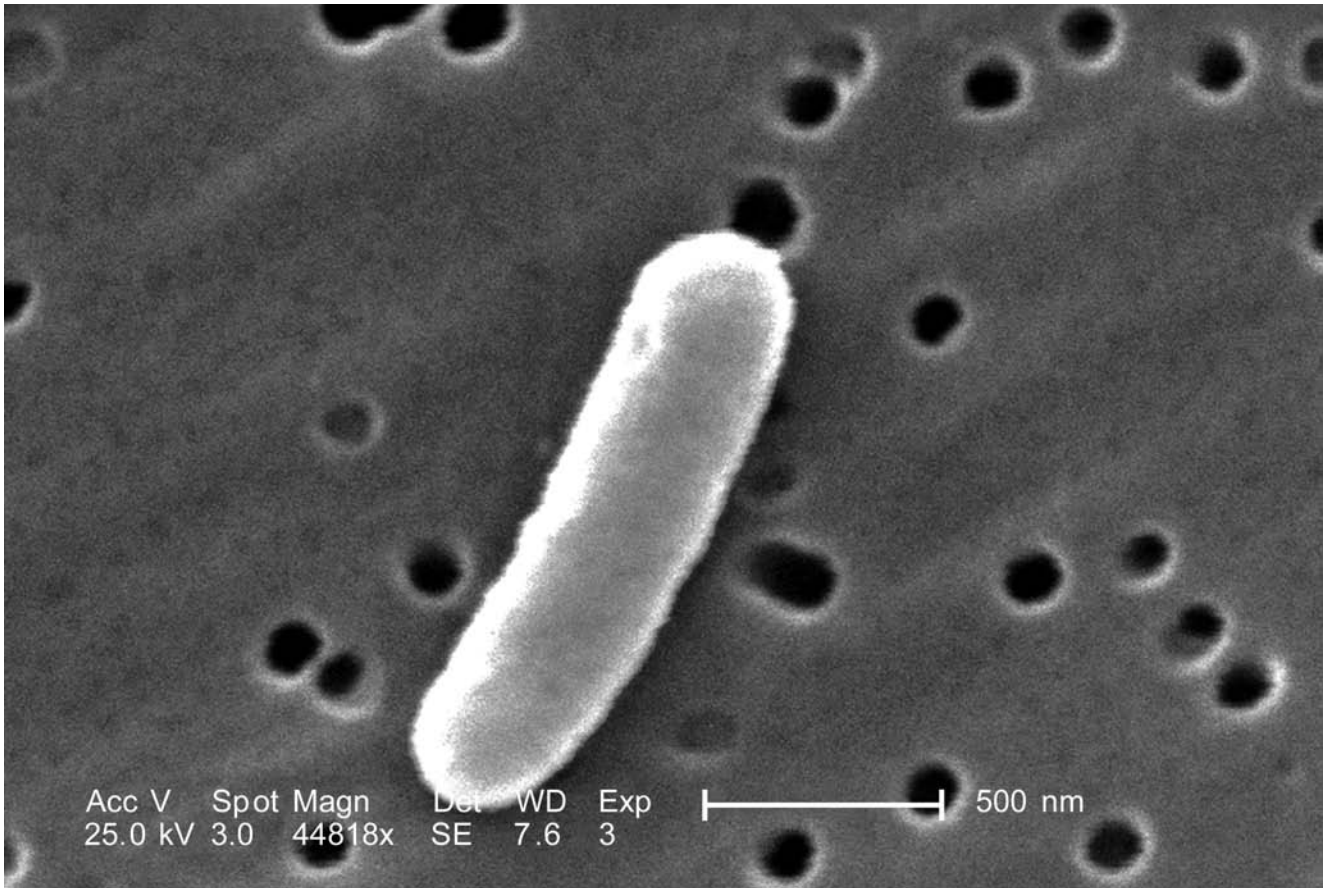
- Standard Method 9222 G *MF Partition Procedures* describes the activities associated with this analysis. This is essentially a continuation of the Standard Method 9222 B described previously, where either the filter itself or selected colonies are transferred to test tubes prepared with EC or NA agar and MUG fluorescent substrate. EC-MUG samples are incubated at 44.5° ± 0.2°C for 24 hours and NA-MUG at 35.0° ± 0.5°C for 4 hours. Colonies are observed under UV light, and blue fluorescence is considered a positive response.[9]

- Standard Method 9223 (described previously under total coliform).

- Also approved are EPA Method 1604 *Total Coliforms and* Escherichia coli *in Water by Membrane Filtration Using a Simultaneous Detection Technique (MI Medium)* and seven commercially available tests.

The regulated limit for total coliform (including fecal coliform and *E. coli*) is 5%. That is, only 5% of samples for a month may be positive or one sample per month for those collecting less than 40 samples. If an elevated level of total coliform is found, the water system will be inspected to determine the source of contamination. If either fecal coliform or *E. coli* is found in drinking water, the health department may call for a boil order for drinking water

Figure 6-2 Scanning electron micrograph of *E. coli* O169:H41 (44,818×). An image bank of full-color photos is available online at http://www.jbpub.com/catalog/9780763771027/. (Courtesy of Centers for Disease Control and Prevention [CDC]/ Janice Haney Carr.)



and alert the effected consumers that the water may not be safe to drink without additional treatment. Consumers may also be advised to use bottled water for drinking until the system is repaired and cleaned.

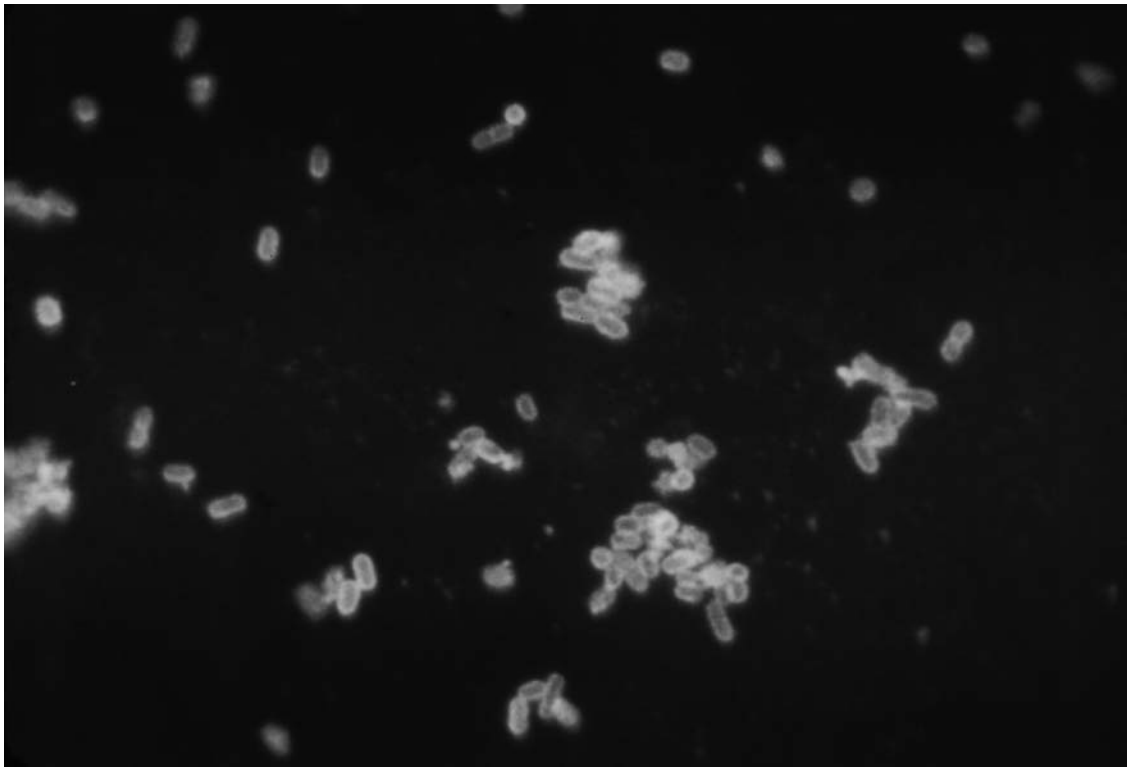
Cryptosporidium and *Giardia lamblia*

These organisms are pathogenic protozoa that cause illness in humans. Illness occurs when people ingest the cysts or oocysts excreted in animal and human feces. The cysts/oocysts have a hard outer shell that affords them much protection. Thus, they can survive outside the body for extended periods of time and are resistant to chlorine disinfectants. For this reason, cryptosporidiosis is one of the most common causes of water-borne disease in the United States, and *Giardia* is the most common intestinal parasite in the world.[9] See Chapter 5, section on protozoal parasites, for more details of their life cycles, characteristics, and associated illnesses.

Sidebar 6-3 *Cryptosporidium* Outbreak[10,11]

One of the largest disease outbreaks associated with a drinking water supply in the United States occurred in Milwaukee, Wisconsin, in 1993. Because of an ineffective filtration system, *Cryptosporidium* oocysts were inadequately removed from the water supply at one of the city's two treatment facilities. It is estimated that 403,000 people (~25% of the population) became ill. Of those, the majority (354,600) did not seek medical attention, approximately 11% (44,000) were seen as outpatients, and 4400 were hospitalized with severe illness. It is estimated that this outbreak incurred $31.7 million in direct medical costs and an additional $64.6 million in lost productivity (e.g., people sick from work). Corso et al. note that the total annual cost of water-borne disease in the United States exceeds $21 billion. The source of the oocysts was

Figure 6-3 Fluorescent antibody stained *E. coli* from a fecal smear obtained from an infant with diarrhea. An image bank of full-color photos is available online at http://www.jbpub.com/catalog/9780763771027/. (Courtesy of CDC/ Bernice Thomason.)



not immediately determined, though heavy rain, cattle manure, and sewage were considered. It was found that oocysts from infected individuals would not infect animals, and they were further identified genetically as being of human origin. This suggests that sewage overflow was the cause of contamination.

EPA requires that 99% *Cryptosporidium* and 99.9% *Giardia* be removed by treatment.[12] *Cryptosporidium* and *Giardia* may be analyzed by the same method.

- EPA Method 1623 Cryptosporidium *and* Giardia *in Water by Filtration/IMS/FA* is a filtration and staining method. A water sample of suitable volume (i.e., sufficient to retain sufficient organisms for analysis) is passed through a commercially available filter with pores of a size adequate to capture the organisms. The captured materials are eluted into tubes and centrifuged to pellet the oocysts and cysts. These are then resuspended with a solution containing anti-*Cryptosporidium* and anti-*Giardia* antibodies bound to magnetic beads. A magnet is then used to separate out the captured oocysts and cysts from the extraneous material; they are freed from the antibodies and placed in a welled slide. Observation via microscope is made after staining with monoclonal antibodies and DAPI. Both organisms fluoresce bright green with this procedure.[8]
- Standard Method 9711 B Giardia *and* Cryptosporidium *Methods* describes general strategies for the sampling and concentration, purification and separation, and assay of sample for these organisms. Collection and concentration methods include flocculation, centrifugation, and various types of filters. Purification and separation methods include density gradient centrifugation, immunomagnetic separation (used previously in Method 1623), and

fluorescence-activated cell sorting. Analysis methods include immunofluorescence (used previously in Method 1623) and PCR. Analysis reagents are commercially available, with PCR components sold as kits.[9]

- Direct microscopic examination is possible. Figures 6-4 and 6-5 show unstained *Cryptosporidium* and *Giardia* organisms. Most often, the analyst will find that other contaminants in water samples make such visual identification difficult at best, if not impossible, without selective staining.

Heterotrophic Plate Count

This is a general test to determine the variety of bacteria present in water. In general, lower concentrations of bacteria indicate better maintained systems. There are no specific illnesses associated with this test and it is used as a gross judge of water quality. Systems are required to purify their water so that there are $<$ 500 bacterial colonies per milliliter of finished water. This test does not differentiate between coliform or other bacteria. There is one EPA-approved method for heterotrophic plate count:

- Standard Method 9215 *Heterotrophic Plate Count* (with R2A medium) describes the activities associated with this analysis. There are three methods of sample preparation (9215 B-D). In the pour plate method, a volume of sample is added to a petri dish and melted media added and mixed, whereas the spread plate method adds the sample to the already solidified media and spreads it across the top. The membrane filter method passes the sample through a filter, which is then rinsed and placed on solidified media. Each method is then followed by incubation at 35°C for 48 hours. The number of colonies visible is then counted and reported as *colony forming units* (CFU).[9]

Legionella

Some *Legionella* species are the causative agents of Legionnaires' disease (also known as Legionellosis). The name comes from a large outbreak at an American

Figure 6-4 Stool smear of *Cryptosporidium parvum*. (Courtesy of CDC/Dr. Peter Drotman.)

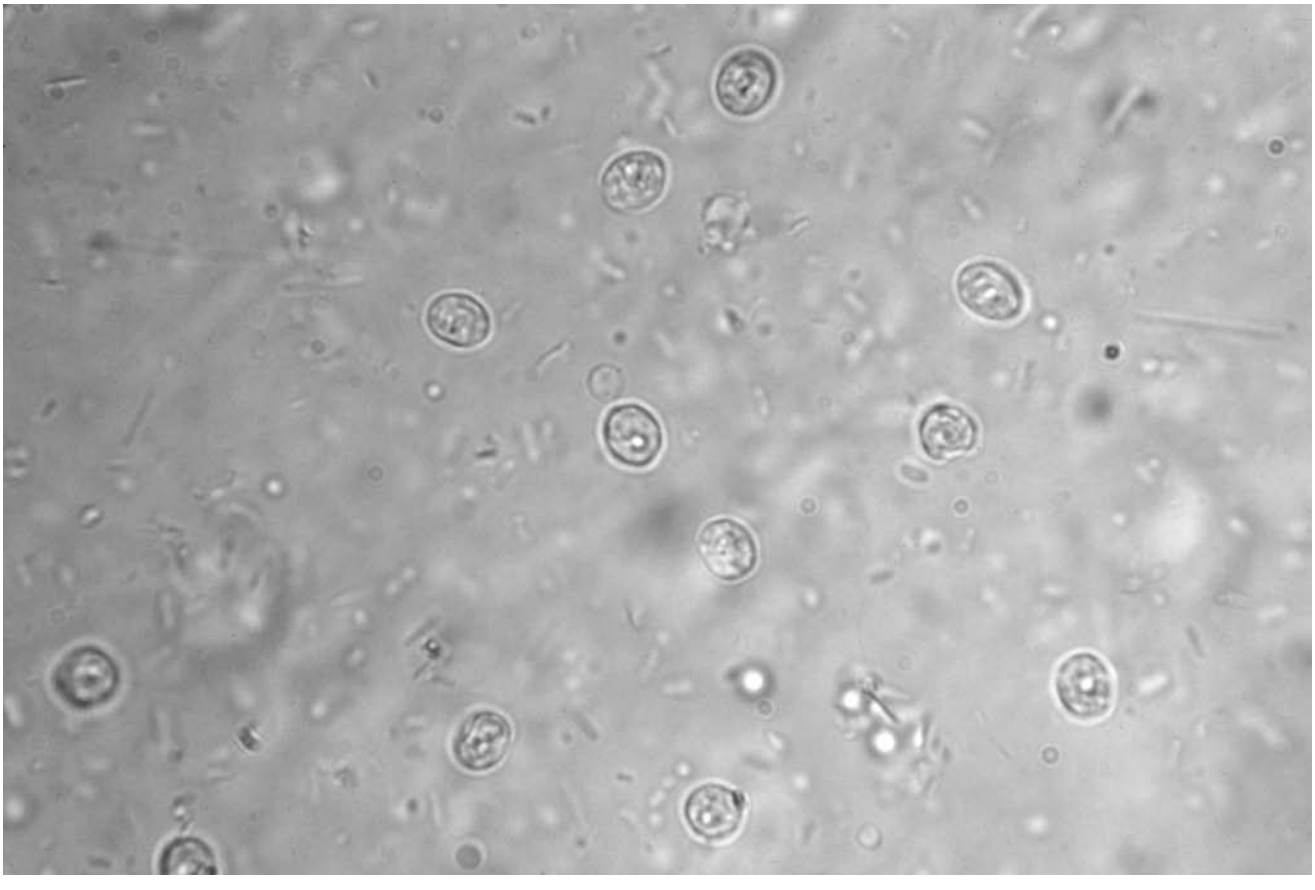
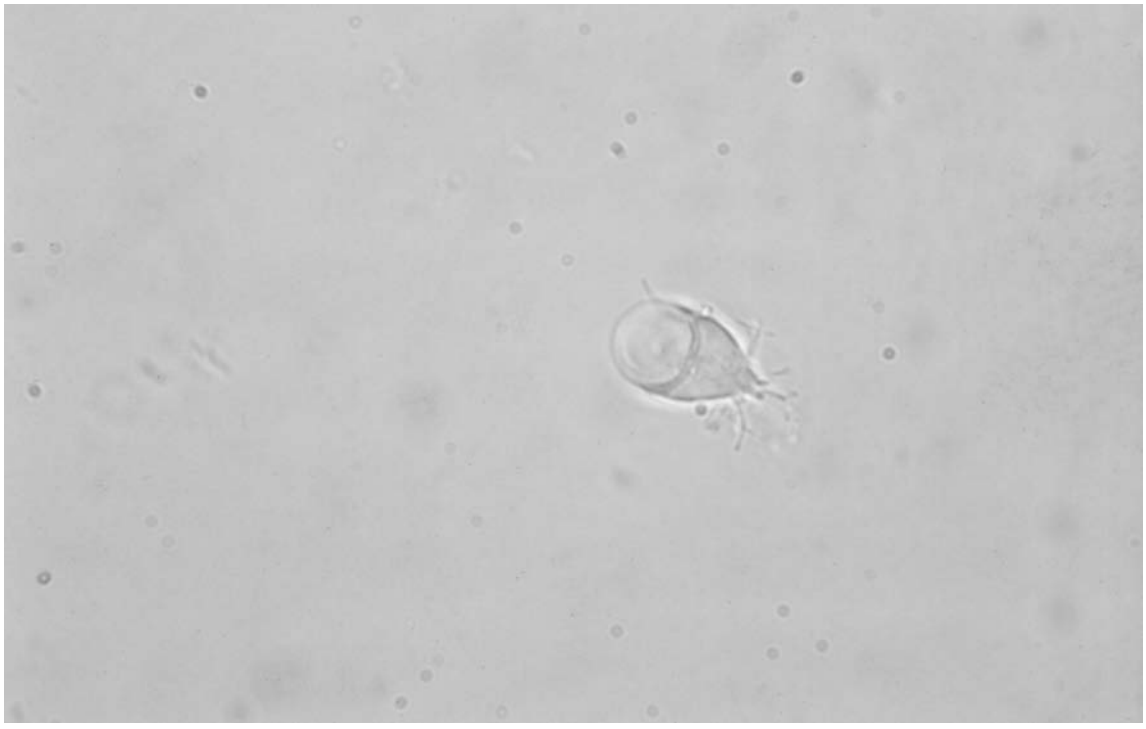


Figure 6-5 Unstained photomicrograph of *Giardia lamblia* trophozoite. (Courtesy of CDC/Dr. Mae Melvin.)



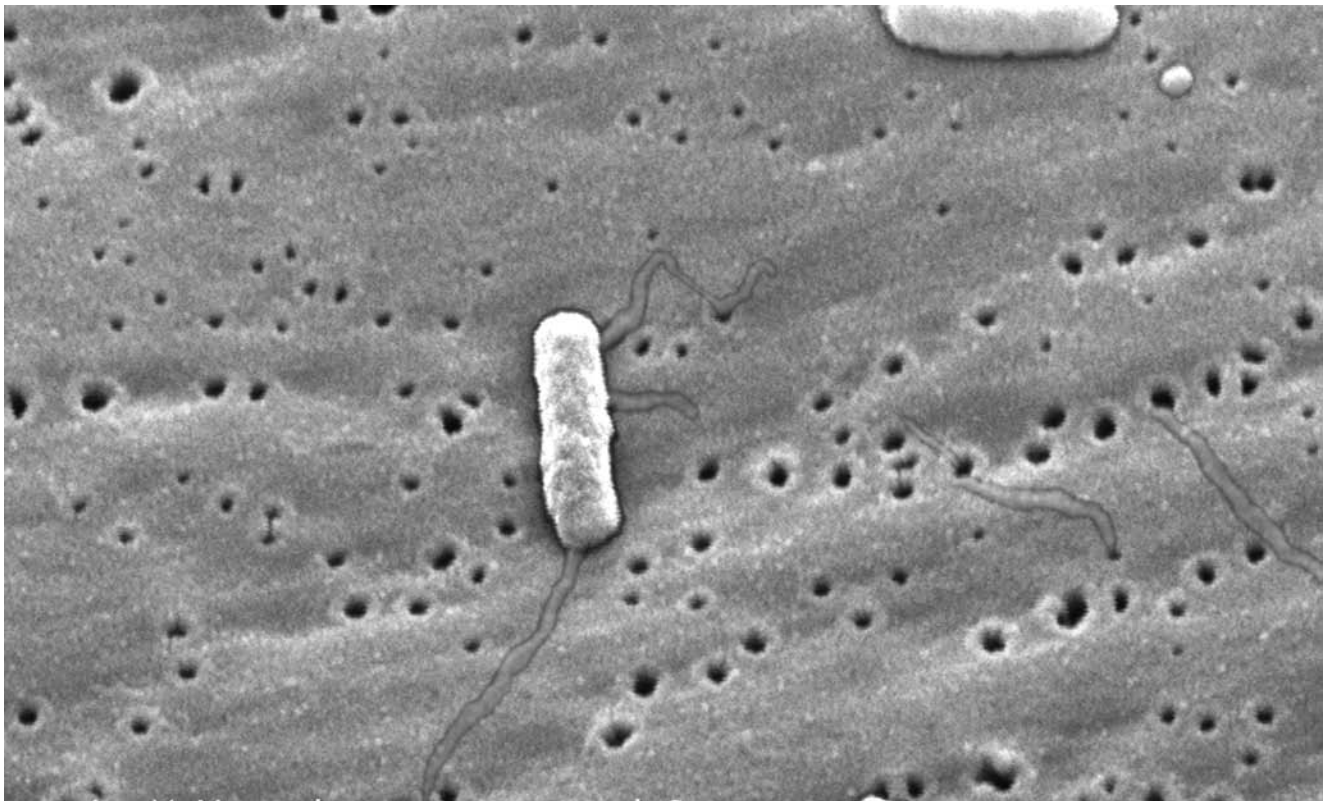
Legion convention in Philadelphia, Pennsylvania, in 1976. Within days of the convention start, many attendees became ill with flulike symptoms. Ultimately, 221 were medically treated and there were 34 deaths. The source of infection was determined to be contaminated condensation on the hotel's air conditioning system. Since that time, surveillance for the disease has increased substantially and 8000 to 18,000 people hospitalized for flulike symptoms annually are found to be infected.[13]

Disease is most often respiratory in nature as the bacteria are transmitted via the air-borne route. There are at least 46 species, with about 20 causing disease in humans. The organisms are Gram-negative, aerobic, non–spore-forming bacteria with flagella. Figure 6-6 shows an example of *Legionella* with the flagella attached. Under certain growth conditions, they may develop a pronounced rod shape and are 0.5 to 0.7 μm wide by 2 to 20 μm long. Many who are infected do not become ill enough to seek treatment. Symptoms that do present usually appear within 2 to 14 days and resemble pneumonia (e.g., fever, chills, cough, and muscle ache). The illness may cause death in 5 to 30% of cases, but is successfully treated with antibiotics. A milder form of illness without the pneumonia symptoms is called Pontiac fever and can resolve within a few days without treatment.[9,13]

Analysis for *Legionella* may be done by several techniques. Culturing is still considered the gold standard but may take up to 10 days. Standard Methods describes different strategies for the analysis of water for *Legionella* species:

- Standard Method 9260 J Legionella describes three techniques. The immunofluorescent procedure concentrates 100 ml of sample by centrifugation, places the concentrate on slides, and stains them with fluorescent antibodies. The test is not terribly specific, and several other bacteria (e.g., *Pseudomona*) cross-react. Positives must therefore be confirmed. The culture method concentrates the samples via filtration or centrifugation. The resultant suspensions are plated on treated and untreated media. Portions of the suspensions are separated and acid treated before plating. Plates are examined every 24 hours (after the first 24) for the appearance of colonies that have a "ground glass" appearance. Figure 6-7 shows a colony with this appearance. Further selective media are then inoculated with these colonies. There are also commercially available PCR kits that are able to detect up to 25 *Legionella* species, and *Legionella pneumophila* specifically.[9]

Figure 6-6 Scanning electron micrograph of *Legionella pneumophila* where polar flagella is observed (some disassociated). (Courtesy of CDC/Margaret Williams, PhD; Claressa Lucas, PhD; Tatiana Travis, BS; Photographer: Janice Haney Carr.)



Turbidity

Clarity is an important indicator of water's quality and taste. While pure water is entirely clear, water drawn for consumption often has fine particles suspended within. Turbidity is a measure of the water's cloudiness and is an indicator of both quality and filtration effectiveness. Thus, water with higher levels of turbidity, indicating less effective filtering and treatment, is more likely to harbor potentially pathogenic organisms. EPA requires that turbidity may never exceed 1 nephelometric turbidity unit (NTU), and must not exceed 0.3 NTU in 95% of daily samples in any month.[3]

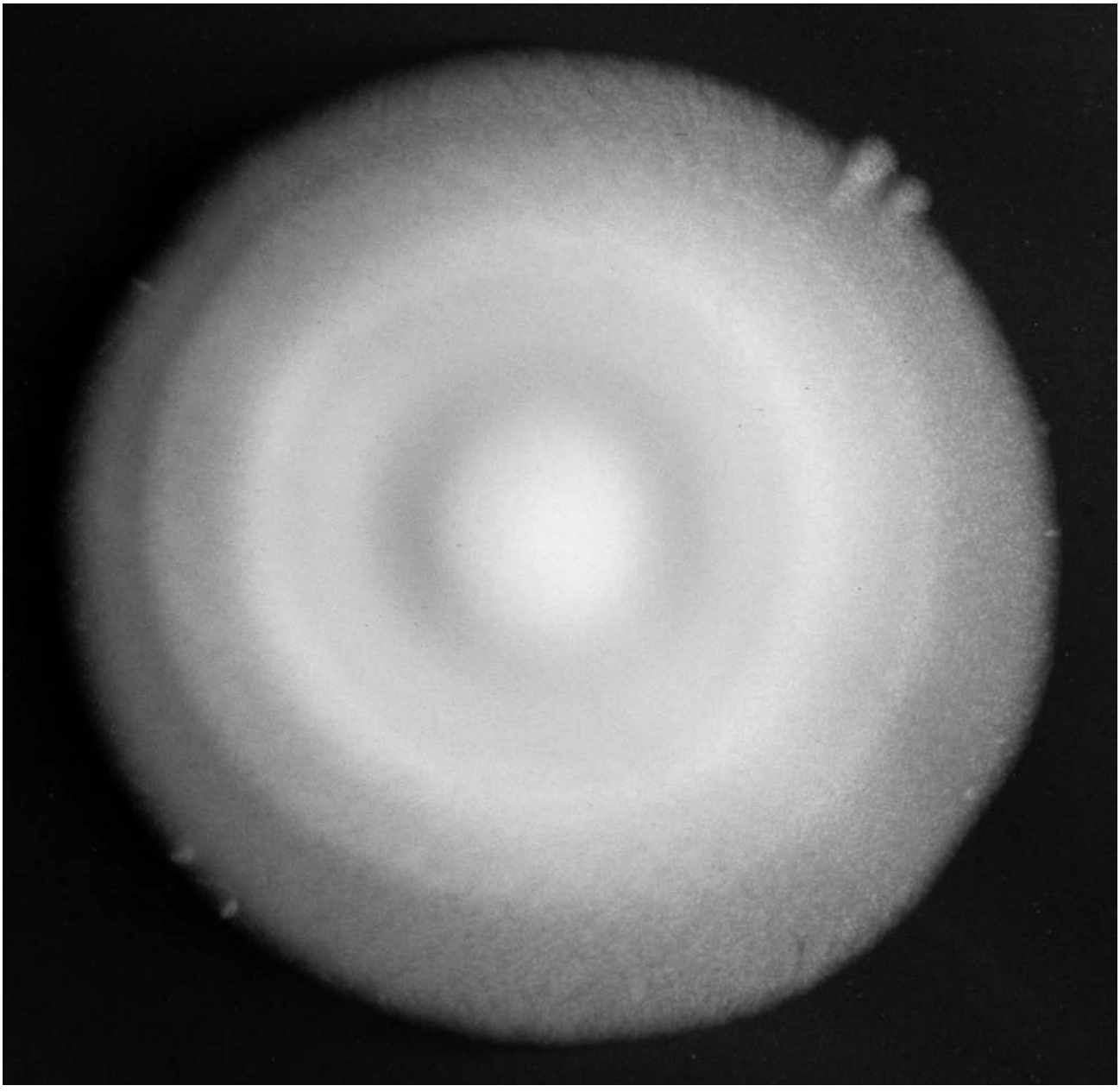
- EPA Method 180.1 *Determination of Turbidity by Nephelometry* requires no sample preparation. The sample is placed in a turbidimeter and a source directs light through the sample. Photodetectors measure the intensity of light scattered at right angles to the path of the incident light. This scattered light intensity is compared to a standard reference unit. Results are reported as NTUs.[12]
- Standard Method 2130 B *Nephelometric Method* is essentially identical to Method 180.1.

Viruses

There are over a hundred viruses associated with feces, and they are collectively referred to as "enteric viruses." Viruses included in this set include polioviruses, coxsackieviruses, enteroviruses, adenoviruses, rotavirus, hepatitis A, and noroviruses. These viruses cannot be assayed as readily as other microbiological contaminants so their prevalence and seasonal variance is largely unknown. Routine examination of water for viruses is not currently recommended. Like *Cryptosporidium*, *Giardia*, and *Legionella*, there is a TT associated with this class of contaminant (99.99% removed/inactivated)[3] and, therefore, no EPA-approved method of analysis. Exposure to enteric viruses may cause illness with symptoms including diarrhea and dehydration, stomach cramps, nausea and abdominal pain, headaches, fevers, and chills.

Viral concentrations in finished water are expected to be extremely low (e.g., 10s to 100s of copies per liter). Concentration of the sample is a critical first step in analysis and may require $>$ 1000 liters of water. Standard Methods describes three concentration methods in Method 9510 B–E *Detection of Enteric Viruses*: microporous filtration

Figure 6-7 Photograph of a single *Legionella* colony grown on agar showing the "ground glass" appearance. (Courtesy of CDC.)



(B–C); aluminum hydroxide adsorption–precipitation (D); and polyethylene glycol hydroextraction–dialysis (E). The actual analysis for specific viruses is beyond the scope of both this book and Standard Methods. Viruses are obligate intracellular parasites and must infect cells to multiply. Their detection is therefore based on the infection and destruction of cell cultures. General techniques include variation of plating infected cells and observing cytopathic effects. Also under development are commercial and experimental immunoassays and PCR tests.

Disinfectants and Disinfectant Byproducts

There are multiple methods whereby water may be treated so it is safe to consume. Systems often use a combination of methods, but general strategies usually include a mix of chemical treatments, aeration, flocculation and sedimentation, and filtration. Of particular interest here is the use of chemicals to disinfect the water by killing any bacteria, viruses, protozoa, etc., that have

managed to survive the other treatments. The disinfectants themselves are not so much a health hazard as a nuisance. They have strong odors, impart a bad taste to the water, and may cause eye and nose irritation and stomach discomfort. Of greater importance in terms of health are the potential products of the reaction of these chemicals with other water contaminants. The disinfectant byproducts can directly impact health, variously leading to increased cancer risk, anemia, and other neurologic problems.

From Table 6-2 we see that there are multiple analysis methods available for use, but none of them can analyze a sample for all four disinfectants. Rather than write a laundry list of all the methods and their differences, we will instead look at two strategies to show they differ:

- EPA Method 327 Rev 1.1 *Determination of Chlorine Dioxide and Chlorite Ion in Drinking Water Using Lissamine Green B and Horseradish Peroxidase With Detection by Visible Spectrophotometry* describes how this is performed in conjunction with daily monitoring. Two samples are collected, one of which has inert gas bubbled through (sparged) to remove chlorine dioxide. The samples have subsequent additions of citric acid buffer and lissamine green B (LGB)/horseradish peroxidase (HRP) reagent accompanied by mixing. The HRP catalyzes the conversion of chlorite to chlorine dioxide, which in turn oxidizes the LGB, reducing its absorption in the red part of the spectrum. This is measured in the spectrophotometer to determine the chlorite concentration. The chlorine dioxide concentration is the difference between the sparged and unsparged samples. Detection limits for chlorite ion and chlorine dioxide are both <0.20 mg/L.[12]
- Standard Method 4500 Cl G *Mercuric Thiocyanate Flow Injection Analysis* is a technique whereby a portion of a sample is introduced into a continuous flow system to which mercuric thiocyanate and ferric nitrate are added. Any chloride ion displaces the thiocyanate anion and complexes with the mercury. The thiocyanate ion then forms a brightly colored ferric thiocyanate complex whose absorbance is measured at 480 nm. The estimated detection limit of 0.07 mg/L Cl^-.[9]
- Both the ASTM method and SM 4500 D are titration methods using an electrode to observe the end point. The other SM 4500 methods are also flow injection analyses.

From Table 6-3 we see that there are multiple analysis methods available for use, but none of them can analyze a sample for all four byproducts. As we did for the disinfectants themselves, we will look at a couple different analysis strategies for disinfectant byproducts to give an idea of how they differ.

- EPA Method 300.1 *Determination of Inorganic Anions in Drinking Water by Ion Chromatography* is a liquid chromatography method utilizing an analytical column to test for various anions. An aliquot of sample is injected into the system and mixes with a sodium carbonate solution mobile phase. The anions are separated during passage through the column and

Table 6-2 Approved Analysis Methods for Water Disinfectants[3]

Method	Chlorine dioxide	Combined chlorine	Free chlorine	Total chlorine
EPA 327 Rev 1.1	Yes	—	—	—
SM 4500-ClO_2 D	Yes	—	—	—
SM 4500-ClO_2 E	Yes	—	—	—
ASTM D1253-03	—	Yes	Yes	Yes
ASTM D1253-86	—	Yes	Yes	Yes
SM 4500 Cl D	—	Yes	Yes	Yes
SM 4500 Cl E	—	—	—	Yes
SM 4500 Cl F	—	Yes	Yes	Yes
SM 4500 Cl G	—	Yes	Yes	Yes
SM 4500 Cl H	—	—	Yes	Yes
SM 4500 Cl I	—	—	—	Yes

Table 6-3 Approved Analysis Methods and MCLs for Water Disinfectant Byproducts[3]

Analyte	300 (0.1)	317	326	Other	MCL (mg/L)
Bromate	X	X	X	D6581, 321.8	0.010
Chlorite (daily monitoring)	X	X	X	327, 4500 ClO_2 E	1.0
Chlorite (distribution system monitoring)	X	X	X	D6581	1.0
Haloacetic acids	—	—	—	552 (.1/.2/.3), SM 6251 D	0.060
TTHs	—	—	—	502.2, 524.2, 551.1	0.080

detected by a conductivity detector as they emerge. Detection limits for bromate and chlorite are 1.44 and 0.89 μg/L, respectively.[14]

- EPA Method 552.1 *Determination of Haloacetic Acids and Dalapon in Drinking Water by Ion-Exchange Liquid–Solid Extraction and Gas Chromatography with an Electron Capture Detector* acidifies the sample, passes it through a solid capture medium (column or disk), elutes the captured compounds, and derivatizes them prior to GC separation and detection by ECD. 552.2 is the liquid–liquid extraction version of this method, and 552.3 uses microextraction. The detection limits for total trihalomethanes (TTHs) range from 0.08 to 0.82 μg/L for 552.2 to 0.024 to 0.97 μg/L for 552.3.[14]
- EPA Method 551.1 *Determination of Chlorination Disinfection Byproducts, Chlorinated Solvents, and Halogenated Pesticides/Herbicides in Drinking Water by Liquid–Liquid Extraction and Gas Chromatography with Electron-Capture Detection* is used to test for TTHs and is also applicable for a wider range of compounds. A sample is extracted once with a solvent (MTBE or pentane) and the extract retained. Without further preparation or cleanup, a portion of the extract is injected onto a GC column for compound separation, followed by ECD detection. Detection limits for individual trihalomethanes are each < 0.1 μg/L.[14]

Inorganic Chemicals

There are a total of 16 inorganic elements and compounds regulated in drinking water by the EPA. As a reminder, the defining characteristic for an "inorganic" compound is that it does not contain carbon. For this section we will further differentiate the inorganic compounds into smaller groups based on similar physical properties, similar analytical methods, or both. We therefore split the list into the elementals, inorganic anions (ions with a negative charge), and asbestos.

Metals Analyses

This first section considers metals analyses. Metals are pervasive in nature and come from the chemical breakdown of minerals. They are also used for many industries and their presence may indicate contamination. They can exist in elemental form, but generally are ionic. There are 11 regulated metals we will consider here, listed in Table 6-4. Various adverse health effects associated with exposure to these metals include kidney and/or liver problems, circulatory and blood problems, skin lesions, and numbness.

As we see in Table 6-4, there is only one analytical method common to all 11 elements and another useful for 9. There are also multiple methods that are unique to individual elements (e.g., ASTM D3559 for lead). We will give a brief description of the three more inclusive methods:

- EPA Method 200.X series
  - 200.5 *Determination of Trace Elements in Drinking Water by Axially Viewed Inductively Coupled Plasma-Atomic Emission Spectrometry* uses acid digestion followed by inductively coupled plasma (ICP) to vaporize and strip the sample constituents and atomic emission spectrometry for detection. Detection limits for these elements range from 0.02 to 1.4 μg/L. 200.7 is a slight variation of this method. Detection limits for these elements range from 0.3 to 20 μg/L.[14]
  - 200.8 *Determination of Trace Elements in Drinking Water by Inductively Coupled Plasma-Mass Spectrometry* uses acid digestion followed by ICP to vaporize and strip the sample constituents and mass spectrometry for detection. Detection limits for these elements range from 0.02 to 7.9 μg/L.[14]

Table 6-4 Approved Analysis Methods and MCLs for Regulated Metals[3]

Compound	200 (0.5/0.7/0.8/0.9)	SM 3113 B	SM 3120 B	Other methods	MCL (mg/L)
Antimony	X	X	—	D3697	0.006
Arsenic	X	X	—	D2972, SM 3114 B	0.010
Barium	X	X	X	SM 3111 D	2
Beryllium	X	X	X	D3645	0.004
Cadmium	X	X	—	—	0.005
Chromium (total)	X	X	X	—	0.1
Copper	X	X	X	D1688, SM 3111 B	TT[8]; Action Level = 1.3
Lead	X	X	—	D3559, Hach Method 1001	TT[8]; Action Level = 0.015
Mercury (inorganic)	X	—	—	245 (.1/.2), D3223, SM 3112 B	0.002
Selenium	X	X	—	D3859 A/B, SM 3114 B	0.05
Thallium	X	—	—	—	0.002

- 200.9 *Determination of Trace Elements by Stabilized Temperature Graphite Furnace Absorption* uses acid digestion followed by stabilized temperature graphite furnace to vaporize and strip the sample constituents and elemental absorbance spectrometry for detection. Detection limits for these elements range from 0.02 to 0.8 μg/L.[14]

- Standard Methods 3113 B *Electrothermal Atomic Absorption Spectrometric Method* utilizes a graphite furnace to vaporize the sample followed by detection with atomic adsorption. The range of detection limits by this method varies by element. Detection limits for these elements range from 0.1 to 2.0 μg/L.[14]

- Standard Methods 3120 B *Metals in Water by Plasma Emission Spectroscopy* is used less frequently and uses a nebulizer to introduce the sample to the ICP, followed by mono- or polychromatic emission spectroscopy. Detection limits for these elements range from 0.3 to 20 μg/L.[14]

Inorganic Anions

This section considers some inorganic anions. These are negatively charged ions that are formed via biological processes, mineral decomposition, and/or industrial uses. There are four regulated ions we will consider here, listed in Table 6-5. Various adverse health effects associated with exposure to these ions include nerve and thyroid problems, bone brittleness, and shortness of breath and "blue baby syndrome." The analysis for NO_2/NO_3 in particular may be the highest-volume chemistry test for some laboratories. The IDPH laboratories performed 8828 of these analyses from July 2007 through June 2008 (unpublished data).

From Table 6-5 we see that there are multiple analysis methods available for use, but none of them can analyze a sample for all four anions. There are also a significant number of methods that are unique to each anion, such as 335.4 for CN^- and D1179 for F^-, and others that are specific for specified commercial venders, such as Bran + Luebbe 380-75WE for F^-. We will briefly describe the three methods that are available for multiple anion analysis:

- EPA Methods 300.0 *Determination of Inorganic Anions by Ion Chromatography* and 300.1 *Determination of Inorganic Anions in Drinking Water by Ion Chromatography* are essentially the same with the exception of different injection volumes and column sizes. Both are liquid chromatography methods utilizing an analytical column to test for various anions. An aliquot of sample is injected into the system and mixes with a sodium carbonate solution mobile phase. The anions are separated during passage through the guard and

Table 6-5 Approved Analysis Methods and MCLs for Regulated Inorganic Anions[3]

Compound	300 (0.0/0.1)	SM 4110 B	SM 4500	Miscellaneous	MCL (mg/L)
Cyanide (as free cyanide)	—	—	X	335.4, D2036, D6888, others	0.2
Fluoride	X	—	X	D1179, B&L 380-75WE, others	4.0
Nitrate (measured as nitrogen)	X	X	—	353.2, D3867, D4327, others	10
Nitrite (measured as nitrogen)	X	X	—	353.2, D3867, D4327, others	1

separator columns and detected by a conductivity detector as they emerge. Method detection limits vary by analyte and instrument. Detection limits for these ions range from 2 to 10 μg/L for 300.0 and from 1 to 9 μg/L for 300.1.[14]

- Standard Methods 4110 B *Ion Chromatography with Chemical Suppression of Eluent Conductivity* follows the same basic principles of 300.0/0.1. Detection limits for these ions range from 2.7 to 3.7 μg/L.[14]
- Standard Methods 4500 offers 15 methods for the analysis of CN^-, variously involving distillation, chemical treatments, flow injection and colorimetric analysis, titration, and ion-specific electrode. 4500-CN^- F is perhaps the easiest to use as there is no sample preparation and detection is by ion-specific electrode. Similar to cyanide, there are multiple (7) variations of the method for F^- analysis. Methods include colorimetric and ion-specific electrode analyses of either the ion itself, or a chemical complex. Detection limits for these ions vary considerably by method and instrument.[14]

Asbestos

Asbestos is best known for its association with the rare lung cancer mesothelioma and with the flulike illness asbestosis, both caused by inhalation of asbestos fibers. It is a naturally occurring silicate fiber, with six defined varieties: chrysotile (white), amosite (brown), crocidolite (blue), tremolite, anthophyllite, and actinolite. There are other forms that are infrequently used and not currently regulated. Asbestos fibers have substantial physical strength and heat, electrical, and chemical resistance. For these reasons, it was heavily used in industry as a flame retardant and in homes and buildings for heating and electrical insulation. Asbestos is much less harmful when ingested, but may still cause illness. Adverse health effects associated with ingesting asbestos include an increased risk of developing benign intestinal polyps. The MCL for asbestos is 7 million fibers per liter.[3] Figures 6-8 and 6-9 are two magnified views of asbestos showing its fibrous characteristics.

There are currently two EPA-approved analysis of asbestos.[12] Both require microscopic examination of the prepared ample. This is because asbestos' adverse health effects lie more with its physical structure and not its chemical constituents. These constituents are also commonly found in other minerals, so analysis for ions or silicon would not necessarily indicate the presence of asbestos per se. For example, the generic structure for chrysotile is Mg3(SiOH5)(OH)4. Magnesium, silicon, and hydroxide ions are all commonly found in water.

- EPA Method 100.1 *Analytical Method for Determination of Asbestos Fibers in Water* passes a sample through a polycarbonate filer to trap any fibers, places a portion of the filter into an electron microscope and dissolves away the filter, and analyzes the remains with transmission electron microscopy. The detection limit for this method is 0.00010 μg/L (0.10 ng/L) for samples with low turbidity (suspended particles).[14]
- EPA 100.2 *Determination of Asbestos Structures Over 10 μm in Length in Drinking Water* uses a different filter and sample preparation technique than 100.1, but analysis is also by transmission electron microscope. The detection limit for this method varies.[14]

Organic Chemicals

There are a total of 53 organic compounds regulated in drinking water by the EPA. As a reminder, the defining characteristic for an "organic" compound is that it

Figure 6-8 Scanning electron micrograph (650×) of raw chrysotile asbestos. (Courtesy of CDC/John Wheeler, PhD; Photographer: Janice Haney Carr.)

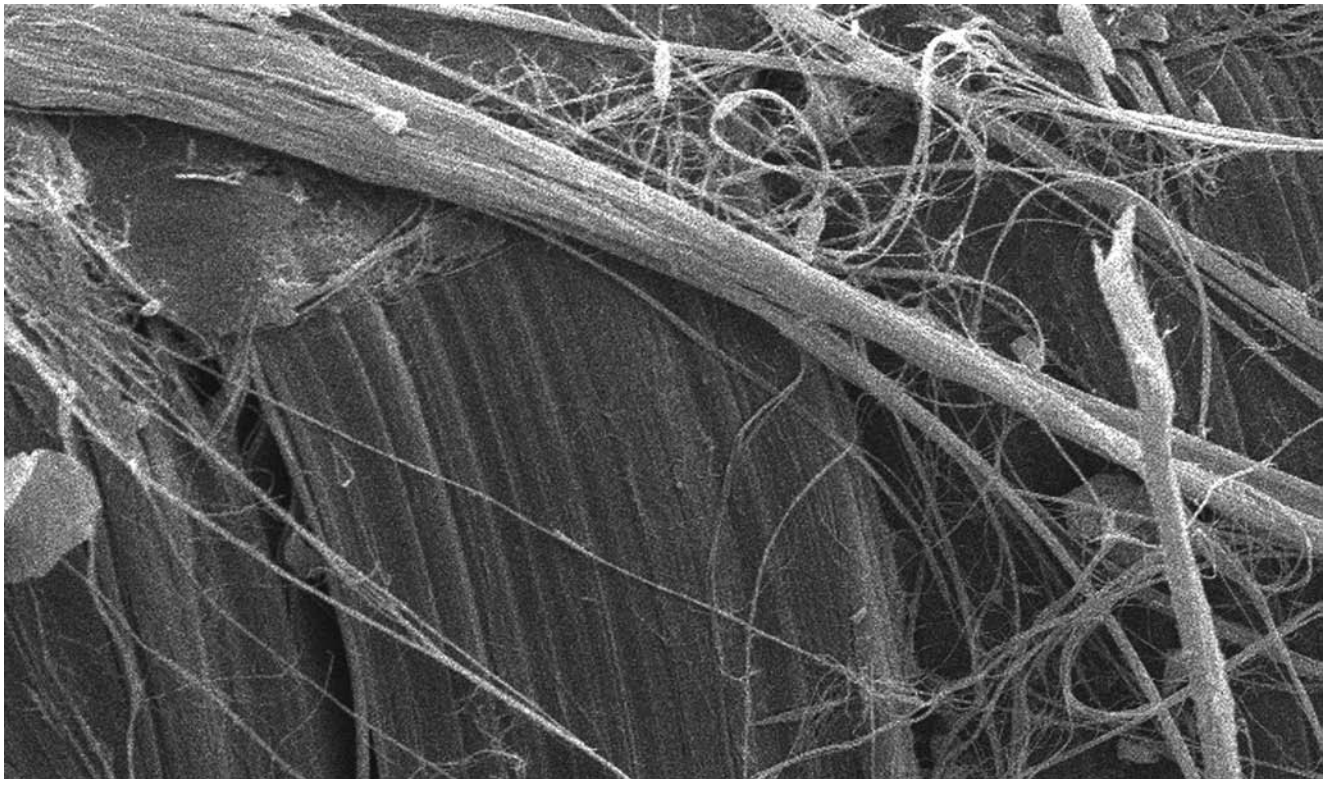
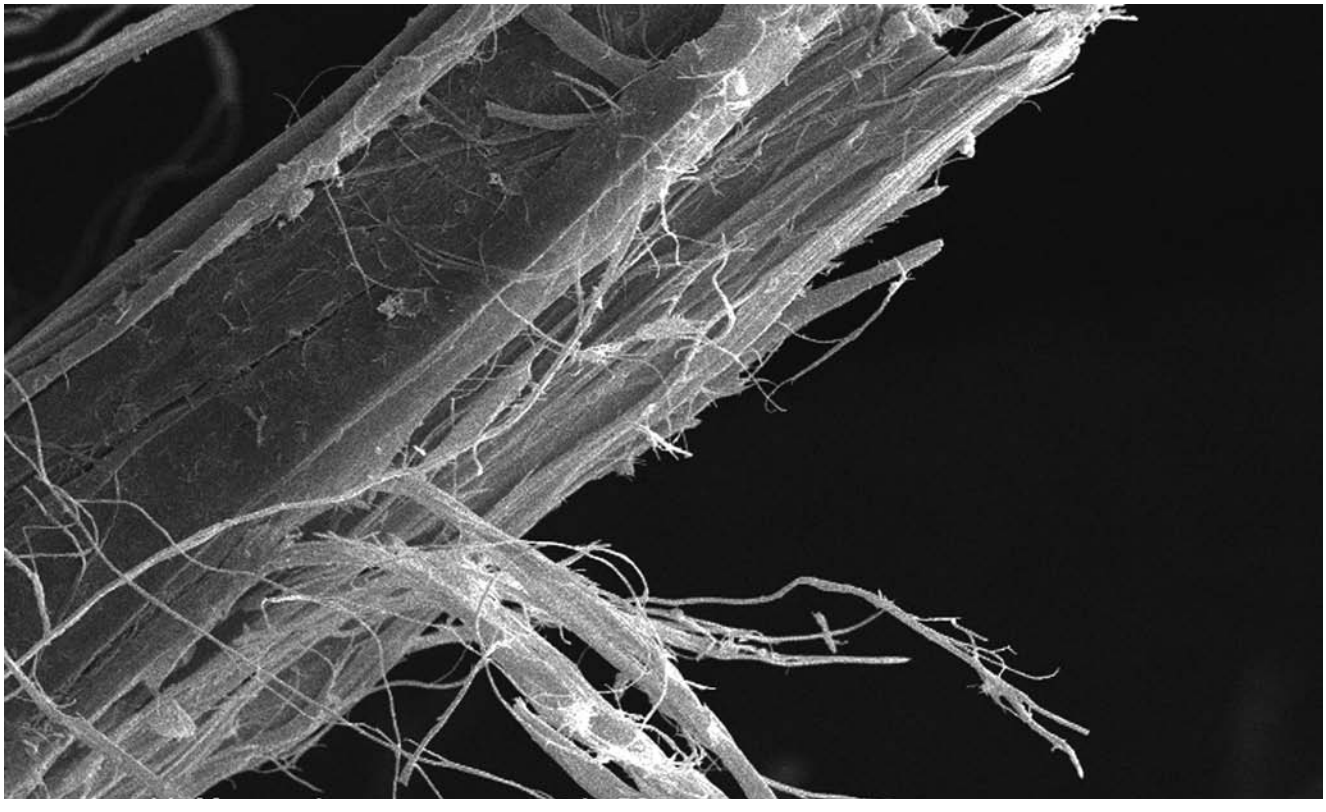


Figure 6-9 Scanning electron micrograph (500×) of raw chrysotile asbestos. (Courtesy of CDC/John Wheeler, PhD; Photographer: Janice Haney Carr.)



does contain covalently bonded carbon. For this section, we will further differentiate the organic compounds into smaller groups based on similar physical properties, similar analytical methods, or both. Two of the compounds listed, acrylamide and epichlorohydrin, will not be discussed as their regulated limits are maintained by TTs (their addition to the water as disinfectants), not analyzed in the finished water as contaminants.

Volatile Organic Compounds

This first section considers the VOCs. Generally speaking, these are compounds that evaporate or volatilize under normal conditions and precise definitions vary with circumstances. There are 21 regulated compounds we will consider here. In Table 6-6 we find all the regulated compounds that match this definition and have similar analytical methods. There are other compounds that may meet the definition of volatile, but require different analytical methods (e.g., 1,2-Dibromo-3-chloropropane). Various adverse health effects associated with exposure to these compounds includes kidney and/or liver problems and increased risk for certain types of cancers.

As we see in Table 6-6, there are two analytical methods common to all 21 compounds and one useful for only 5. We will give a brief description of each method.

- EPA Method 524.2 *Measurement of Purgeable Organic Compounds in Water by Capillary Gas Chromatography/Mass Spectroscopy* uses high-purity helium to purge volatile compounds from a water sample, which are then trapped on a sorbent material. This is then heated to elute the compounds and they are transferred to a capillary column for separation by GC followed by detection and identification by mass spectroscopy (MS). Detection limits for these compounds range from 0.02 to 0.1 μg/L.[14]
- EPA Method 502.2 *Volatile Organic Compounds in Water by Purge and Trap Capillary Column Gas Chromatography with Photoionization and*

Table 6-6 Approved Analysis Methods and MCLs for Volatile Organic Compounds[3]

Compound	502.2	524.2	551.1	MCL (mg/L)
1,1,1-Trichloroethane	X	X	X	0.2
1,1,2-Trichloroethane	X	X	X	0.005
1,1-Dichloroethylene	X	X	—	0.007
o-Dichlorobenzene	X	X	—	0.6
p-Dichlorobenzene	X	X	—	0.075
1,2,4-Trichlorobenzene	X	X	—	0.07
1,2-Dichloroethane	X	X	—	0.005
1,2-Dichloropropane	X	X	—	0.005
Benzene	X	X	—	0.005
Carbon Tetrachloride	X	X	X	0.005
Chlorobenzene	X	X	—	0.1
cis-Dichloroethylene	X	X	—	0.07
Dichloromethane	X	X	—	0.005
Ethylbenzene	X	X	—	0.7
Styrene	X	X	—	0.1
Tetrachloroethylene	X	X	X	0.005
Toluene	X	X	—	1.0
trans-Dichloroethylene	X	X	—	0.1
Trichloroethylene	X	X	X	0.005
Vinyl Chloride	X	X	—	0.002
Xylenes (total)	X	X	—	10

Electrolytic Conductivity Detectors in Series uses high-purity helium to purge volatile compounds from a water sample, which are then trapped on a sorbent material. This is then heated to elute the compounds, and they are transferred to a capillary column for separation by GC followed by detection with two detectors placed in series (photoionization [PID] and electrolytic conductivity [ELCD]). PID is extremely sensitive to aromatic compounds, while ELCD is sensitive to electronegative compounds. Detection limits for these compounds range from 0.01 to 0.06 μg/L (PID) and 0.010.1 μg/L (ELCD).[14]

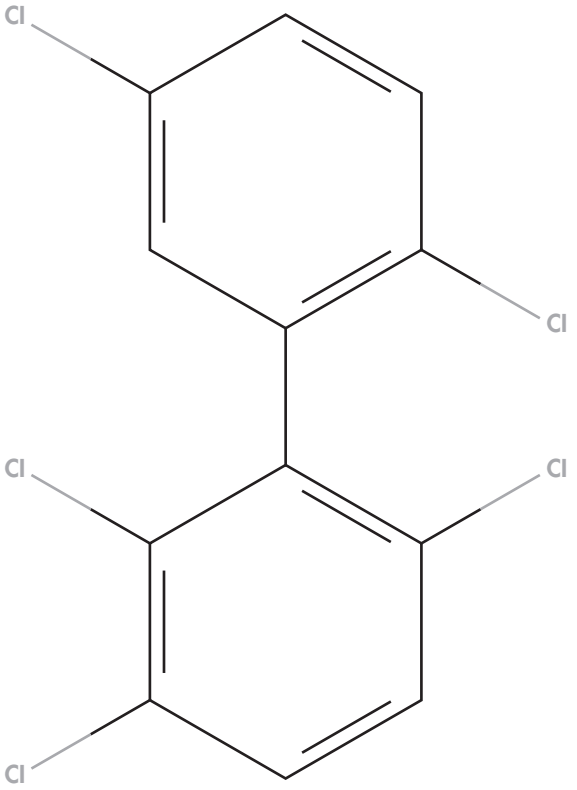
- EPA Method 551.1 *Determination of Chlorinated Disinfection Byproducts, Chlorinated Solvents, and Halogenated Pesticides/Herbicides in Drinking Water by Liquid–Liquid Extraction and Gas Chromatography with Electron-Capture Detection* extracts the sample a single time with solvent followed by separation by GC and detection with ECD. The range of detection limits for compounds analyzed by this method is quite wide with significant instrument and analyst dependence. Detection limits for these compounds range from 0.002 to 0.01 μg/L.[14]

Organochlorine and Organohalide Pesticides/Herbicides

This section considers organic compounds that are often used as pesticides and herbicides. Most are chlorinated, though other halogens may also be used. There are 13 regulated compounds we will consider here (with all possible polychlorinated biphenyl [PCB] congeners consolidated into one class). PCB consists of biphenyl molecule to which is attached anywhere from 1 to 10 chlorines. In theory, there are 209 possible PCB structures, though only about 130 are usually found in commercial mixtures. Figure 6-10 shows the structure of one congener, 2,2',3,5',6-pentachlorobiphenyl, with chlorine atoms in 5 of 10 possible locations. In Table 6-7 we find the list of these regulated compounds that have similar analytical methods. Various adverse health effects associated with exposure to these compounds includes kidney, liver, and/or other organ problems; reproductive problems; and increased risk for certain types of cancers.

As we see in Table 6-7, there are three analytical methods common to all 13 compounds and one useful for only 3. We will give a brief description of each method.

Figure 6-10 Structure of 2,2',3,5',6-pentachlorobiphenyl. (Courtesy of National Center for Biotechnology Information [NCBI].)



- EPA Method 508.1 *Determination of Chlorinated Pesticides, Herbicides, and Organohalides by Liquid–Solid Extraction and Electron-Capture Gas Chromatography* passes the sample through a C-18–impregnated disk or column to remove the target compounds. These are then eluted with solvent and transferred to a capillary column for separation by GC followed by detection by ECD. Detection limits for these compounds range from 0.001 to 0.13 μg/L.[14]
- EPA Method 525.2 *Determination of Organic Compounds in Drinking Water by Liquid–Solid Extraction and Capillary Column Gas Chromatography/Mass Spectrometry* passes the sample through a C-18–impregnated disk or column to remove the target compounds. These are then eluted with solvent and transferred to a capillary column for separation by GC followed by detection and identification by MS. Detection limits for these compounds range from 0.07 to 1 μg/L.[14]

Table 6-7 Approved Analysis Methods and MCLs for Organochlorine Pesticides[3]

Compound	505	507	508	508.1	525.2	551.1	MCL (mg/L)
Alachlor	X	X	—	X	X	X	0.002
Atrazine (also Syngenta immunoassay)	X	X	—	X	X	X	0.003
Chlordane	X	—	X	X	X	—	0.002
Endrin	X	—	X	X	X	X	0.002
Heptachlor	X	—	X	X	X	X	0.0004
Heptachlor epoxide	X	—	X	X	X	X	0.0002
Hexachlorobenzene	X	—	X	X	X	X	0.001
Hexachlorocyclopentadiene	X	—	X	X	X	X	0.05
Lindane	X	—	X	X	X	X	0.0002
Methoxychlor	X	—	X	X	X	X	0.04
Polychlorinated biphenyls (PCBs)	X	—	X	X	X	—	0.0005
Simazine	X	X	—	X	X	X	0.004
Toxaphene	X	—	X	X	X	—	0.003

- EPA Method 505 *Analysis of Organochlorine Pesticides and Commercial Polychlorinated Biphenyl (PCB) Products in Water by Microextraction and Gas Chromatography* extracts the sample a single time with solvent followed by separation by GC and detection with ECD. The range of detection limits for compounds analyzed by this method range is wide with significant instrument and analyst dependence. The range of detection limits varies by matrix and instrumentation. Detection limits for these compounds range from 0.002 to 1 μg/L.[14]
- Less frequently used are:
  - Method 507 *Determination of Nitrogen- and Phosphorous-Containing Pesticides in Water by Gas Chromatography with a Nitrogen–Phosphorous Detector,* which utilizes liquid–liquid extraction followed by GC separation and NPD detection. The range of detection limits varies by matrix and instrumentation.
  - Method 508 *Determination of Chlorinated Pesticides in Water by Gas Chromatography with a Electron-Capture Detector,* which utilizes liquid–liquid extraction followed by GC separation and ECD detection. The range of detection limits varies significantly by analyte.
  - Method 551.1 *Determination of Chlorinated Disinfection Byproducts, Chlorinated Solvents, and Halogenated Pesticides/Herbicides Drinking in Water by Liquid–Liquid Extraction and Gas Chromatography with Electron-Capture Detection* (described previously with VOCs). The range of detection limits varies significantly by analyte.

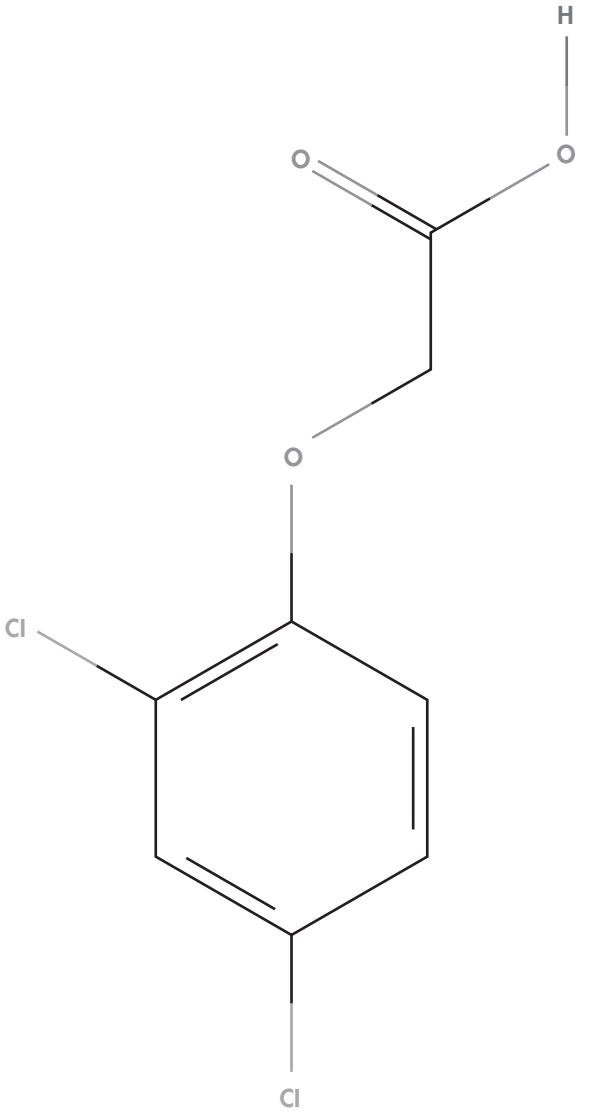
Phenoxy Acid Herbicides/Chlorinated Organic Acids

This section considers pesticides and herbicides that are chemically distinct from those discussed previously. These are structurally acids, which means that they are more water soluble than the organohalides discussed previously and must often be chemically altered (hydrolyzed, hybridized) as part of the extraction and analysis process. There are six regulated compounds we will consider here, listed in Table 6-8. Various adverse health effects associated with exposure to these compounds essentially mirror those for chlorinated pesticides and include kidney, liver, and/or other organ problems; reproductive problems; and increased risk for certain types of cancers. Figures 6-11 and 6-12 show the structures for 2,4-D and Endrin to highlight the difference in structure between the organochlorides discussed previously and the phenoxy acid herbicides discussed here.

Table 6-8 Approved Analysis Methods and MCLs for Phenoxyacid Herbicides[3]

Compound	515 (0.1/0.2/0.3/0.4)	525.2	552 (0.1/0.2/0.3)	555	ASTM D5317	MCL (mg/L)
2,4-D	X	—	—	X	X	0.07
Dalapon	X	—	X	—	—	0.2
Dinoseb	X	—	—	X	—	0.007
Pentachlorophenol	X	X	—	X	X	0.001
Picloram	X	—	—	X	X	0.5
2,4,5-TP (Silvex)	X	—	—	X	X	0.05

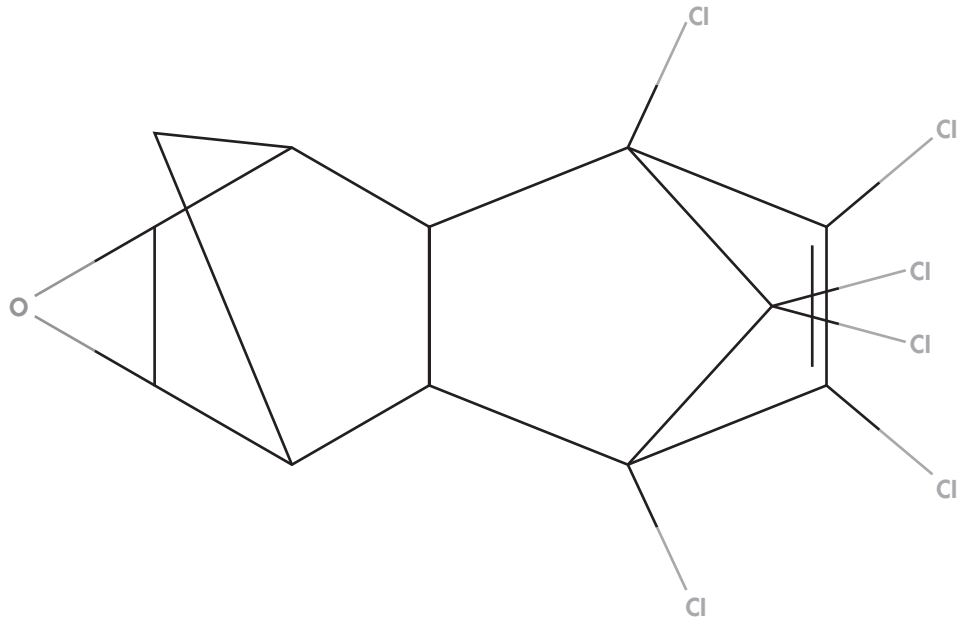
Figure 6-11 Structure of 2,4-dichlorophenoxyacetic acid. (Courtesy of NCBI.)



As we see in Table 6-8, there is only one analytical method common to all six compounds and two useful for only one each. We will give a brief description of the two more widely used:

- EPA Method 515.2 *Determination of Chlorinated Acids in Water Using Liquid–Solid Extraction and Gas Chromatography With Electron-Capture Detector* hydrolyzes the sample with base and extracts other organic compounds with solvent. The sample is re-acidified and passed through a resin-impregnated disk to capture the target compounds. These are converted to their methyl ester forms with diazomethane or trimethylsilyldiazomethane (TMSD), transferred to a capillary column for GC separation, followed by detection with ECD. The range of detection limits for compounds analyzed by this method range is wide with significant instrument and matrix dependence. 515.1 is the liquid–liquid extraction version of this method, and 515.3 and 515.4 are variants using 40 ml of sample versus 1 liter. Detection limits for these compounds range from 0.06 to 0.35 μg/L for 515.2 and from 0.032 to 1.3 μg/L for 515.1.[14]
- EPA Method 555 *Determination of Chlorinated Acids in Water by High Performance Liquid Chromatography, with a Photodiode Array Ultraviolet Detector* hydrolyzes the sample with base for 1 hour followed by acidification with H_3PO_4. An aliquot is introduced to the HPLC with a capture column (filled with C-18) followed in line with a separation column. Detection follows with a UV spectrometer. Detection limits for these compounds range from 0.5 to 1.8 μg/L.[14]

Figure 6-12 Structure of endrin. (Courtesy of NCBI.)



Less frequently used are:

- EPA Method 525.2 is the liquid–solid extraction described previously in the organochlorine pesticide section and is fairly useful for pentachlorophenol (detection limit of 1 μg/L).[14]
- EPA Method 552.1 *Determination of Haloacetic Acids and Dalapon in Drinking Water by Ion-Exchange Liquid–Solid Extraction and Gas Chromatography with an Electron-Capture Detector* acidifies the sample, passes it through a solid capture medium (column or disk), elutes the captured compounds, and derivatizes them prior to GC separation and detection by ECD. 552.2 is the liquid–liquid extraction version of this method, and 552.3 uses microextraction. The detection limits for dalapon range from 0.11 to 0.32 μg/L for these methods.[14]
- ASTM D5317 *Standard Test Method for Determination of Chlorinated Organic Acid Compounds in Water by Gas Chromatography with an Electron-Capture Detector* is essentially the same as 515.1. The range of detection limits for compounds analyzed by this method range from 0.08 to 0.2 μg/L.[14]

Carbamates/Water-Soluble Pesticides and Herbicides

This section considers pesticides and herbicides that are chemically distinct from those discussed previously and are generally quite water soluble. There are five regulated compounds we will consider here, listed in Table 6-9. Various adverse health effects associated with exposure to these compounds include cataracts and blood, nervous system, stomach, intestinal, and reproductive problems. Figures 6-12 and 6-13 show the structures for glyphosate and endrin to highlight the difference in structure between the organochlorides discussed previously and those discussed here.

As we see in Table 6-9, there is no single analytical method common to all five compounds and two useful for only two each. In particular, we notice some of the difficulties inherent with performing chemical analyses versus microbiological or radiological. Many methods for chemical analyses are only applicable for a single regulated compound. The analyst or laboratory must have the capability and proficiency for at least four distinct analytical methods for the analysis of these five compounds. This quickly becomes expensive and very time-consuming.

- EPA Methods 531.1 and 531.2 are both titled *Measurement of N-Methylcarbamoyloximes and N-Methylcarbamates in Water by Direct Aqueous Injection HPLC With*

Table 6-9 Approved Analysis Methods and MCLs for Water-Soluble/Other Pesticides[3]

Compound	531 (0.1/0.2)	547	548.1	549.2	SM 6610 B	SM 6651	MCL (mg/L)
Carbofuran	X	—	—	—	X	—	0.04
Diquat	—	—	—	X	—	—	0.02
Endothall	—	—	X	—	—	—	0.1
Glyphosate	—	X	—	—	—	X	0.7
Oxamyl (Vydate)	X	—	—	—	X	—	0.2

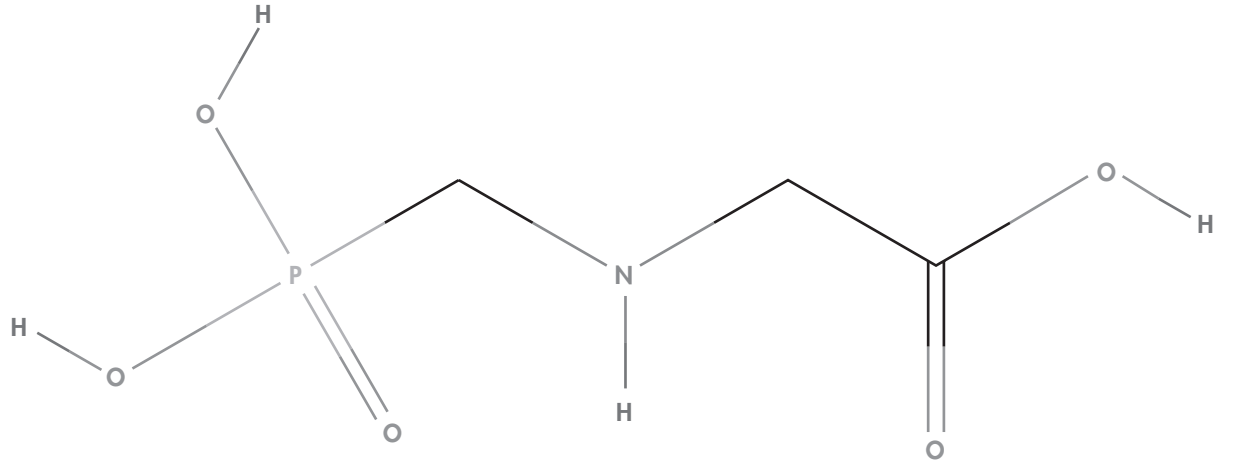
Postcolumn Derivatization and both inject a sample onto an HPLC system with a reversed phase column. As the compounds are eluted, they are derivatized in a postcolumn and this product reacted with other compounds to form a highly fluorescent isoindol product, which is detected via fluorescence. Standard Method 6610 B *High-Performance Liquid Chromatographic Method* follows the same basic principles as 531. Detection limits for carbofuran and oxamyl are 0.52 and 0.86 μg/L by 531.1, 0.043 and 0.065 μg/L by 531.2, and 1.5 and 2.0 μg/L by 6610 B.[9,14]

- EPA Method 547 *Determination of Glyphosate in Drinking Water by Direct-Aqueous-Injection HPLC, Post Column Derivatization, and Fluorescence Detection* uses a similar principle as for 531.1, with the differences of using a cation exchange column instead of reversed phase, and postelution reaction with a different compound before reaction to form a highly fluorescent product. Standard Method 6651 *Glyphosate Herbicide* follows the same basic principles as 547. Detection limit for glyphosate by 547 is 6 μg/L.[14]
- EPA Method 548.1 *Determination of Endothall in Drinking Water by Ion-Exchange Extraction, Acidic Methanol Methylation, and Gas Chromatography/Mass Spectrometry* uses liquid–solid extraction followed by acid treatment, solvent exchange and reduction, separation by GC, and detection and identification by either MS or FID. The detection limit for the MS analysis is 1.79 μg/L and 0.7 by FID.[14]
- EPA Method 549.2 *Determination of Diquat and Paraquat in Drinking Water by Liquid–Solid Extraction and High-Performance Liquid Chromatography with Ultraviolet Detection* uses liquid–solid extraction followed by acid treatment, separation by HPLC, and detection and identification by UV fluorescence and/or PDA. The detection limit for diquat by this method is 0.72 μg/L.[14]

Remaining Organic Compounds

The six remaining organic compounds escape neat categorization so they are all included here to wrap up the section. They represent a mix of characteristics. For example,

Figure 6-13 Structure of glyphosate. (Courtesy of NCBI.)



DBCP could be considered a volatile compound, and can be analyzed by 551.1 like other VOCs, but is not suitable to purge-and-trap extraction as described in 502.1 and 524.2. It is therefore usually analyzed separately from the other VOCs. Dioxin has an exceedingly low MCL, and meeting this needs a level of sophistication and expense not required by other compounds. Various adverse health effects associated with exposure to these compounds include liver, stomach, kidney, and reproductive problems; weight loss; and increased risk of cancer.

Unlike the previous sections, we will discuss the methods as they relate to the analytes. With the exception of Method 525.2, the methods listed in Table 6-10 are unique to specific analytes/pairs of analytes.

- Benzo(a)pyrene is a polycyclic aromatic hydrocarbon (PAH) analyzed by two methods:
  - EPA Method 525.2 *Determination of Organic Compounds in Drinking Water by Liquid–Solid Extraction and Capillary Column Gas Chromatography/Mass Spectrometry* was discussed with VOC analysis. The detection limit for this compound by 525.2 is 0.032 μg/L.[14]
  - EPA Method 550.X *Determination of Polycyclic Aromatic Hydrocarbons in Drinking Water by* (*Liquid–Liquid* [550] or *Liquid–Solid* [550.1]) *Extraction and HPLC with Coupled Ultraviolet and Fluorescence Detection* utilizes either liquid–liquid (550) or liquid–solid (550.1) phase extraction followed by separation by HPLC and detection by UV absorbance and fluorescence. The detection limits for this compound is 0.029 and 0.016 μg/L for 550 and 550.1, respectively. [14]
- DBCP was a pesticide that was phased out in 1979, and EDB is used as an antiknock agent in aviation fuels, in some industries, and as a pesticide. These are often analyzed together by the following two methods.
  - EPA Method 504.1 *1,2-Dibromomethane (EDB), and 1,2-Dibromo-3-chloropropane (DBCP), and 1,2,3-Trichloropropane (123TCP) in Water by Microextraction and Gas Chromatography* uses a single extraction of a small sample followed by separation by GC and detection by ECD. The detection limits for both compounds by this method is 0.01 μg/L. [14]
  - EPA Method 551.1 *Determination of Chlorination Disinfection Byproducts, Chlorinated Solvents, and Halogenated Pesticides/Herbicides in Drinking Water by Liquid–Liquid Extraction and Gas Chromatography with Electron-Capture Detection* was discussed with disinfectant byproduct analysis. The detection limit for DBCP and EDB are 0.009 and 0.008 μg/L, respectively.[14]
- Di(2-ethylhexyl) adipate and di(2-ethylhexyl) phthalate are both widely used as plasticizers. That is, they help make plastic products flexible. These are also often analyzed together by the following two methods.
  - EPA Method 506 *Determination of Phthalate and Adipate Esters in Drinking Water by Liquid–Liquid Extraction of Solid–Liquid Extraction and Gas Chromatography with Photoionization Detection* uses either extraction method followed by extract concentration, separation by GC, and detection with PID. The detection limits for these compounds by this method are 11.82 and 2.25 μg/L, respectively. [14]

Table 6-10 Approved Analysis Methods and MCLs for Remaining Compounds[3]

Compound	504.1	506	525.2	550/.1	551.1	1613	MCL (mg/L)
Benzo(a)pyrene (PAHs)	—	—	X	X	—	—	0.0002
1,2-Dibromo-3-chloropropane (DBCP)	X	—	—	—	X	—	0.0002
Ethylene dibromide (EDB)	X	—	—	—	X	—	0.00005
Di(2-ethylhexyl) adipate	—	X	X	—	—	—	0.4
Di(2-ethylhexyl) phthalate	—	X	X	—	—	—	0.006
Dioxin (2,3,7,8-TCDD)	—	—	—	—	—	X	0.00000003

- EPA Method 525.2 *Determination of Organic Compounds in Drinking Water by Liquid–Solid Extraction and Capillary Column Gas Chromatography/Mass Spectrometry* was discussed with organochlorine pesticides. The detection limits for these compounds by this method are 0.09 and 0.46 μg/L, respectively. [14]

- Dioxins are a class of compounds formed during the manufacture of some organochlorine compounds/pesticides, during the burning of transformers containing PCBs, and when wood is burned in the presence of chlorine. 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) is one of 75 possible forms of dioxin and became widely known as a contaminant of Agent Orange used in Vietnam. The structure of 2,3,7,8 TCDD is shown in Figure 6-14 and shows chlorine occupying four of eight possible sites on the base molecule. Dioxins are remarkably persistent in the environment and are analyzed by the following method.

  - EPA Method 1613 *Tetra-through Octa-Chlorinated Dioxins and Furans by Isotope Dilution HRGC/HRMS* allows for either liquid–liquid or liquid–solid phase extraction followed by concentration and separation by high-resolution GC and detection and identification by high-resolution MS. The detection limit for this compound by this method is 10 pg/L, equivalent to 0.000010 μg/L.[14]

Radionuclides

There are four different types of radioactive parameter regulated in drinking water by the EPA. Unlike previous sections, these are not always specifically associated with a specific component. The inorganic compounds are very specific about which compounds are regulated, but we find here a mixture of specifics and classes of elements. The two elements that are specified are radium (both the 226 and 228 isotopes) and uranium. The two distinct classes are total alpha emitters, and combined beta and photon emitters. There are any number of radionuclides that could be present and result in a positive alpha particle test, and the test does not distinguish between them, just the total amount emitted (activity). Also different from other analytes is the high degree of specialization by analytical method. There is very little overlap of methods adequate for multiple analytes, and a great number of approved methods. We will therefore discuss each regulated class and some of their associated test methods individually. The list of regulated contaminants and their MCLs are shown in Table 6-11.

Alpha and Beta Particles

The nature of radioactive decay was discussed in Chapter 4 and will not be described again here. In brief, alpha particles are identical to helium nuclei (two protons and two neutrons) and are easily stopped within 2 centimeters of air or by a sheet of paper. Purely external exposure is therefore not of great concern. However, alpha particles may wreak great havoc on cellular DNA when placed in immediate proximity to living cells. This may occur via inhalation (potentially causing lung cancer) or ingestion.

Beta particles, on the other hand, are simply electrons. They are typically less energetic than alpha particles but travel farther and are more difficult to stop because of their much smaller size. They can travel several feet in air and may penetrate skin. They are stopped by a plexiglas shield or sheet of aluminum. They are also less likely to cause damage to cells and internal exposure is not as much of a concern as it is for alpha particles. Adverse health effects associated with exposure to these particles includes an increased risk of cancer. There are multiple tests approved for their analysis and we will discuss three of them.

Figure 6-14 Structure of 2,3,7,8-TCDD showing chlorines at four of eight possible positions.

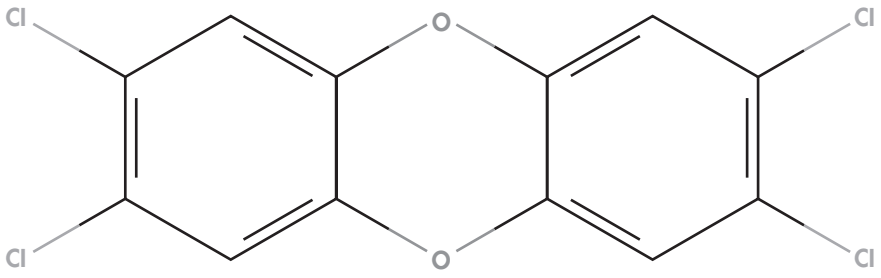


Table 6-11 Regulated Radionuclides and Their MCLs[3]

Compound	Method	MCL
Alpha particles	see later for each analyte	15 pCi/L (equivalent to ~33 disintegrations/minute/liter)
Beta particles and photon emitters		4 millirems per year
Radium-226 and radium-228 (combined)		5 pCi/L
Uranium		30 ug/L

- Standard Methods 7110 B *Gross Alpha and Gross Beta Radioactivity* evaporates a sample to dryness and measures activity via gas proportional counting until the desired sensitivity is achieved.[9,14]
- Standard Methods 7110 C *Coprecipitation Method for Gross Alpha Radioactivity in Drinking Water* mixes the sample with barium sulfate and iron hydroxide to cause the alpha-emitting radionuclides of interest to precipitate out of solution. They are then filtered out of the sample, dried, and measured by either an alpha scintillation or gas proportional counter until the desired sensitivity is achieved.[9,14]
- EPA Method 900.0 *Gross Alpha and Gross Beta Radioactivity in Water* evaporates a preserved sample to dryness and measures activity via gas proportional counting until the desired sensitivity is achieved.[14]
- Other methods include EPA 00-02 in EPA 520/5-84-006 and USGS R-1120-76.

Photon (Gamma) Emitters

Photons (gamma rays) are massless particles of great energy and penetrating power. They are only stopped by a thickness of lead or several feet of concrete. They are thus a great health hazard over substantial distances. Exposure to gamma rays leads to an increased risk of cancer. There are multiple methods for their analysis:

- Standard Methods 7120 B *Gamma Spectroscopic Method* places a sample in the instrument's sample holder and measures activity with either an NaI(Tl) (scintillation) or Ge(Li) (semiconductor) detector until the desired sensitivity is achieved.[9,14]
- EPA Method 901.1 *Gamma Emitting Radionuclides in Drinking Water* places a sample in the instrument's sample holder and measures activity with either an NaI(Tl) (scintillation) or Ge(Li) (semiconductor; preferred) detector until the desired sensitivity is achieved.[14]
- Other methods include ASTM D3649 and D4785, Department of Homeland Security 4.5.2.3 and Ga-01-R, and Standard Methods 7500 series.

Radium 226 and Radium 228 (Combined)

Radium is a widely naturally occurring radionuclide that can be found in almost any environmental sample. It is produced by the decay of uranium (radium-226) and thorium (radium-228) and exposed by natural erosion. Radium-226 is the more common isotope and is primarily an alpha emitter, while radium-228 is a beta emitter. Both isotopes decay to form isotopes of radon. Because of its ubiquity and its radioactive products, radium is of significant health concern and exposure leads to increased risk of cancer. This element is tested for specifically by many approved methods.

- Radium-226
  - ASTM D3454 *Standard Test Method for Radium-226 in Water* precipitates the radium with barium sulfate and acid-treated to form barium–radium salts, these are dissolved, stored for "growth" of radium-222, and purged with gas into a scintillation counting chamber for alpha activity counting.[14]
  - EPA Method 903.0 *Alpha-Emitting Radium Isotopes in Drinking Water* precipitates the radium with barium and lead sulfate and EDTA, this is then filtered out and activity measured by either alpha scintillation or proportional counting until desired sensitivity is achieved.[14]
  - Other radium-226 methods include EPA 903.1 and Ra-04; Standard Methods 304, 305, 7500-Ra series; and USGS R-1140-76 and R-1141-76.
- Radium-228
  - Standard Methods 7500-Ra D *Radium in Water by Sequential Precipitation and Internal Propor-*

tional Counter or Scintillation Counter precipitates the radium with barium and lead sulfates and EDTA, removes the actinium-228 product of radium-228 with yttrium oxalate after 36 hours, and measures the beta activity by either beta scintillation or proportional counting until the desired sensitivity is achieved.[14]

- EPA Method 904.0 *Prescribed Procedures for Measurement of Radioactivity in Drinking Water* uses the same principles as 7500-Ra D.
- Other methods include EPA Ra-05 and 600/4-75-008, USGS R-1142-76, and methods from the Georgia Institute of Technology, New Jersey Department of Environmental Protection, and the New York Department of Health.

Uranium

Uranium is also a widely occurring element that is often found during testing. The two most common isotopes are uranium-238 (> 99%) and uranium-235 (< 0.8%). Both isotopes are alpha emitters. Uranium is thus a health risk because it both emits alpha particles and forms uranium oxide. Exposure leads to increased risk of cancer and potential kidney damage. This element is tested for specifically by many approved methods.

- ASTM D5673 *Standard Test Method for Elements in Water by Inductively Coupled Plasma-Mass Spectrometry* uses a nebulizer to introduce a sample aliquot into the ICP where the constituents are atomized and ionized and then detected and identified by MS. The detection limit for uranium by this method is 0.8 μg/L. EPA Method 200.8 uses the same basic principles and has a detection limit of 0.1 μg/L.[14]
- Standard Methods 7500-U B *Radiochemical Method* acidifies the sample and precipitates out the uranium with ferric hydroxide, this is separated out and passed through an anion-exchange column, then eluted with acid, evaporated to near dryness, converted to nitrate salt, and activity measured by alpha scintillation or proportional counting.[9,14]
- Other methods include ASTM D2907, D3972, and D5174; Department of Homeland Security U-02 and U-04; EPA 520/5-84-006 and 600/4-80-032; Standard Methods 3125; and USGS R-1180-76, R-1181-76, and R-1182-76.

Secondary Drinking Water Standards

As was mentioned at the beginning of this chapter, the secondary drinking water standards are not enforceable. They concern 15 parameters of drinking water that are not thought to have associated risks to health and are listed in Table 6-12. While the EPA recommends that systems check water for these parameters, it is not required and there is no testing schedule. States may decide to adopt these standards. Since these are not regulated, there are no methods specifically approved for their analyses. Of course, that does not mean there are no methods in existence, just that the laboratory may choose the method of its convenience. Often the choice of analytical method is a compromise between the skills and resources of the laboratory, the needs of the customer, and the expected frequency of requests for a particular analyte.

Aesthetic effects are those that affect the water's taste, appearance, or odor. The parameters associated with aesthetic effects are chloride, copper, foaming agents, iron, manganese, pH, sulfate, odor, total dissolved solids, and zinc. Measuring these parameters is still somewhat subjective and unacceptable levels vary significantly by parameter and consumer. Some odor-producing compounds may be present in extremely small quantities and still be noticeable. However, what level is bothersome varies from person to person. Color may indicate higher levels of dissolved organic material or metals (e.g., iron). Foaming agents cause the water to foam at a point of agitation (such as a faucet) and is often caused by detergents and similar compounds.

Cosmetic effects are those that affect consumers without actual harm to health, such as tissue discoloration. Skin discoloration may be caused by silver ingestion, though this has not been known to occur by water ingestion in the United States. This standard is set as many home purification systems utilize silver as an antibacterial agent. Tooth discoloration and/or pitting may occur in the teeth of children because of high-fluoride content. Fluoride has both primary and secondary standards. The primary is set for those systems which add fluoride to their water (to promote tooth health) and the secondary is set for those systems drawn from water with naturally high-fluoride levels.

Technical effects are those that damage water delivery or treatment systems, such as hard water deposits. The corrosion of iron and copper may not only weaken pipes but discolor water and stain fixtures. Scaling and sedimentation may clog pipes and reduce fixture and water heater use. Parameters associated with technical effects are chloride, copper, corrosivity, iron, manganese, pH, total dissolved solids, zinc, and aluminum.

Table 6-12 Secondary Drinking Water Parameters[3]

Parameter	Regulation	Noticeable Effects if Exceeded
Aluminum	0.05 to 0.2 mg/L	noticeable color
Chloride	250 mg/L	salty taste
Color	15 (color units)	noticeable color
Copper	1.0 mg/L	metallic taste; staining
Corrosivity	noncorrosive	metallic taste; corroded pipes; fixture staining
Fluoride	2.0 mg/L	tooth discoloration/pitting
Foaming agents	0.5 mg/L	foam, noticeable taste/odor
Iron	0.3 mg/L	metallic taste; reddish color/staining
Manganese	0.05 mg/L	bitter metallic taste; black color/staining
Odor	3 threshold odor number	rotten egg, musty, chemical odor
pH	6.5–8.5	low: bitter metallic taste; corrosion high: soda taste, deposits; slippery feel
Silver	0.10 mg/L	skin discoloration
Sulfate	250 mg/L	salty taste
Total dissolved Solids	500 mg/L	salty taste; color/staining; hardness and deposits
Zinc	5 mg/L	metallic taste

As mentioned previously, there are no federally mandated analysis methods associated with these parameters. Therefore laboratories have freedom to choose their own methods. These may range from the simple to the complex. pH, for example, may be analyzed by the use of test strips or a hydrogen electrode (pH meter; EPA Method 150.2 *pH, Continuous Monitoring*). Sulfate may be measured gravimetrically through precipitation or via flow injection analysis (Standard Methods 4500-$SO4^{2-}$). Analytical methods for all these parameters may be found in either the Standard Methods, in various EPA Methods, and through commercially available products and instrumentation.

Finally, it should be noted that PHLs are often asked to perform analyses for nonregulated parameters. These requests are usually associated with a specific source of water or specific potential contaminant. For example, farmers in agricultural communities frequently use large amounts of different pesticides during the year, and there is potential for rain runoff or misapplication resulting in surface and/or ground water contamination. Laboratories may be requested to test water for a specific compound that may be contaminating the water, or test a specific well for different potential contaminants. Analysts typically use the EPA analysis method whose target compounds most closely match the contaminant of interest.

Sidebar 6-4 Pesticide-poisoned Personal Well

Chlorpyrifos is a registered termiticide that is applied around a building foundation with a wand that is inserted into the ground. It is not regulated as a drinking water contaminant. However, sometimes, things go wrong. A homeowner in Illinois contacted his county health department about a bad odor that soon developed in his well water after his home was treated for termite prevention. He suspected that "somehow" the chemical used (chlorpyrifos has a distinctive, sulfur-type odor) leaked into the well. The IDPH laboratory provided water collection materials to the health department sanitarian who collected a water sample. The laboratory tested the water and found PPM (parts per million; mg/L) levels of chlorpyrifos in the water. As the instrumentation was set for analysis at the PPB (parts per billion; µg/L) level, the instrument was overloaded and contaminated. It took some time to clean up the instrument and a large dilution of the sample extract to obtain results in the proper calibration range. Apparently, the "wand" was used around the house near the well and at least one point penetrated the well, directly introducing chlorpyrifos into the home's drinking

water supply. The laboratory results brought together the homeowner, the county health department, the Illinois EPA (a possible chemical spill), Illinois Department of Agriculture (registers termiticides), IDPH laboratory, IDPH Program, and the manufacturer of the insecticide. Once the entire group was convinced that the documentation of the laboratory testing (quality controls, review of paper records and chromatograms, logs of reference standards, etc.) was accurate, then clean up became the focus. Decontamination of the well involved the introduction of high levels of chlorine to the well followed by several exchanges of water until the compound and its breakdown product were no longer detected.

Discussion Questions

1. Describe the rules associated with the Safe Drinking Water Act and the contaminants they regulate.
2. For what reasons might the federal government have mandated drinking water standards rather than allowing each provider to set determine their own?
3. What are the differences between the primary and secondary drinking water standards, and between the MCL and MCLG?
4. Why are no MCLs set to zero?
5. Describe the differences in purpose between the total coliform and *E. coli* tests. What information does each relay that the other may not?
6. How might microorganism testing of drinking water be inadequate for immunocompromised consumers?
7. Fluoride is the only regulated parameter where there can not only be too much, but also too little (concentration below recommended levels). Why is this, and why might there be too much fluoride?
8. Recreational waters are often implicated in illness. What are potential sources of contamination for these sources?
9. Go on the Internet and access PubMed (http://www.ncbi.nlm.nih.gov/PubMed). Enter "leptospirosis 1998 Springfield" as a search. Choose one or more articles and describe the outbreak, how/if standard water testing would have detected the contaminant, and determine why/why not leptospirosis testing should be included in standard testing.
10. Go on the Internet and find three good sources for detailed information concerning drinking water safety and/or water-borne contaminants. They cannot be government sponsored (.gov) or listed under Additional Resouces. Briefly describe what they provide and why they are good sources of reliable information.

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Additional Resources

Cotruvo, J. A., Dufour, A., Rees, G., Bartram, J., Carr, R., Cliver, D. O., et al. (Eds.). (2004). *Waterborne zoonoses: Identification, causes and control*. London: IWA Publishing.

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7 Food Testing

Introduction

In this chapter we will discuss food-borne pathogens and testing methodologies used to determine their presence. In many ways, food is the most complex sample matrix to test because of the wide variety of microbiological and chemical constituents that are commonly found in different foods. Such constituents may include fats, lipids, proteins, simple and complex sugars, and outright microbes (legitimately present in some food such as yogurt). The question is not only how to separate the chemicals from the bacterium from the viruses, but also how to differentiate the good from the bad? Consistency in methodology effectiveness is also an issue. Whereas one may confidently assume that a chemical extraction method good for one water sample is similarly good for the next, what about the determination of *Salmonella* spp. in turkey breast meat versus soup? And testing is only part of the difficulty.

The Centers for Disease Control and Prevention (CDC) reports that there are over 250 known food-borne diseases causing in excess of 76 million cases of disease annually in the United States alone. Some of these are associated with the 400 to 500 food-borne outbreaks investigated by state and local health departments each year and reported to the CDC. While the majority of these illnesses are quite mild and resolve within 1 to 2 days, there remain ~325,000 hospitalizations and ~5000 deaths each year.[1] It is in fact the relative mildness of most infections that leads to severe underreporting of disease, with the causative agent unknown for 82% of all illnesses and hospitalizations and 64% of deaths.[2] For example, only 1 of every 38 estimated *Salmonella* illnesses is diagnosed and reported. The pathogens most commonly recognized to cause food-borne illness include *Campylobacter*, *Salmonella*, *Escherichia coli* O157:H7, norovirus, and *Giardia lamblia*.[1] Bacterial pathogens are the most commonly confirmed, representing 257 of 507 confirmed etiology outbreaks in 2007 associated with 6410 identified cases. They are followed closely by viruses with 199 confirmed outbreaks and 6120 associated cases. Parasites and chemicals were confirmed for 5 and 34 outbreaks associated with 65 and 141 cases, respectively. Furthermore, bacteria were the suspect agent(s) in an additional 61 outbreaks, viruses in 127 outbreaks, parasites in 1 additional outbreak, and chemicals in 15.[3]

While some diseases caused by pathogenic organism infection may have some distinctive clinical presentations, the great majority present very similar initial symptoms. Since the exposure is via ingestion, the immediate effects are usually centered around the digestive tract and include nausea, vomiting, and diarrhea. The incubation period, the time between ingestion, and the appearance of symptoms does vary somewhat, but not necessarily enough for a diagnosis. In fact, the similarity of symptoms often means that a definitive diagnosis associated with a particular pathogen is not possible without laboratory identification. This is in spite of the fact that some pathogens remain within the intestine, others propagate to other parts of the body, and yet others produce toxins that may enter the bloodstream.

There are a variety of ways food may become contaminated with a human pathogen. Many live in otherwise healthy food animals, often within their intestines. If improperly slaughtered, these microbes may contaminate

meat products. Some types of *Salmonella* may infect the ovaries of poultry, such that eggs are contaminated from creation. Fruits and vegetables may become contaminated by exposure to natural elements and contaminated irrigation. Food may also be improperly processed (e.g., poor sanitation) or handled by contaminated workers. Finally, it may be improperly stored and transported, allowing conditions whereby a few pathogens initially present may multiply into millions. Since different steps in food production often occur in different places and by different companies and people over a possibly extended time period, discovering the source of contamination is not always simple. Food is often distributed over a wide geographic area, and even identifying an outbreak (as opposed to sporadic single cases) is difficult as there may be individuals across several states infected by the same source. Local outbreaks based on a local source such as a potluck supper are much easier, by comparison, to identify and address. There are a number of national-level initiatives that have been established to assist in the identification of food-borne outbreaks, tracking the appearance of specific pathogens (trends), and the identification of specific cases associated with an outbreak spread across multiple states.

Enterics Testing

As mentioned in Chapter 5, there is often close association between food-borne pathogen testing and enterics testing. It often occurs that an enterics sample positive for a pathogen instigates an investigation that may result in food samples being submitted as part of the effort to trace the source of exposure. This food testing is thus a critical component of the investigation and assists investigators in determining how the individual may have been exposed, how the food may have been contaminated, and what steps or remediation may need to be taken to avoid this scenario in the future. Food is often tested as part of routine surveillance not associated with any outbreak to avoid such occurrences. We will therefore discuss food testing algorithms used in the laboratory that differ slightly based on its use as part of an outbreak investigation or routine surveillance.

FoodNet

FoodNet is the component of the CDC's Emerging Infections Program (EIP). Federal partners include the CDC, the US Department of Agriculture (USDA), and the US Food and Drug Administration (FDA). There are also 10 state sites (California, Colorado, Connecticut, Georgia, Maryland, Minnesota, New Mexico, New York, Oregon, and Tennessee; Figure 7-1). This catchment area encompasses 15.1% of the US population.[4] The purpose of FoodNet is the active surveillance of food-borne diseases in these areas, specifically *Campylobacter*, *Cryptosporidium*, *Cyclospora*, *E. coli O157:H7*, *Listeria*

Figure 7-1 Map of FoodNet partner sites. (Courtesy of CDC.)

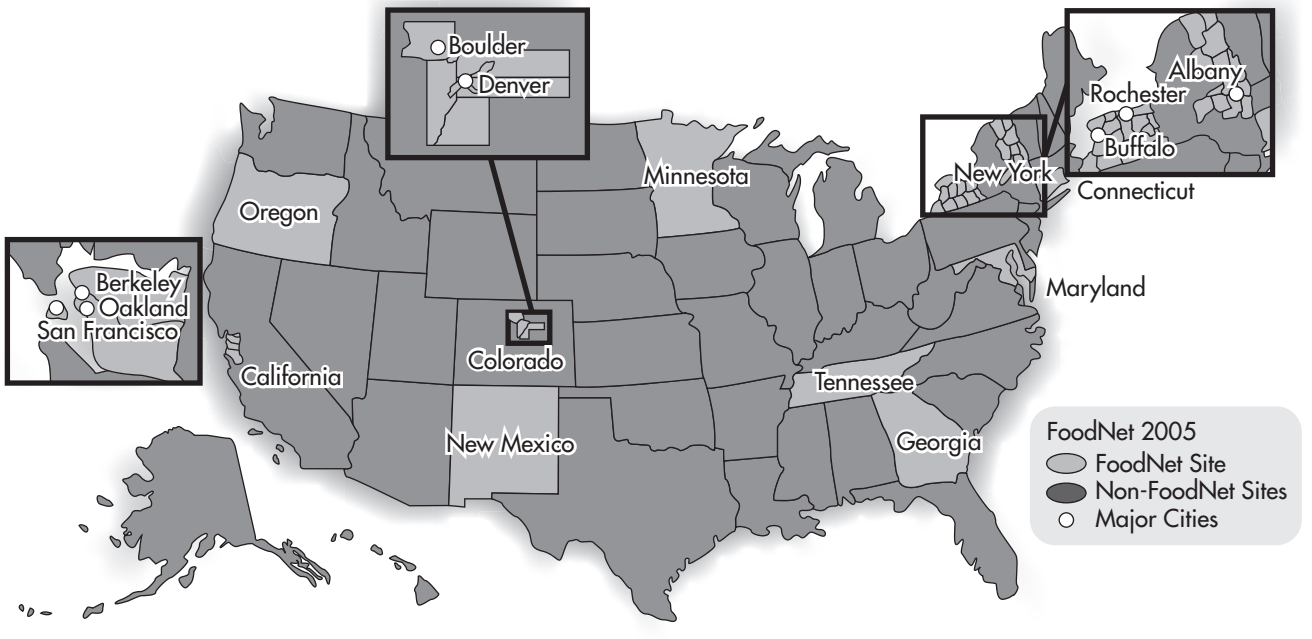
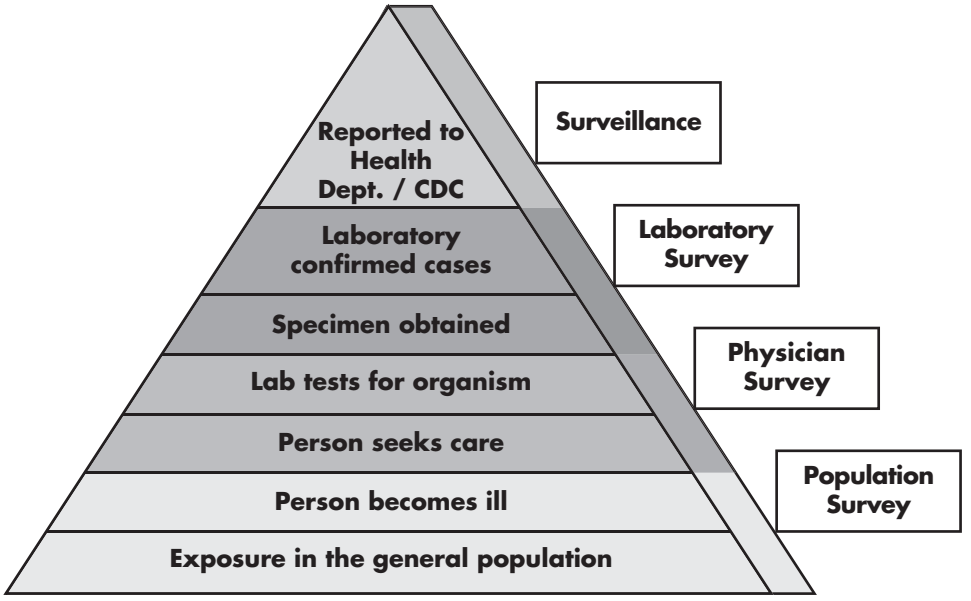


Figure 7-2 FoodNet's Burden of Illness Pyramid showing the events leading from exposure to confirmed case reporting. (Courtesy of CDC.)



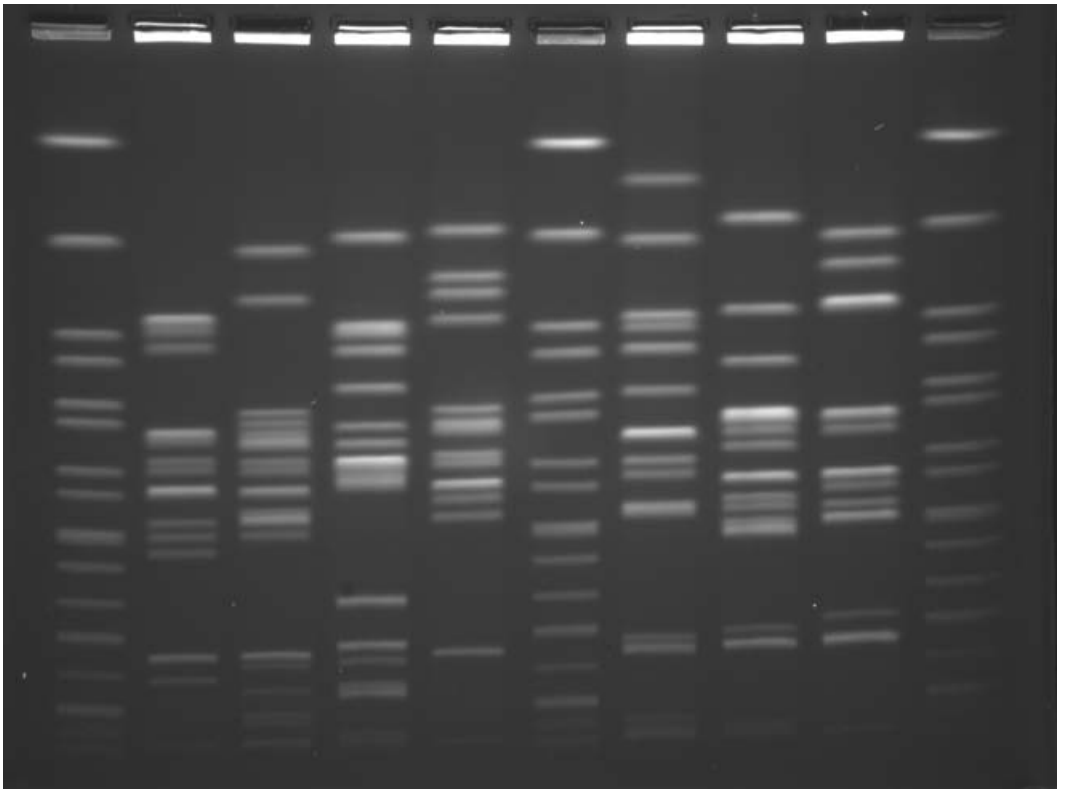
monocytogenes, *Salmonella*, *Shigella*, *Vibrio*, and *Yersinia enterocolitica*. Hemolytic uremic syndrome (HUS), a result of infection with some specific strains of *E. coli*, is also monitored in nine states. The "active" part of the surveillance entails public health officers regularly contacting the approximately 650 laboratories that test stool samples within the EIP area to collect data concerning diagnoses of diarrheal illness.[5] These data are then transmitted directly to the CDC. Passive reporting relies on local physicians/health departments sending such data to the state health department and from there to the CDC. There are any number of ways this chain of information transfer may be disrupted, so active surveillance is a much better, though more costly, method of collecting such data.

The burden of illness pyramid (Figure 7-2) shows the weakness of passive surveillance. There may be a great many people exposed to a pathogen in a population. For example, all 200 people at a hypothetical church supper may have eaten the *Salmonella*-contaminated egg salad. However, not all become ill. Of these, even fewer seek treatment (third level). Of those seeking care, not all provide a stool sample for analysis (some treated symptomatically). Of these collected samples, not all will actually be analyzed (for any number of reasons). The laboratory tests the submitted samples, but sometimes it cannot identify a causative agent and cannot confirm the case (sixth level). Finally, not all data may be reported to the CDC (again, for any number of reasons). What we find is that for 200 people exposed to the pathogen, and a large number becoming ill, the CDC would be informed of only a handful of confirmed cases. Smaller outbreaks might be missed entirely. Not only would there be a missed chance to identify a source of contamination and provide guidance on how to avoid it in the future, but a new pathogen, not identified before, would remain unidentified. Thus, FoodNet's active surveillance is key to food-borne disease identification and prevention. More information on FoodNet and its history and activities can be found on its Web site at http://www.cdc.gov/foodnet/.

PulseNet

PulseNet was developed by the CDC in collaboration with the Association of Public Health Laboratories (APHL). Its purpose is to coordinate the identification and response to food-borne outbreaks by sharing highly specific pathogen subtype information. This information is acquired from the use of pulsed-field gel electrophoresis (PFGE), a molecular analysis technique whereby an organism's DNA is cut into ~10 to 20 large pieces by a restriction enzyme and separated by travel through an agarose gel using variable electric current as the motive force. The pieces travel faster/slower depending on size and the result is a "ladder" pattern (Figure 7-3).

Figure 7-3 Example of a PFGE analysis of *Salmonella* strains showing the characteristic "ladder" patterns. Lanes 1, 6, and 10 are marker lanes and the others are different samples. All were cut with the restriction enzyme XbaI. (Courtesy of Illinois Department of Public Health, Division of Laboratories.)



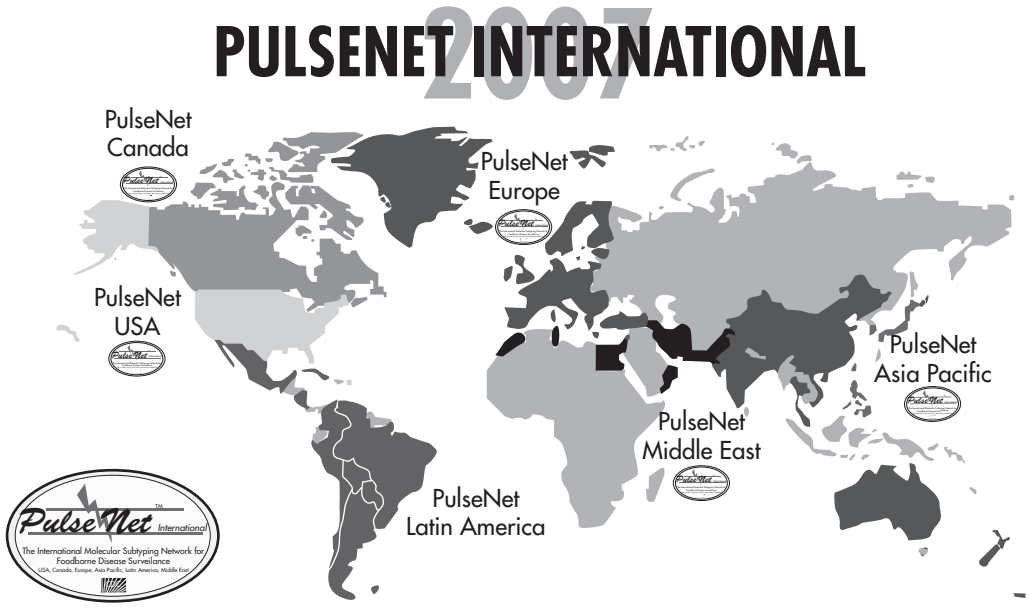
In theory, the sizes of the DNA pieces (and thus the ladder pattern) will be unique to an organism and is considered its "fingerprint," different even between similar strains. The technique is described in more detail in Chapter 2. Epidemiologists can tell whether two individuals with salmonellosis were infected with the same exact strain (indicating a common source of exposure) or different strains. In this manner, officials can match infected individuals from around the country who might have been exposed to the same source and look for as-yet-unidentified outbreaks by matching individually submitted patterns.

Many of the patterns submitted to PulseNet come from state public health laboratories (PHLs). They do not necessarily analyze all samples suspected of containing a food-borne pathogen. Likewise, they do not perform PFGE on all organisms identified by standard means. Specifically targeted organisms include *Salmonella* spp., *C. jejuni*, *L. monocytogenes*, and *Shigella* spp. To increase the utility and quality of PulseNet data, submitters use CDC-approved methods of analysis, undergo training, and participate in proficiency studies. Also, while PulseNet began as a primarily North American entity, it has expanded to include partners around the world as seen in Figure 7-4. More information on PulseNet and its history and activities can be found on its Web site at http://www.cdc.gov/pulsenet/.

Food Emergency Response Network

The Food Emergency Response Network (FERN) is analogous to the Laboratory Response Network (LRN) and fills a gap in state and national laboratory preparedness activities. The CDC, EPA, and other agencies have done much to coordinate and enhance PHL capacity at the state and national level, but they are limited (for the most part) to the analyses of clinical and environmental samples. Food remains a large potential target for terrorism and is vulnerable to naturally occurring disease outbreaks. The FERN works with state and national laboratories, whether public health, agricultural, veterinary, or other, to coordinate their activities and enhance communication so that

Figure 7-4 Map of PulseNet partner laboratories worldwide. (Courtesy of CDC/Dr. Philip S. Brachman.)



they are better prepared to respond to food emergency and can support each other as surge capacity as needed.

FERN activities are coordinated around four objectives concerning PHL infrastructure. *Prevention* is addressed through the establishment of a national surveillance program for food-borne pathogens. *Preparedness* involves augmenting laboratory skills and capacity to respond to emergency events. *Response* is the coordination of PHL capacities to provide analytical surge capacity and facilitate communication. *Recovery* entails continuous and effective monitoring after an event to bolster public confidence. Other FERN activities, not duplicated elsewhere, include the development and validation of methods for food analysis, laboratory staff training, and the provision of proficiency testing. This last often tests general state-of-the-nation capability for laboratory analysis versus mere technical proficiency of individual analysts. More information on FERN and its history and activities can be found on its Web site at http://www.fernlab.org/index.cfm/.

Sidebar 7-1 Vulnerability of Food to Intentional Poisoning[6]

Eighteen people from four families became ill after the consumption of ground beef purchased in a Michigan supermarket between December 31 and January 1, 2003. Reported symptoms included a burning sensation in the mouth, nausea and vomiting, and dizziness. Laboratory tests ordered by the supermarket determined that the causative agent was nicotine. This led to the recall of 1700 pounds of ground beef and an additional 36 people reporting that they or family members had become ill after consuming the product. Investigation found that the contamination was limited to the one store and was not associated with the chain of stores or beef supplier. A second laboratory analysis reported a nicotine concentration of 300 mg/kg in ground beef samples, and this high level suggested purposeful contamination. Pesticide contamination was considered as nicotine is sometimes used as an additive in formulations. The USDA and the Federal Bureau of Investigation (FBI) were brought into the investigation because of the possible transportation across state lines and criminal activity. A total of 148 people reporting illness were interviewed, 92 had illness consistent with the case definition, and 4 sought medical treatment. As a result of the investigation, a former employee of the supermarket was indicted for the intentional poisoning of 200 pounds of meat with the pesticide Black Leaf 40. This case highlights the potential ease with which food may be contaminated and the vigilance required by many parties to ensure its safety.

OutbreakNet and Council to Improve Food-borne Outbreak Response

OutbreakNet is a CDC team that assists in the coordination and investigation of food-borne, water-borne, and other enteric illnesses. They rely in part on data from PulseNet and partner with a network of epidemiologists and federal agencies such as the USDA and FDA. Their Web site is http://www.cdc.gov/foodborneoutbreaks/index.htm. The Council to Improve Foodborne Outbreak Response (CIFOR) is a collaborative effort to share resources and expertise nationwide to reduce the overall burden of food-borne illness. One of its major products is the *Guidelines for Foodborne Outbreak Response*. These guidelines are for use by public health personnel at all levels to assist in all aspects of food-borne outbreak investigation and response. CIFOR also is a clearinghouse for many educational tools created by and for members and the general public. Items available include case studies, models, job descriptions, and training materials. The co-chairs of CIFOR are the Council of State and Territorial Epidemiologists (CSTE) and the National Association of County and City Health Officials (NACCHO). They are supported by the CDC, and have nine other national level partners. They can be found at http://www.cifor.us.

Grade "A" Pasteurized Milk Ordinance, 2007

The *Grade "A" Pasteurized Milk Ordinance* (PMO) is a standard voluntarily adopted by states to produce greater uniformity and a higher level of excellence in milk production. Milk sanitation is one the US Public Health Service's oldest programs, and the first PMO was created in 1924. Since that time, the proportion of all food-borne disease outbreaks attributed to milk has dropped from 25% to $<$ 1% (2007).[7] Raw milk is still a significant source of infection, and the CDC records 69 outbreaks associated with raw milk from 1993 to 2006, resulting in 1505 illnesses, 185 hospitalizations, and 2 deaths.[8]

The PMO provides the requirements necessary for the production of milk and milk products from the cow to the dairy shelf. Methods for the testing of milk are recognized by the National Conference of Interstate Milk Shippers (NCIMS) and subject to provisions of the PMO. The PMO standards for Grade A pasteurized milk and milk products include temperature (cooled to 7°C and maintained), total bacteria (maximum of 10,000/ml), and coliform bacteria (maximum of 10/ml). Appendix G of the PMO specifies some of the chemical tests to be performed. This appendix also specifies the regular testing of milk for both a broad chemical spectrum and chlorinated hydrocarbon pesticides (e.g., heptachlor) performed by many PHLs.[7] Most of the routine testing of milk and milk products, such as cheese and ice cream, is performed by PHLs to ensure these standards are met.

In comparison to milk, overall food sampling and testing is much more varied in part because of the almost limitless potential types of food; their methods of production, transportation, and storage; and the multiple agencies that may have oversight. A general set of guidelines for food quality has been developed and published by the USFDA. The *2005 Food Code* may be found at http://www.fda.gov/Food/FoodSafety/Retail FoodProtection/FoodCode/FoodCode2005/default.htm. Its effects are usually realized by food producers and do not consistently impact PHLs.

Sidebar 7-2 *Campylobacter* Contamination in Milk and Cheese[9]

An insular religious community living in a rural Kansas county held a community fair to celebrate their pioneer heritage on October 20, 2007. One of the activity stations used unpasteurized milk and rennet extract to make soft cheese (where the milk is curdled and the whey drained with little additional processing). This cheese was retained during the day and served during the community dinner held that evening. Of the 101 people who ate the cheese, 67 became ill with 66 reporting watery diarrhea. The only food associated with illness was the cheese, and samples were collected for analysis from the freezer of the community church. *Campylobacter jejuni* was isolated from the three stool samples collected, but not from any of the cheese samples. *Campylobacter* from cow feces or colonized cow teats has been known to contaminate dairy products and thus the cheese is suspected to be the source of contamination even though the samples were negative for the organism. This case highlights the need for both sanitation and pasteurization where possible. It also highlights one of the inherent difficulties of food testing. Unlike almost any other sample matrix discussed in this text, food contaminants are NOT likely to be homogeneously distributed throughout a sample. It is frequently the case that contaminants may become localized within portions of a food product (e.g., 20-lb wheel of cheese), and that subsamples from that product may not contain detectable levels of the organism even though it is present in other portions of sufficient quantity to cause illness. This difficulty with food sampling is discussed in more detail later in the chapter.

Important Bacterial, Parasitic, and Viral Agents

In this section we will discuss some of the more common pathogenic organisms that are the target of testing in food in PHLs. It is by no means exhaustive of all potential pathogens, but does describe some of those most commonly found. It should also be noted that just because a pathogen is common does not also imply that tests for its presence are equally common. Norovirus is an example of a very common pathogen for which there are no reliable tests for its presence in food. Likewise, there may be specific levels of expertise and/or equipment required for other pathogens that some laboratories do not maintain because of costs, lack of personnel, or infrequent requests. Should a sample then be submitted for this type of analysis, it may be sent to another government lab that does maintain the test or to a commercial laboratory.

Bacterial Pathogens

Campylobacter spp. were the confirmed etiology in 20 food-borne outbreaks in the United States in 2007 (16 were *C. jejuni* and the other 4 were unknown species).[3] Campylobacteriosis is the illness caused by infection with *C. jejuni* and is one of the more common sources of diarrheal illness, causing an average of 13 cases per 100,000 people annually, effecting over 2.4 million people each year in the United States.[10] Illness usually occurs within 2 to 5 days of exposure and symptoms may include diarrhea, abdominal pain, nausea, fever, and vomiting. The disease usually lasts 1 week, and most people recover without specific treatment, though antibiotics may be effectively prescribed to shorten the disease course. Infection with *Campylobacter* has been associated with subsequent development of Guillain–Barré syndrome (GBS; acute flaccid paralysis). Serologic studies have shown that up to 30% of individuals with GBS were recently infected by *Campylobacter*, though other agents are also implicated.[2]

Campylobacter is a Gram-negative, slender, motile rod requiring reduced oxygen levels for growth (Figure 7-5). It is estimated that 400 to 500 organisms are necessary to induce disease, and it is a frequent contaminant of poultry where studies have shown that 20 to 100% of retail chickens are contaminated.[11] Further details of *Campylobacter* were discussed in Chapter 5 and will not be repeated here.

Clostridium spp. were the confirmed etiology in 34 food-borne outbreaks in the United States in 2007 (31 were *C. perfringens* and the other 3 were *C. botulinum*).[3] Perfringens food poisoning is the term associated with illness because of *C. perfringens* infection and it is estimated that there are 250,000 cases annually in the United States.[2] Illness is associated with the toxin (enterotoxin)

Figure 7-5 Scanning electron micrograph of *Campylobacter jejuni* (12,123×). (Courtesy of CDC/ Dr. Patricia Fields; Dr. Collette Fitzgerald; Photographer: Janice Carr.)

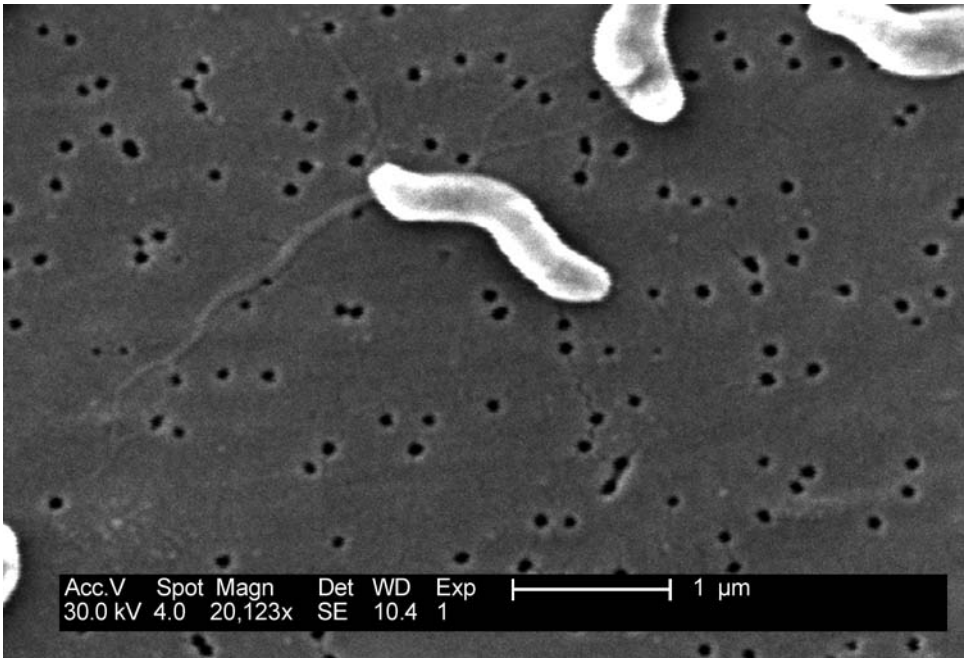
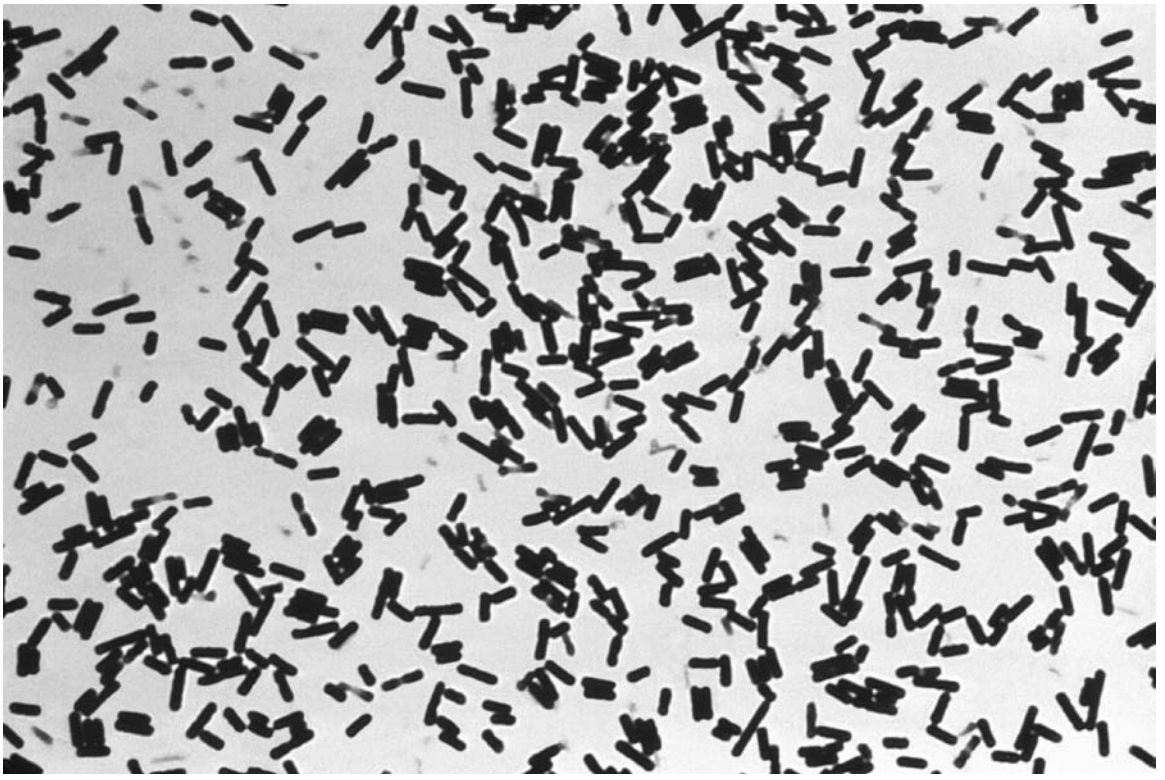


Figure 7-6 ***Clostridium perfringens*** **seen after Gram staining (1000×). (Courtesy of CDC/Don Stalons.)**



produced and usually occurs within 1 to 3 days of exposure. Symptoms include diarrhea and intense abdominal pain. The disease usually lasts 24 hours and most people recover without specific treatment. Some individuals may experience symptoms for up to 2 weeks, and others may develop necrotic enteritis (pig-bel), which is often fatal.[11] Botulism, associated with poisoning with *C. botulinum* toxin is discussed in Chapter 9.

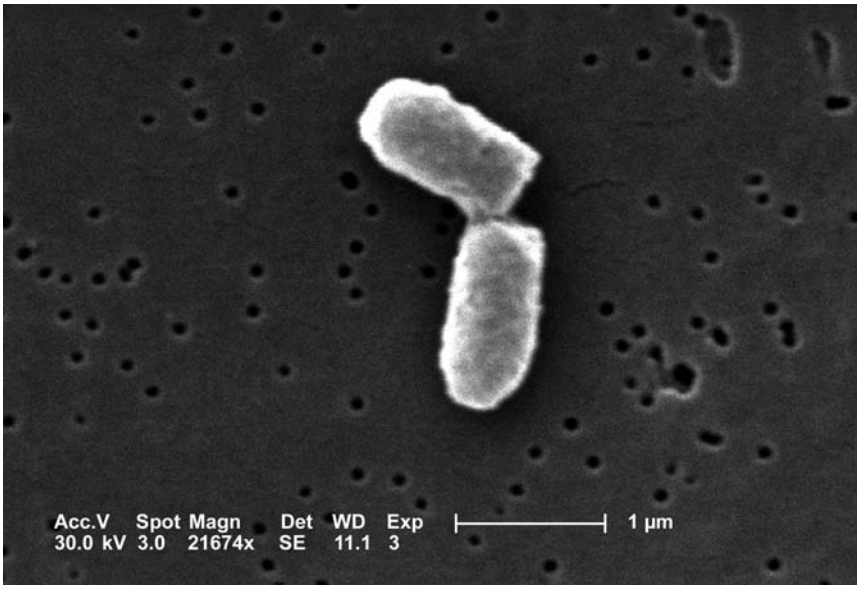
C. perfringens is a large, Gram-positive, anaerobic, nonmotile, spore-forming rod (Figure 7-6). There are five different types of exotoxin produced and α-toxin is the most important. On blood agar, colonies show two zones of hemolysis (complete associated with α-toxin and a further partial hemolysis associated with α-toxin). Growth in broth is accompanied by the production of hydrogen and carbon dioxide.[12] It is estimated that a large number (in excess of 100,000) organisms are necessary to induce disease, and it is widely distributed in the environment and animals.[11] Diagnosis of illness is based on stool sample analysis and source contamination by the analysis of implicated foods.

E. coli O157:H7 was the confirmed etiology in 41 food-borne outbreaks in the United States in 2007 (39 of which were O157:H7 variants, one of O111 and another unspecified).[3] It is also estimated that there are 70,000 O157 infections in the United States each year.[13] STEC infection symptoms usually develop 3 to 4 days after exposure and vary by individual but often include severe stomach cramps, bloody diarrhea, vomiting, and mild fever (if present). Illness usually lasts 5 to 7 days and resolves without treatment. However, 5 to 10% of those diagnosed with STEC infection go on to develop HUS, a potentially life-threatening illness.[13]

E. coli are Gram-negative, facultative rods that ferment lactose and glucose and are oxidase negative (Figure 7-7). The infective dose may be as low as 10 organisms. Almost all documented outbreaks, and many individual cases, have been associated with the consumption of raw or undercooked hamburger.[11] Further details of *E. coli* are discussed in Chapter 5.

Listeria monocytogenes is the causative agent of Listeriosis and was the confirmed etiology in 1 food-borne outbreak in the United States in 2007.[3] Illness caused by *L. monocytogenes* infection has one of the highest rates of food-borne–associated hospitalizations (92.2% of reported cases) and deaths (20% case fatalities).[2] Approximately 2500 individuals in the United States become ill with Listeriosis annually and pregnant women are especially susceptible, being 20 times more likely to become ill and representing almost one third of cases.[14]

Figure 7-7 Scanning electron micrograph of *E. coli* undergoing cell division (21,674×). (Courtesy of CDC/Evangeline Sowers; Janice Haney Carr; Photographer: Janice Haney Carr.)



L. monocytogenes is common in soil and water and is carried by many animals. Exposure may come from contaminated raw foods and unpasteurized foods. The time between exposure and the onset of symptoms is thought to range from a few days to 3 weeks. Gastrointestinal symptoms common to other food-borne pathogens are less frequent, but may precede more serious conditions. Spread of the infection to the central nervous system (CNS) may lead to headache, stiff neck, loss of balance, and confusion. Listeriosis is often discovered by the complications, which may include septicemia (~50% fatal), encephalitis, meningitis (~70% fatal), and intrauterine or cervical infections (~80% fatal) in pregnant women.[11] Infection during pregnancy may have serious impact, leading to miscarriage, stillbirth, premature delivery, or infant infection. Antibiotics (e.g., penicillin, ampicillin) may be prescribed to pregnant women to prevent the spread of infection to the fetus.

L. monocytogenes is a Gram-negative, motile rod (Figure 7-8). *Listeria* grow small β-hemolytic colonies that are catalase positive. There are at least 11 recognized serotypes of *L. monocytogenes*, but three (1/2a, 1/2b, and 4b) cause the majority of human illness.[15] It is estimated that less than 1000 organisms are necessary to induce disease, and studies suggest that 1 to 10% of humans carry *L. monocytogenes* in their intestines.[11] Diagnosis of illness is based on identification of the organisms in either blood or cerebrospinal fluid. Stool samples are difficult to analyze and have limited utility.

Figure 7-8 Electron micrograph of *Listeria monocytogenes* with flagella visible (41,250×). (Courtesy of CDC/ Dr. Balasubr Swaminathan; Peggy Hayes; Photographer: Elizabeth White.)

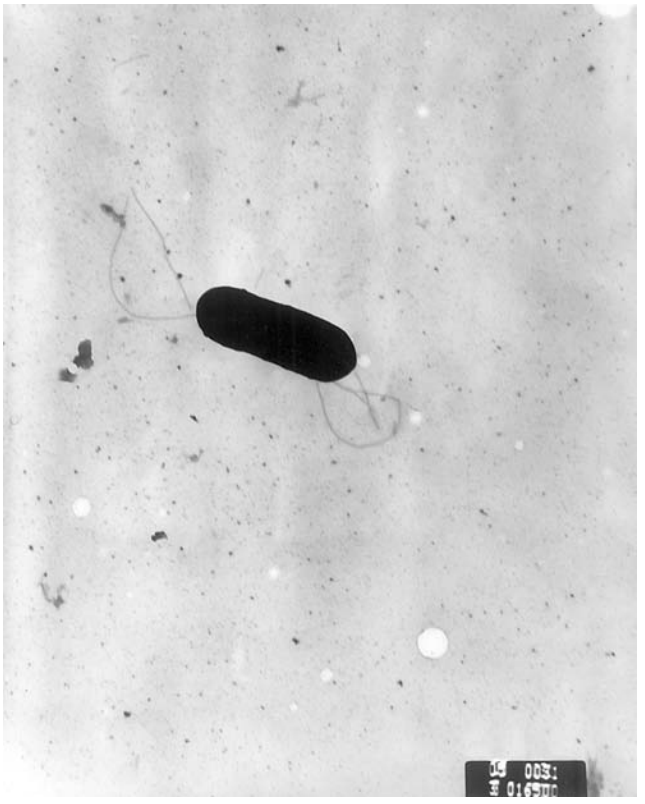
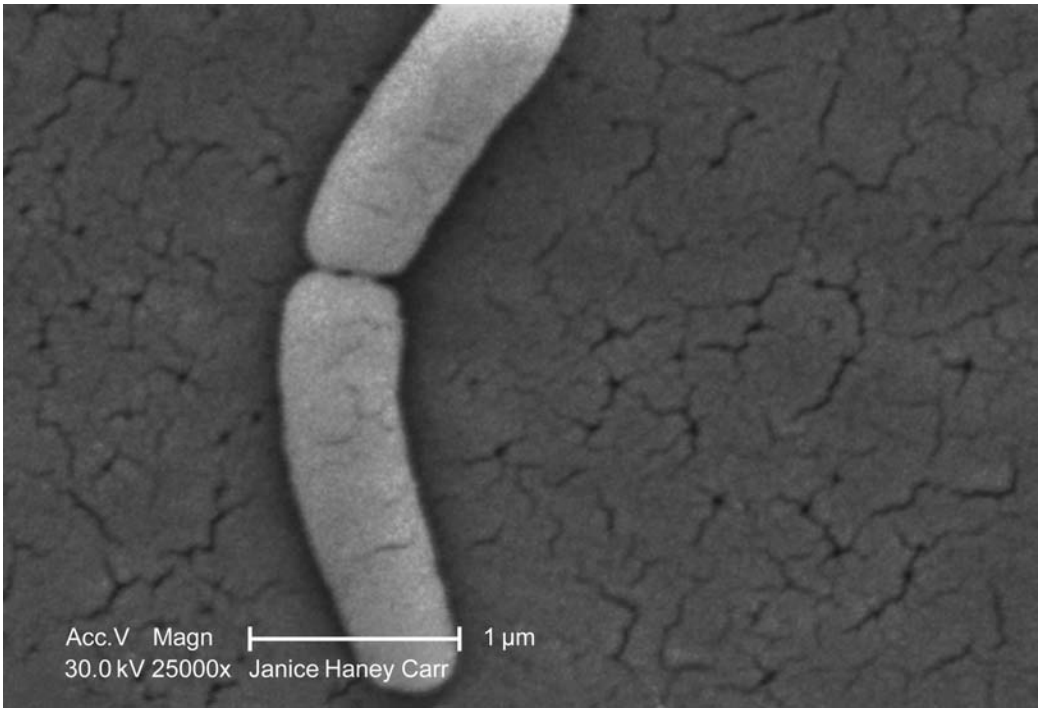


Figure 7-9 Scanning electron micrograph of *Salmonella typhimurium* undergoing cell division (25,000×). (Courtesy of CDC/Bette Jensen; Photographer: Janice Haney Carr.)



Salmonella spp. are the causative agents of salmonellosis and were the confirmed etiology in 135 food-borne outbreaks in the United States in 2007 (including 28 *S. enteritidis*, 20 *S. typhimurium*, and 17 *S. newport*).[3] There were a reported 6655 cases of *Salmonella* spp. reported in the FoodNet areas in 2006. Ninety percent were serotyped, with *S. typhimurium*, *S. enteritidis*, and *S. newport* accounting for 47% of those subtypes (with 1157, 1109, and 531 cases, respectively).[4] In total, ~40,000 cases of salmonellosis are reported each year in the United States, with 400 deaths. Since many cases are mild and therefore not diagnosed and reported, it is estimated that the number of actual illnesses exceeds 1.2 million.[16] It should be noted that the hospitalization rate for reported cases of *Salmonella* Typhi is more than three times greater than that for nontyphoidal strains (0.750 versus 0.221).[2] Illness usually occurs within 12 to 72 hours of exposure and symptoms presented by most people include diarrhea, abdominal pain, and fever. The disease usually lasts 4 to 7 days and most people recover without specific treatment, though antibiotics may be effective if the infection spreads into the bloodstream. A small number of infected individuals develop Reiter syndrome. This is characterized by pain in the joints and eyes and painful urination. Symptoms may persist for months or years, and potentially lead to chronic arthritis.[16]

Salmonella is a Gram-negative, motile rod (with some nonmotile exceptions) with widespread occurrence in swine, poultry, water, and soil (Figure 7-9). It is estimated that only 15 to 20 organisms are necessary to induce disease, and it is a frequent contaminant of poultry products and reptiles. The largest food-borne outbreak of salmonellosis occurred in 1985 with > 16,000 confirmed cases in 6 states associated with milk from a Chicago dairy.[11] Further details of *Salmonella* are discussed in Chapter 5 and will not be repeated here.

Sidebar 7-3 *Salmonella* Contamination of Raw Produce[17]

The largest food-borne disease outbreak in the United States in the past 10 years was caused by the contamination of raw produce with *Salmonella*. Recognition of the outbreak began on May 22, 2008, when the New Mexico Department of Health informed the CDC of four individuals infected with PFGE-indistinguishable strains of *Salmonella* Saintpaul and an additional 15 people whose strains had not yet been characterized. Saintpaul strain is relatively rare in the United States, causing only 1.6% of all laboratory-confirmed *Salmonella* infections. As of August

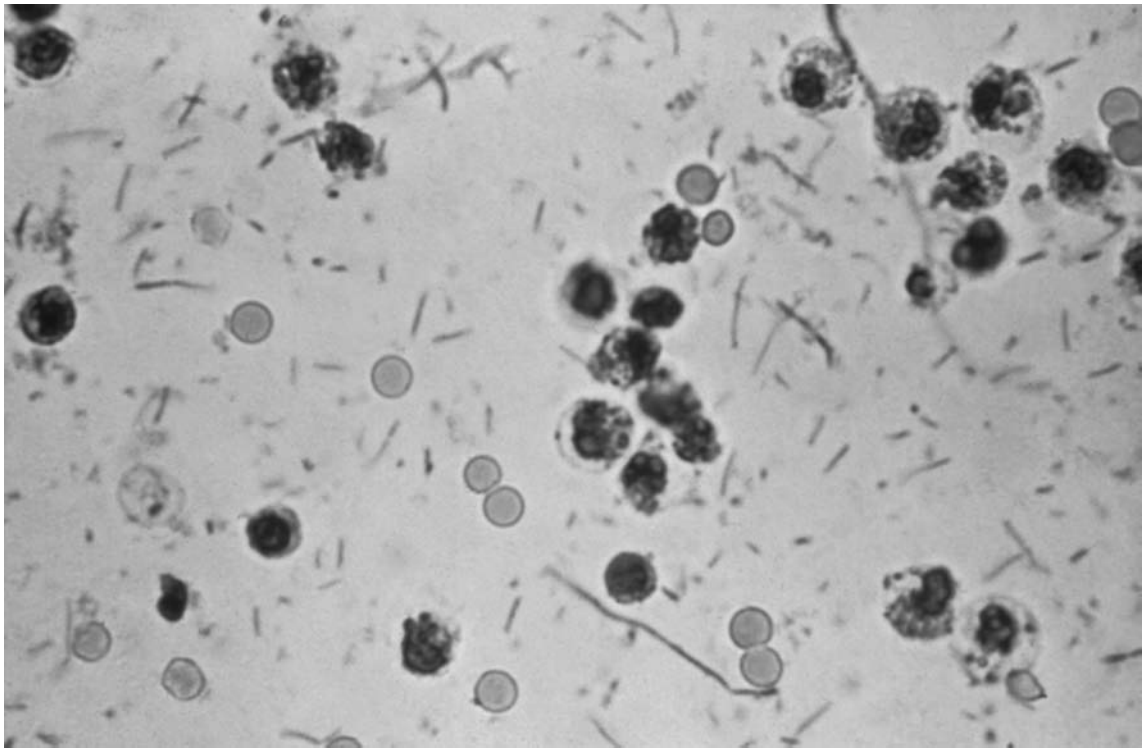
2008, there had been 1442 confirmed cases whose *Salmonella* serotype matches the case definition PFGE pattern *Xba*I JN6X01.0048. These cases were distributed through 43 states and the District of Columbia.

Epidemiologic investigations found an association between illness and eating in Mexican-style restaurants. Food types consumed within these restaurants and found to be associated with illness included raw tomatoes, salsa (containing tomatoes and jalapeno peppers), pico de gallo, and other items. Over the course of investigating several outbreaks, the list of potential sources was narrowed down to raw tomatoes and jalapeno and serrano peppers. The FDA performed a traceback for the source of suspect tomatoes, but did not identify a single distributor, packer, or grower. Laboratory cultures of tomatoes randomly obtained from multiple distributors were negative for *Salmonella*. A similar traceback for jalapeno and serrano peppers identified two farms in Mexico. Laboratory analysis of jalapeno peppers from one farm and irrigation water from the other were positive for *Salmonella* Saintpaul. This case highlights the importance of robust laboratory analysis to both accurately identify strains associated with the outbreak and pinpoint the source of contamination.

Shigella spp. are the causative agent for shigellosis and were the confirmed etiology in 10 food-borne outbreaks in the United States in 2007 (9 were *S. sonnei* and the other was unknown species).[3] Annually, there are ~14,000 cases reported in the United States (300,000 estimated), and children aged 2 to 4 are the most likely to get shigellosis.[11,18] *Shigella* is present in the feces of those infected and fecal contamination is the primary method of exposure (e.g., unwashed hands of contaminated sewage on fruits and vegetables). Illness usually occurs within 1 to 2 days of exposure and symptoms may include diarrhea (often bloody), abdominal pain, and fever. The disease usually lasts 1 week and most people recover without specific treatment. Antibiotics such as ampicillin and ceftriaxone may be prescribed to shorten the disease course, though some bacterium have become resistant.

Shigella spp. are Gram-negative, nonmotile, non–spore-forming rods and there are four species (*S. dysenteriae* [subgroup A], *S. flexneri* [subgroup B], *S. boydii* [subgroup C], and *S. sonnei* [subgroup D]; example of *S. boydii* shown in Figure 7-10). It is estimated that only 10 organisms are necessary to induce disease, but rarely occurs in animals.[11] Further details of *Shigella* are discussed in Chapter 5 and will not be repeated here.

Figure 7-10 Photomicrograph of a stool sample containing *Shigella*. (Courtesy of CDC.)



There are, of course, many other bacteria that are able to infect humans via the consumption of contaminated food. Another important enterotoxin-producing food-borne organism frequently tested for along with *C. perfringens* is *Bacillus cereus*. Other bacteria include *Yersinia enterocolitica* and food-borne infections with *Staphylococcus* and *Streptococcus*. Other pathogens with high rates of toxicity, as measured by high hospitalization rates for known cases, include *Vibrio vulnificus* at 91% of reported cases and *Brucella* spp. at 55%. Fortunately, these last two are relatively infrequent causes of illness and are often transmitted by means other than food. For all food-borne illnesses with an identified etiology, bacteria are the causative agent 30.2% of the time, are responsible for 59.9% of food-borne illness-related hospitalizations, and cause 71.7% of food-borne illness-related deaths.[2]

Protozoan Pathogens

Cryptosporidium spp. were the confirmed etiology in three food-borne outbreaks in the United States in 2007.[3] Human surveys of infection show that ~2% of those in the United States are infected with *Cryptosporidium* and ~80% show serologic evidence of past infection. Oocysts are shed in feces and the fertilization of salad vegetables with manure is a possible source of human infection (Figure 7-11).[11] Illness usually occurs within 1 week of exposure and symptoms may include watery diarrhea, dehydration, abdominal pain, nausea, and weight loss. The disease usually lasts 1 to 2 weeks and resolves without treatment.[19] Other properties of *Cryptosporidium* are discussed more completely in Chapter 5 and will not be repeated here.

Sidebar 7-4 Food-borne Outbreak of Cryptosporidiosis[20]

An investigation into a possible food-borne outbreak began on December 29, 1997, after many members of a group attending a banquet on December 18, 1997, became ill with acute gastroenteritis. Of 62 people attending the banquet, 87% (54) met the case definition, with 98% of those experiencing diarrhea. The median incubation period was 6 days and median length of illness 5 days (though two later reported intermittent symptoms for 4+ weeks). Eight of 10 stool samples tested positive of *Cryptosporidium* with both modified acid-fast and auramine-rhodamine staining. While no single food item was associated with illness, there was a small association between the consumption of any food containing unwashed green onions and illness. The food preparers were tested, and stool samples from 2 of the 15 workers were positive for *Cryptosporidium*. This case highlights some of the distinctive characteristics of *Cryptosporidium* illness (prolonged diarrhea, longer incubation times, and high attack rates) that would lead a physician to request a test for this microorganism. The high attack rate also hinders identification of the specific manner of contamination/transmission, and this was never completely determined for this outbreak.

Cyclospora

Cyclospora cayetanensis is a single-celled parasite infecting the intestinal tract of humans. Oocysts are shed in feces and these may then contaminate water used to irrigate fruits and vegetables (Figure 7-12). Illness usually occurs within 1 week of exposure and symptoms may include watery diarrhea and frequent stools, flatus, abdominal pain, nausea, loss of appetite, and weight loss. The disease may last anywhere from a few days to more than a month. There are effective treatments, including trimethoprim-sulfamethoxazole (TMP-SMX).[21] Other

Figure 7-11 **Wet mount of *Cryptosporidium parvum* oocysts observed by differential interference contrast microscopy. (Courtesy of CDC/Division of Parasitic Diseases [DPDx].)**

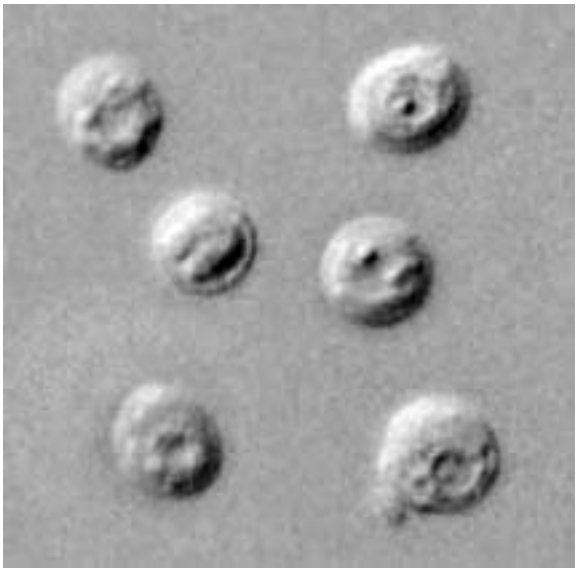
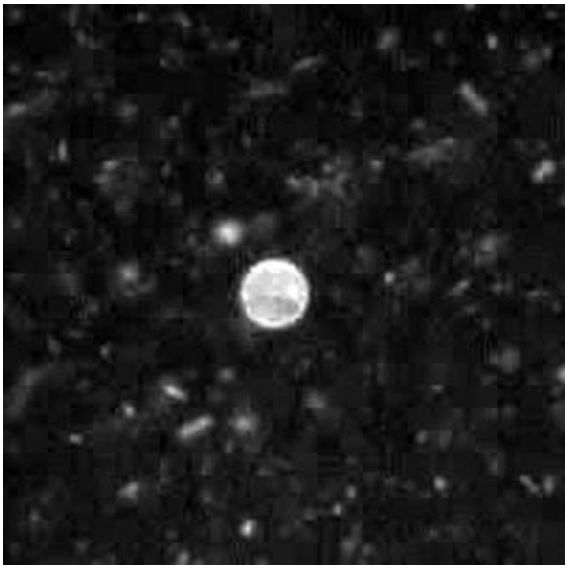


Figure 7-12 ***Cyclospora cayetanensis*** **oocyst autofluorescing under ultraviolet microscopy. (Courtesy of CDC/DPDx.)**



properties of *Cyclospora* are discussed more completely in Chapter 5 and will not be repeated here.

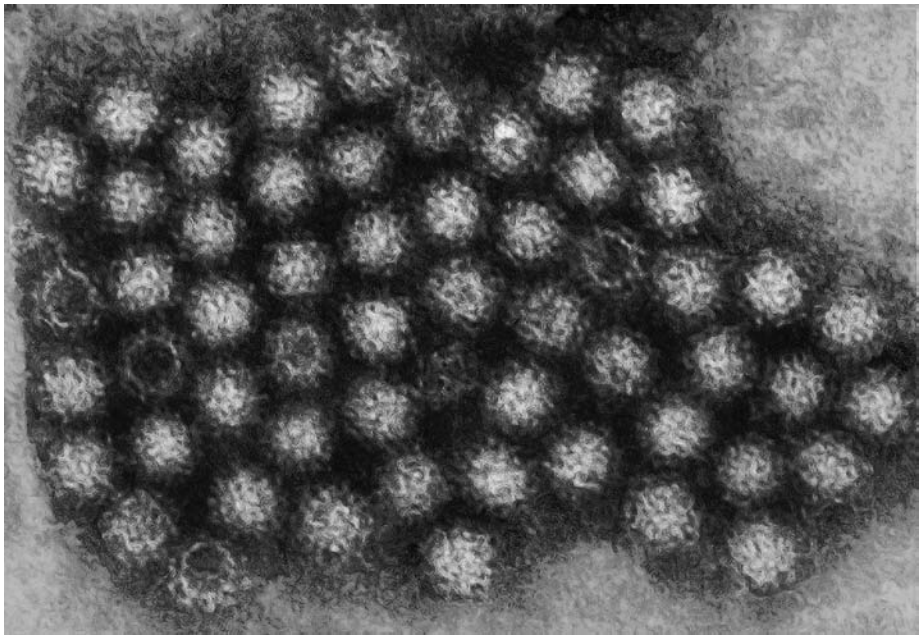
There are other protozoa that are able to infect humans via the consumption of contaminated food. *Giardia lamblia* was discussed in Chapter 6. While it is a major cause of parasitic protozoal illness, it is infrequently transmitted in food. *Toxoplasma gondii* and *Trichinella spiralis* are also potential agents. For all food-borne illnesses with an identified etiology, parasitic protozoa are the causative agent only 2.6% of the time, are responsible for 5.3% of food-borne illness-related hospitalizations, and cause 21.1% of food-borne illness-related deaths.[2]

Viral Pathogens

Norovirus is the official genus name for the group temporarily termed Norwalk-like virus and was the confirmed etiology in 199 food-borne outbreaks in the United States in 2007.[3] The CDC estimates that 50% of food-borne outbreaks of gastroenteritis can be attributed to this group, causing 23 million cases.[22] Norovirus is present in the feces of those infected and fecal contamination is the primary method of exposure (e.g., unwashed hands or contaminated sewage on fruits and vegetables). Illness usually occurs within 1 to 2 days of exposure and symptoms usually include water diarrhea, abdominal pain, nausea, and vomiting. The disease usually lasts 2 to 5 days and dehydration is the most common complication.

There are five main groups of Norovirus (GI to GV), further divided into at least 31 genetic clusters. An example is shown in Figure 7-13. They are also highly contagious with as few as 10 viral particles thought required to induce infection.[11] Diagnosis of illness is based on reverse transcriptase polymerization chain reaction (RT-PCR) analysis

Figure 7-13 Transmission electron micrograph of Norovirus virions. (Courtesy of CDC/Charles D. Humphrey.)



of stool samples. The ease of dissemination and high infection rate were exemplified in a Michigan restaurant in 2006. Several of the cooking staff reported to work while ill (one vomited on the premises) and at least 364 patrons subsequently became ill during the time period of January 19 to February 3. All 13 stool samples obtained from patrons and employees tested positive for norovirus by RT-PCR and were identified as genotype GI/4 Chiba by sequencing.[23] Other properties of norovirus were discussed more completely in Chapter 5 and will not be repeated here.

There are other viruses that are able to infect humans via the consumption of contaminated food. These include rotavirus, astrovirus, and hepatitis A, though they are all infrequently transmitted in food. In total, noroviruses alone are the causative agents of 66.6% of reported food-borne illnesses with a known etiology, with the other viruses causing 0.6% combined. Combined, they are the cause of 34.8% of food-borne illness-related hospitalizations and 7.1% of food-borne illness-related deaths.[2]

Chemical Contaminants

These will not be discussed in depth as they are relatively infrequent and each event may differ by contaminant. That is, there are no trends or reliable method of predicting, based on prior data, what compounds may contaminate food, when, and where. Each event is essentially unique and must be dealt with on a case-by-case basis. One such case occurred in Illinois in November 2003 when number of students and staff at two elementary schools in became ill after eating lunch. Symptoms included headache and gastrointestinal tract irritation. The state health department conducted an investigation involving cohort epidemiology, environmental, and laboratory components. There were 312 individuals interviewed and 157 were ill. Furthermore, 91% of students reported that their chicken tenders smelled unusual and eating tenders with unusual smell was strongly associated with becoming ill. Laboratory analysis showed the presence of ammonia in uncooked tenders with levels as high as 2468 ppm (parts per million). Ammonia is a commonly used warehouse refrigerant and the chicken became contaminated as the result of a leak. This is the first reported instance of ammonia poisoning solid food.[24]

Food Sample Collection

The collection of food samples for analysis is at once as easy as, or more complex than, the collection of clinical and water samples described in the previous chapters. They are as easy in the sense of how clinical samples are packaged and shipped. We read in Chapter 5 that, in general, clinical samples are collected with a swab, cup, or vacutainer and packaged to protect the sample during shipment. The volume may be unspecified or general, such as 20+ ml urine, and they are usually cooled to ~4°C if they cannot be analyzed immediately. Food samples are much the same in that the general requirements are nearly identical. General recommendations are that they be placed into a container that will protect them during shipment and handling. These are often clean plastic cups, jars, or even resealable plastic bags. They are then refrigerated (but not frozen) to < 4.4°C to preserve the sample and reduce microbial growth. Foods that are frozen when collected should be kept frozen with dry ice until analysis. Finally, they should be analyzed within 36 hours of collection.[25] These general requirements are in sharp contrast to the very specific requirements associated with water samples. Those are often collected to meet state and/or federal regulations and must comply with the specific analysis method by which they will be analyzed. These methods are usually quite specific about sample container type, color, and size as well as shipping and time constraints.

Another significant difference between the collection of food samples as compared to others is the manner in which samples are chosen. With most other sample types, it is assumed that any potential agents targeted for analysis are either homogeneously distributed within the sample or are most likely concentrated at the site of sample collection. Water drawn from a home tap and venous blood are examples of samples where the expectation is that they are representative of the entire site. That is, water drawn from a different tap or blood drawn from a different vein is expected to yield the same analytical results. The analyst may therefore offer quantitative results based on the extrapolation of the amount of agent in the sample to the whole. Other samples, such as swabs and wipes, are taken from areas where the potential agent is most likely to be found. The purpose is simply detection of the agent, and quantification of the whole from which the sample was obtained is not a goal.

Food samples present a challenge in that the distribution of a pathogen is not expected to be homogeneously distributed but a quantitative result may still be needed. For many samples taken as part of an outbreak investigation, this may not be problem when the main difficulty may be finding any remaining food at all. The investigator is left with collecting the half-sandwich or leftover casserole and sending it all to the laboratory for

analysis. The analysis of larger samples (e.g., the 100 pounds of recalled beef or 200-gallon vat of peanut butter) is much more difficult to handle and sampling plans must be devised. The premise of a sampling plan is that the investigator determines where in the entire sample to collect a subsample and how many to collect. The number of subsamples is determined (in part) by the degree of confidence that the investigator wants. For example, a single 25-g subsample from a 10-kg sample may contain a detectable level of the pathogen $< 5\%$ of the time. There is therefore low confidence in a negative result. However, if six subsamples are taken, the probability of detecting the pathogen may increase to $> 50\%$. There are other factors involved in sample plan determination as well and these determine the number of food samples a laboratory may receive. Sampling plans associated with food processing also have a time component, such as sample grabs every 2 hours of operation. A detailed description of sampling plans and methods is beyond the scope of this book, but may be found in Chapter 2 of the *Compendium of Methods for the Microbial Examination of Foods*, 4th edition.

Food Analysis Algorithms

Food Implicated by Epidemiologic Study

There are a number of reasons why food products may be considered a source of actual or potential exposure to a pathogen. Stool sample(s) may be positive for a foodborne organism or the testing of food preparation supplies may indicate contamination. In the event, there are times when food samples are collected. In PHLs, these are most commonly from leftovers associated with an epidemiologic investigation. The investigation will help in determining which particular food should be examined. Often there are few if any leftovers from an event or single meal and investigators must make due with whatever is available. When possible, multiple samples are taken and tests performed on implicated food to increase chances of finding organisms of interest. This takes into account the likely uneven distribution of the pathogen in the food and also the fragile/stressed condition of the organisms.

Testing laboratories often prepare a protocol or standard operating procedure (SOP) for the initial testing of food samples, which will hopefully lead to either a negative result (no pathogens found) or a preliminary identification. This is somewhat similar to the testing algorithm described in Chapter 6. For the IDPH laboratory, an informal SOP for food analysis is based on a combination of methods from the *Bacteriological Analytical Manual* (BAM), FERN, and the LRN. It makes use of rapid and/or automated instruments to quickly screen samples for preliminary positives. The following instrumentation and test kits may be used.

- Neogen enzyme-linked immunosorbent assay (ELISA): Reveal ELISA for *E. Coli* O157, *L. Monocytogenes*, and *Salmonella*
- Biocontrol ELISA: Listeria VIP
- Pathatrix Immuno Magnetic capture utilizes magnetic beads coated with organism-specific antibodies. After an initial incubation period, the sample enrichment broth is recirculated with the beads. The beads bind to the specific organism targeted by their attached antibodies (if present) and the complex is magnetically retained while the rest of the sample is rinsed away. The retained sample may then be subjected to further processing and PCR, or allowed to grow into colonies for identification by specific media.
- TECRA Unique: This is an automated ELISA for the rapid identification of *Salmonella* spp. The sample is incubated with enrichment broth, then an aliquot placed in the instrument with the preprepared reagents and broth. While the instrument can only analyze for one organism at a time (e.g., *Salmonella*), it offers the advantage of a continuous digital readout of results versus a simple positive/negative. In this way, the analyst can see whether there is an indication of organism presence that is not sufficient to trigger an outright positive response. Samples that indicate organism present, but are not themselves method-positive, may be subjected to further testing.
- PCR may be performed for any number of specified organisms. This is not usually used for screening as the primers used must be for a specific organism. However, if there are reasons to suggest a specific organism, such as patient symptoms, likely contamination source, or associating with a known outbreak, the method provides much more swift results. Whereas most automated and rapid methods still require 12 to 24+ hours to allow for enrichment and analysis, PCR utilizing existing DNA may be completed in as little as 3+ hours under optimal conditions. Unfortunately, food samples, even those that have been extracted and prepared, often

contain substances that inhibit the polymerization process such that PCR is not always possible.

From these tests, the analyst can make one of two conclusions (assuming all controls were maintained). If the sample tests negative for any pathogen then the testing may be completed and the negative result returned. Alternatively and depending on circumstances, further attempts may be made based on likelihood of pathogen presence (though not initially detected) and sample availability.

Positive results, on the other hand, cause an additional series of tests to be performed. An aliquot from the enrichment is streaked onto selective agar for isolation of typical colonies (e.g., Hektoen for *Salmonella*). If there is good isolation from the streak, colonies will be plucked and subjected to identification confirmation. There are several instrument platforms that may be used. The following tests require pure colony isolates.

- VITEK 2 Compact 30: This is a fairly complex instrument and the analysis whereby isolated colonies are picked and brought to a standard cell density and then are automatically inoculated by the instrument into specific cards (Gram-negative, Gram-positive, yeast) that contain multiple tests that are run concurrently. The tests are a series of fluorescent immunoassays and biochemical reactions and results are read by the instrument every 15 minutes. In this manner, the organism may be identified and speciated. The API 20E is similar in some ways, but the tests must be manually performed. On the other hand, it offers a few species-tests not available with the VITEK.
- MicroGen: This utilizes a strip of prepared media that is manually inoculated and incubated for the identification of *L. monocytogenes* strains using vender-supplied software.

In addition, growth from purity plate may be further characterized by PFGE. These patterns are then compared with those obtained from other patients or other isolates from similar food types. Matches will results in wider testing and potential recalls based on the nature of the exposure source. Occasionally, the streak will refuse to yield good colonies (e.g., overgrowth). Various measures such as reinoculation will be attempted to obtain zcolony isolation, and analysis with a Pathatrix may be attempted. Ultimately, there may be failure for isolation and identification, in which case the positive result is not confirmed and the sample is assumed to contain artifacts that interfere with testing, resulting in spurious results.

Routine Surveillance

Many types of food are routinely tested during their production and shipment. This may be done at the instigation of the producer or a requirement of a regulating agency or rule. The PMO is an example that requires the testing of milk from producers on a routine basis. The actual sampling is usually conducted as part of routine inspection, and each sample may be represented by multiple subsamples depending on the level of confidence limits (usually 95%) prescribed. That is, it is assumed that a particular pathogen will not be homogeneously distributed within a food batch, and that taking a single scoop out of a 2500-pound grain bin might easily miss a contaminant. At the laboratory, analyses are either done singly to each sample or subsamples or they may be pooled for economy. In general, 25 or 375 g of sample are weighed out for testing. Unlike testing done to support epidemiologic investigations, obtaining a suitable amount of sample is not a problem.

The screening algorithm and isolation characterization and identification are essentially the same as described previously. A minor difference is that a negative test is usually not pursued any further and accepted at face value. It may be of interest to the reader to note that, unlike almost any other type of analysis described in this text, the context of sample submission bears a great deal on how the results are received by the submitter. Consider a gallon of milk that is found to contain *Salmonella*. If submitted as part of an outbreak investigation, the epidemiologist may be thrilled to have found the source of exposure that explains the illnesses. If that same gallon of milk was submitted as part of routine surveillance, the milk producer would not receive this information as good news as it might have a negative impact on their business.

Culturing and Other Analysis Methods

In the algorithms just discussed, the reader may have noticed a couple glaring omissions. One is that there is no provision for parasitic contamination. The tests utilized are not amenable for these organisms and tests for their presence must be expressly requested by the submitter based on diagnostic clues from the patient(s) or prior laboratory analyses. In that event, they must be tested for specifically and some of the available methods are mentioned here. Also, viruses are not mentioned because there are no reliable methods for their analysis in food. Finally, chemical contaminants have not been described or their methods discussed. Again, these are quite rare

in occurrence and methods of analysis tend to be quite specific to certain compounds/compound classes and food types. Some methods for the analysis of chemical compounds in food may be found in the FDA's *Chemistry Laboratory Guidebook* (e.g., R33 Screen for Halogenated Environmental Contaminants) and *Pesticide Analytical Manual* (PAM).

Bacterial Pathogens

The *Bacteriological Analytical Manual* Chapter 7 describes a method for the isolation, culturing, and identification of *Campylobacter* from food and water samples. Samples (25–50 g of food or 2–4 liters of strained water) are mixed with antibiotic-enhanced Bolton broth. This is preincubated for 4 to 5 hours and fully incubated for another 20 to 44 hours at 42°C and under low oxygen conditions (microaerobic). Streaks are made at 24 and 48 hours of incubation onto isolation agar. Colonies of *Campylobacter* are then observed (shown in Figure 7-14).[26] Another method is the *Microbiology Laboratory Guidebook* Chapter 6.

The *Bacteriological Analytical Manual* Chapter 16 describes a method for the isolation, culturing, and identification of *C. perfringens* from food samples. Samples (25-g food) are mixed with peptone dilution solution and homogenized in a blender. Dilutions are made and added to TSC agar plates (options with or without egg yolk). Additional TSC agar is added and mixed with the inoculums, allowed to solidify, and incubated at 35°C for 20 to 24 hours. An example of *C. perfringens* in egg yolk agar is seen in Figure 7-15. Suspect colonies are inoculated into liquid thioglycollate broth for further incubation and microscopic observation using Gram stain. This may be followed by the iron-milk media presumptive test, confirmatory tests using other selective media (e.g., motility nitrate [buffered] and lactose gelatin), and further tests for sporulation and enterotoxin production.[26] Another method is the *Microbiology Laboratory Guidebook* Chapter 13.

The *Bacteriological Analytical Manual* Chapter 4a describes methods for the isolation and identification of pathogenic *E. coli* from food samples. Samples are diluted 1:10 with BHI broth, mixed, and allowed to settle. The media is decanted and incubated for 3 hours to resuscitate cells, then mixed with TP broth and incubated at high temperature for 20 hours, followed by streaking on L-EMB and MacConkey agars (shown in Figure 7-16).

Figure 7-14 Examples of *Campylobacter fetus* (ss. *jejuni*) grown on Skirrow's and Butzler's medium. (Courtesy of CDC/Sheila Mitchell.)

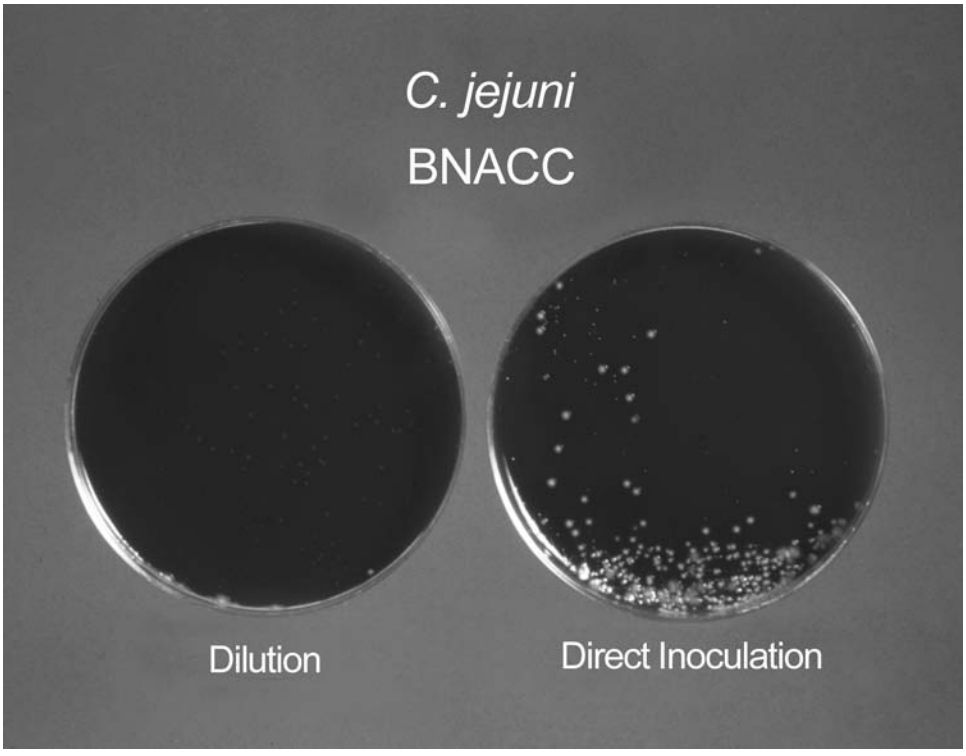


Figure 7-15 ***Clostridium perfringens*** **colonies observed on egg yolk agar. (Courtesy of CDC.)**

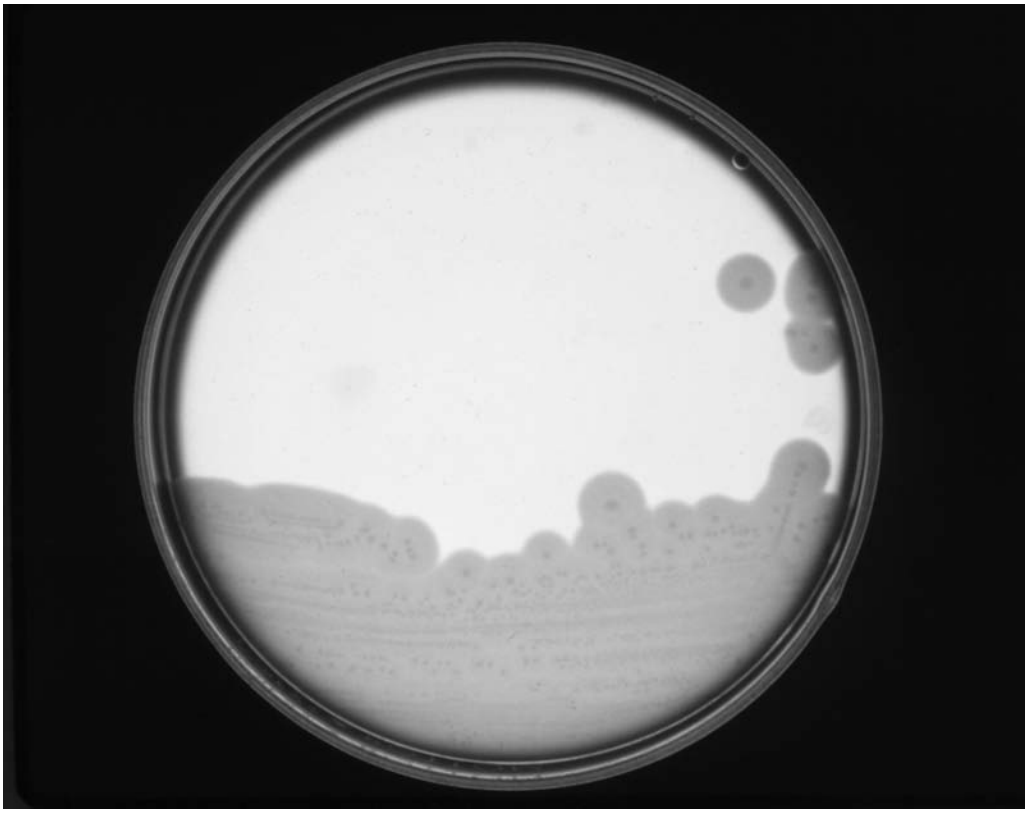
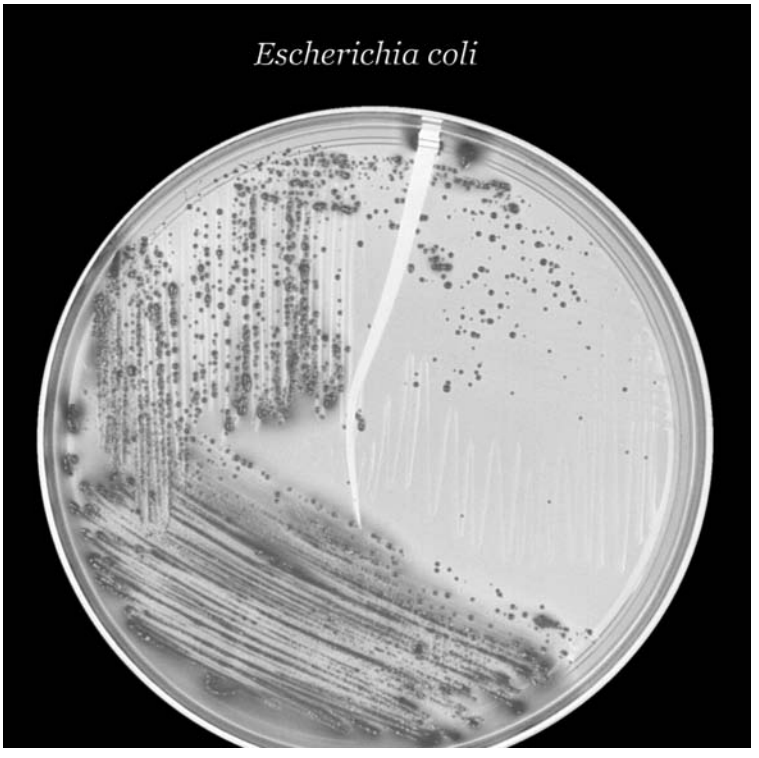


Figure 7-16 ***Escherichia coli*** **colonies observed on MacConkey agar. (Courtesy of CDC.)**



Suspicious colonies are selected for further biochemical screening and identification.[26] Some of these specific tests were described in Chapter 5. Other methods in the *Microbiology Laboratory Guidebook* are Chapters 5A.01 and 5.04.

The *Bacteriological Analytical Manual* Chapters 10 and 11 describe methods for the isolation, culturing, and identification of *L. monocytogenes* from food. Food samples (25-g food) are incubated for 4 hours in buffered *Listeria* enrichment broth (BLEB). Selective agents (e.g., acriflavine) are then added and enrichment incubation continues for an additional 44 hours. Differential-selective agars (e.g., Oxford, PALCAM) are streaked at 24 and 48 hours with the enrichment cultures to isolate *Listeria* species. The CAMP test (Christie–Atkins–Munch–Peterson) can be done when blood agar test results are equivocal. Parallel streaks of β-hemolytic *S. aureus* and *R. equi* are made on a sheep's blood agar plate, streaks of test cultures made between them, and the plates incubated for 48 hours. *L. monocytogenes* (and *L. seeligeri*) show enhanced hemolytic reactions in the areas influenced by the *S. aureus.* Chapter 11 discusses how to serotype *L. monocytogenes* based on its flagella (H) and somatic (O) types using agglutination methods.[26] Other methods in the *Microbiology Laboratory Guidebook* are Chapters 8.06 and 8A.03.

The *Bacteriological Analytical Manual* Chapter 5 describes methods for the isolation, culturing, and identification of *Salmonella* spp. from food. Food samples (25-g food) are prepared, mixed with broth (e.g., sterile lactose), and incubated for 24 hours. Depending on the food type and expected microbial load, portions of the incubated sample are inoculated into selenite cystine (SC) broth, Rappaport–Vassiliadis (RV) medium, and/or tetrathionate (TT) broth and incubated for another 24 hours. Inoculates are then streaked onto selective media such as XLD or Hektoen enteric agar and incubated another 24 hours. Plates are examined for the presence of *Salmonella* isolates (e.g., XLD; seen below in Figure 7-17). Colonies may be selected and subject to somewhat extensive identification procedures.[26] Some of these specific tests were described in Chapter 5. Figure 7-18 shows a micrograph of *Salmonella* from the tetrathionate enrichment broth and treated with fluorescent antibodies. Other methods in the *Microbiology Laboratory Guidebook* are Chapters 4.04 and 4C.04.

The *Bacteriological Analytical Manual* Chapter 6 describes methods for the isolation, culturing, and

Figure 7-17 ***Salmonella*** **spp. colonies observed on a XLD agar. (Courtesy of CDC.)**

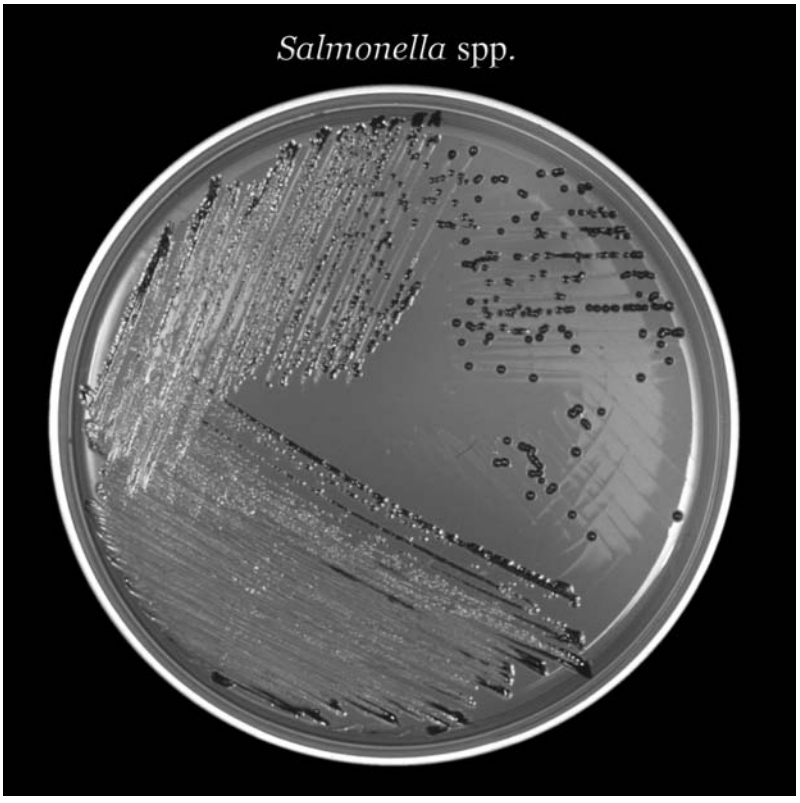
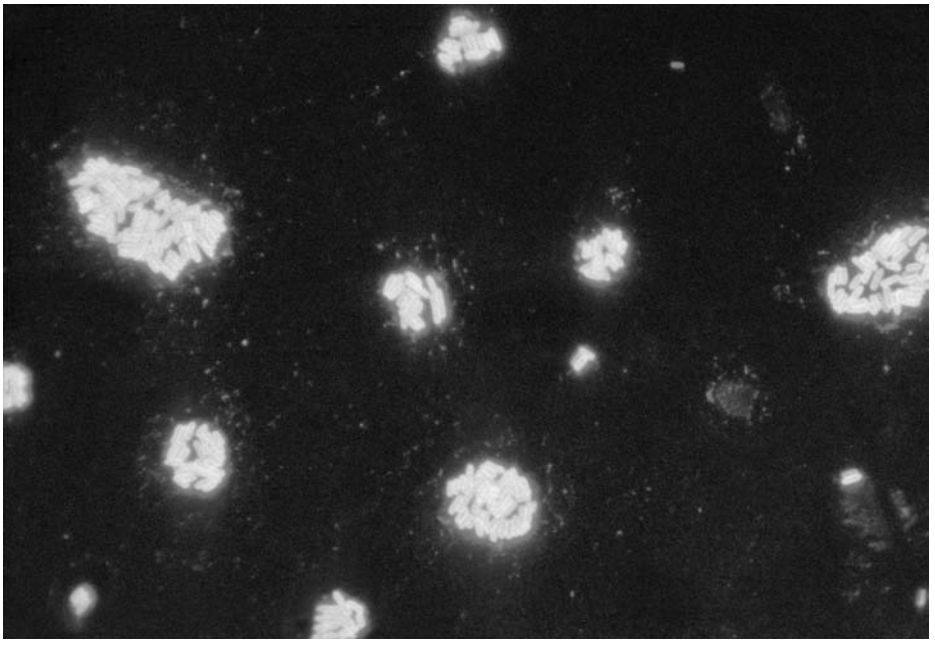


Figure 7-18 Microscopic observation of *Salmonella* spp. enriched and stained with fluorescent antibodies. (Courtesy of CDC/B. Thomason.)



identification of *Shigella* spp. from food. Food samples (25-g food) are mixed with 225-ml *Shigella* broth (to which novobiocin has been added) and allowed to settle for 10 minutes. The supernatant is poured into an Erlenmeyer flask and incubated anaerobically at 42°C for 20 hours (44°C for *S. sonnei*) before streaking onto MacConkey agar. Potential *Shigella* colonies then selected and inoculated into further selective media (e.g., glucose broth, TSI agar slant, or others; Figures 7-19 and 7-20). Alternatively, the sample may be mixed with trypticase soy broth with yeast extract, the supernatant retained and incubated 20 to 24 hours at 35° to 37°C, and used for DNA hybridization.[26] Some of these specific tests were described in Chapter 5.

Figure 7-19 Colonies of *Shigella boydii* observed on Hektoen enteric agar. (Courtesy of CDC.)

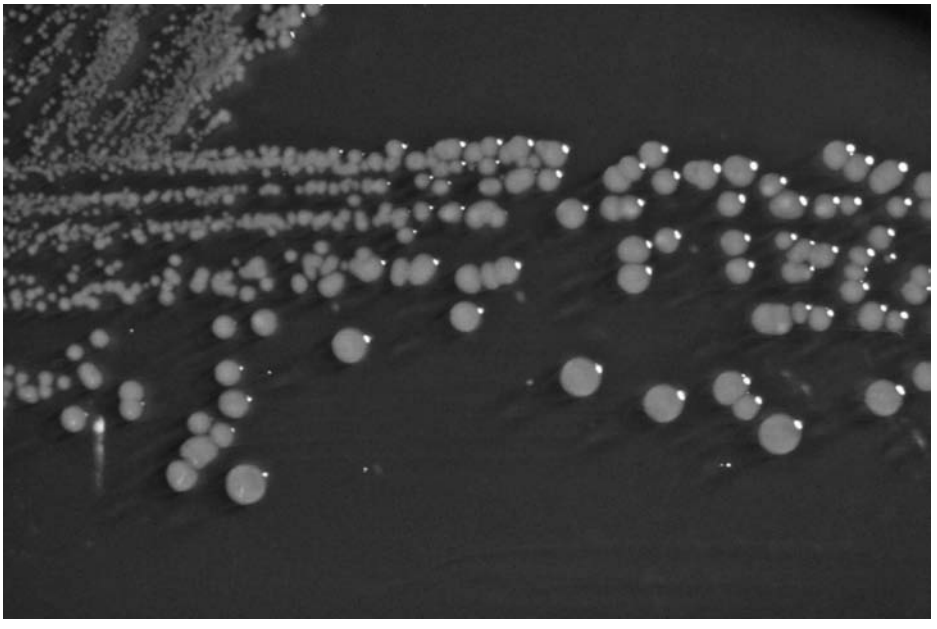
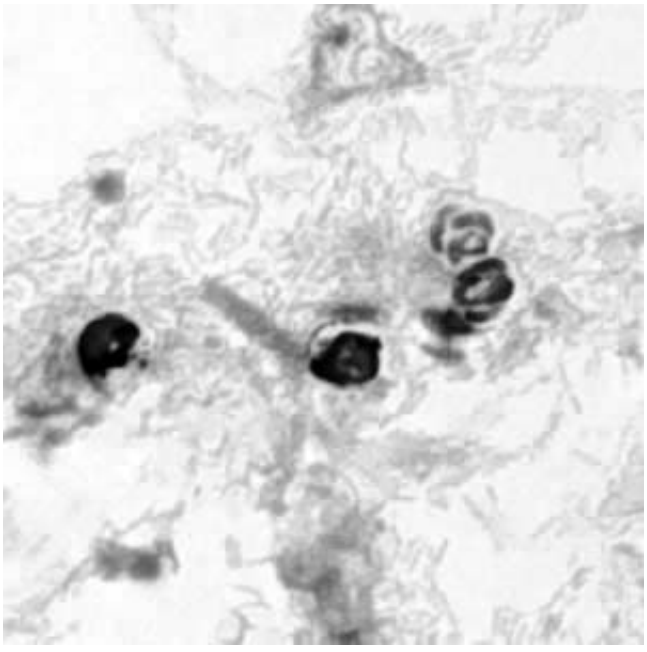


Figure 7-20 Colonies of *Shigella boydii* observed on blood agar. (Courtesy of CDC.)

Protozoan Pathogens

The *Bacteriological Analytical Manual* Chapter 19a describes methods for the isolation and identification of *Cyclospora* and *Cryptosporidium* from food and water samples. Samples are treated and filtered to isolate the parasite. The method also employs immunomagnetic bead separation and immunofluorescent antibody staining. Duplicate slides are made to observe *Cyclospora's* characteristic blue autofluorescence. Another aliquot of the prepared samples may be analyzed using PCR.[26] Figures 7-21 through 7-24 show the identification of *C. parvum*, *Cyclospora*, *Cryptosporidium*, and *Giardia* by isolation and staining. Figures 7-25 and 7-26 show the identification of *C. parvum* and *Cyclospora* by PCR.

Figure 7-21 *Cryptosporidium parvum* oocysts observed after acid-fast staining with sporozoites visible. (Courtesy of CDC/DPDx.)



Viral Pathogens

There are no general and reliable methods for the analysis of norovirus in food. There are some RT-PCR methods used for some seafood/shellfish, but these must be validated for each food type and are infrequently performed.

The analysis of food samples for human pathogens is both critically important and terribly complex. There are often no easy answers when it comes to some analyses (such

Figure 7-22 Four *Cyclospora cayetanensis* oocysts seen in a stool sample after acid-fast staining (note variable staining). (Courtesy of CDC/DPDx; Melanie Moser.)

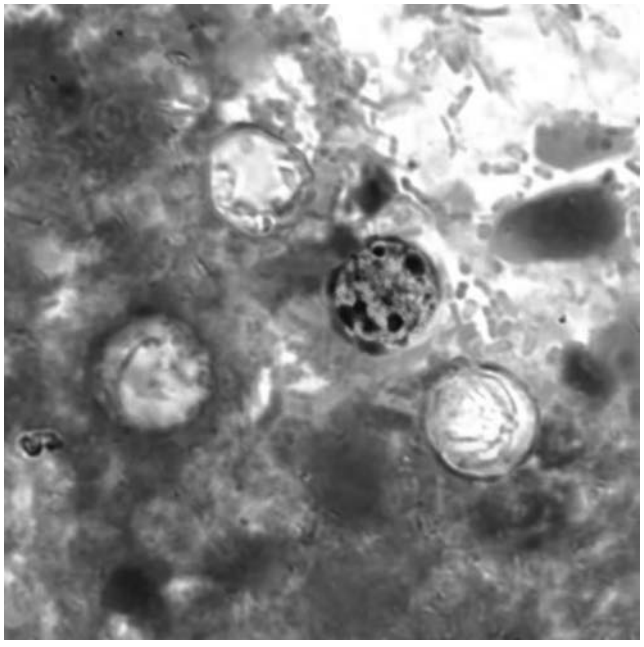


Figure 7-23 Oocysts of *Cryptosporidium* (upper left; *Giardia intestinalis*, lower right) visible after immunofluorescent antibody staining. (Courtesy of CDC/DPDx.)

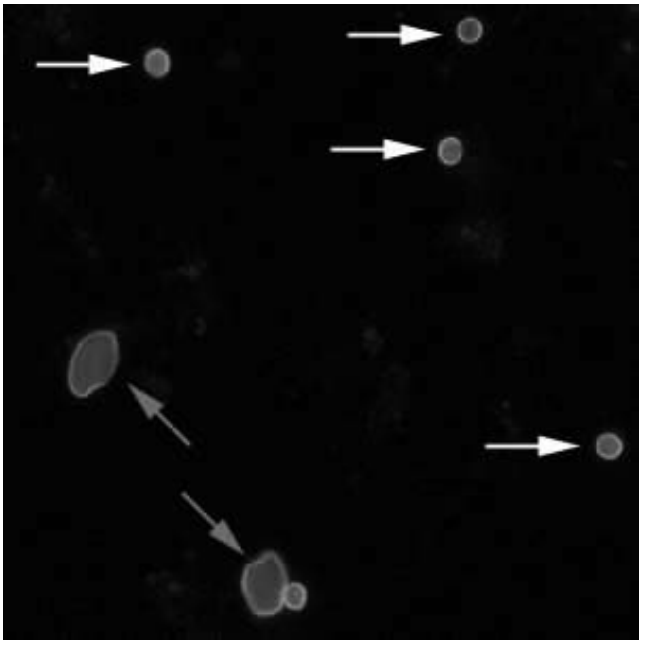


Figure 7-24 Three *Cyclospora cayetanensis* oocysts seen in a stool sample after safranin staining (note uniform staining). (Courtesy of CDC/DPDx; Melanie Moser.)

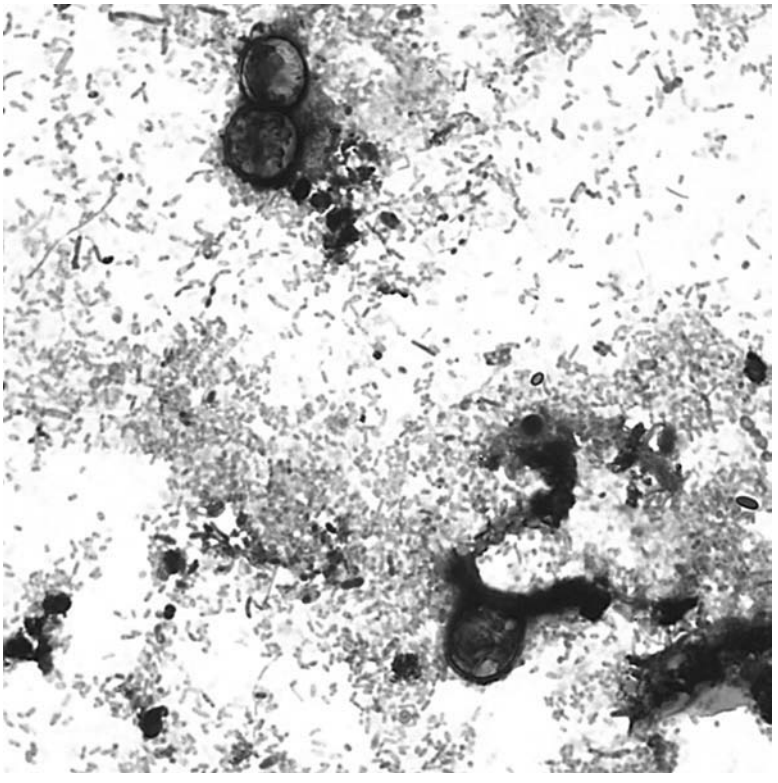


Figure 7-25 **Agarose gel analysis of PCR test for *Cryptosporidium parvum* showing the diagnostic band at 435 bp. (Courtesy of CDC/DPDx.)**

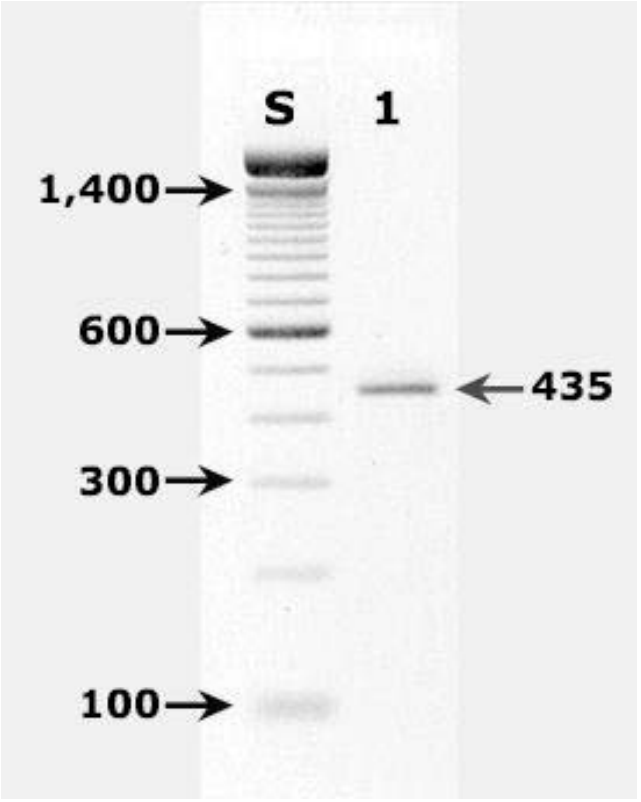
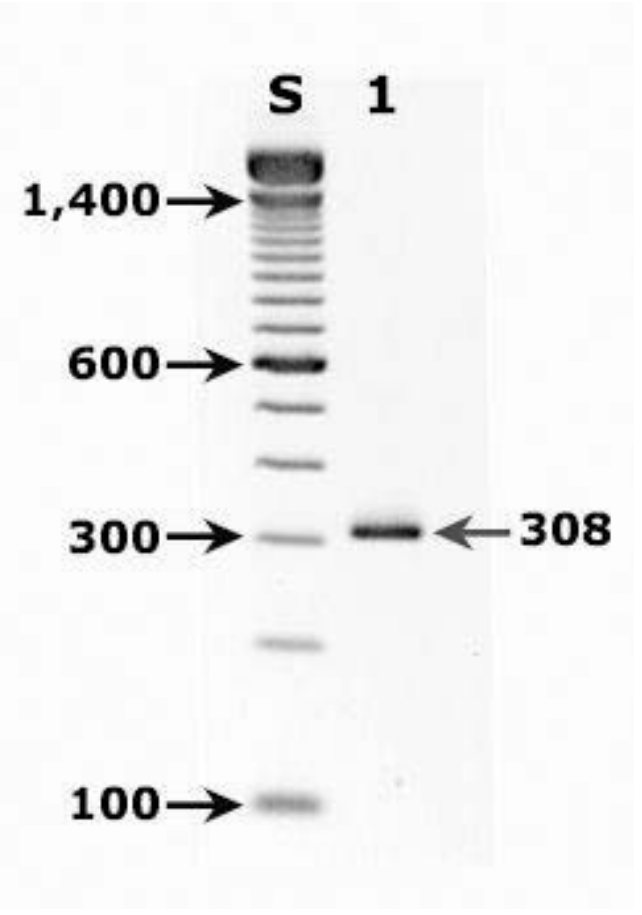


Figure 7-26 **Agarose gel analysis of nested PCR test for *Cyclospora cayetanensis* showing the diagnostic band at 308 bp. (Courtesy of CDC/DPDx.)**



as viruses and trace level chemical compounds). The identification of bacterial contaminants has been immeasurably aided by the development of rapid and automated techniques. What once may have taken an analyst several days to determine for a single sample may now be done for a panel, with potentially less ambiguity of results interpretation offered by automated detection. Furthermore, the ability to consistently use more defining tests allows for more consistent organisms identification. This, coupled with DNA fingerprinting now done on some bacterial strains, assists in the investigation of food-borne outbreaks, linking together multiple samples separated by time and place.

Discussion Questions

1. Describe the extent to which FoodNet, FERN, CIFOR, PulseNet, and PHLs complement or overlap each other.
2. What makes food a difficult sample matrix to analyze for bacterial contaminants?
3. PFGE fingerprinting is performed on some bacterial strains but not others. Why might this be the case?
4. Given the importance of food to human health, describe some reasons why all food is not tested all the time. What are the inherent difficulties?
5. Why are so few cases of food poisoning (by any pathogen) recognized and reported? What steps might be taken to increase identification and reporting?
6. What might be the reason for a dearth of analysis methods for viruses in food?
7. Go on the Internet and find three good sources for detailed information concerning food safety and/or food-borne contaminants. They cannot be government-sponsored (.gov) or listed under Additional Resources. Briefly describe what they provide and why they are good sources of reliable information.

8. Many food-borne pathogens are killed or inactivated by sufficient heat (cooking). Why do many people still undercook food, and what may public health officials do to intervene?

9. How might rules associated with restaurant cleanliness minimize the chance of food contamination with pathogens? Describe some rules associated with either federal standards or local health department regulations.

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Additional Resources

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8

Air Testing

Introduction

Air is the highest priority necessity for life, yet many of us are fortunate enough that we seldom have cause to regard its quality. Those who live in highly polluted areas, or who suffer from respiratory illnesses, are much more sensitive to pollutants that may be present and how they affect our quality of life. Air quality is critically important to health because the average individual inhales in excess of 3000 gallons of air each day. In addition, almost 30 million people suffer from asthma, and many air pollutants trigger attacks.[1] Studies have shown how some pollutants contribute to changing the natural environment through such mechanisms as acid rain and the greenhouse effect. Much information concerning air quality may be found on the US Environmental Protection Agency's (EPA) Clean Air Act Web site at http://www.epa.gov/air/caa/.

The intersection of air quality and public health is somewhat nebulous in comparison to other sample matrices and methods. Air-borne contaminants may have a high potential for health risk because of fairly direct ingestion into the body and adsorption to the bloodstream. On the other hand, attempting to measure just what someone may have inhaled after the fact is near impossible because of the constantly changing makeup of localized air. One would have to carry an air sampling/analysis device with them to discover the quality of inhaled air (and this is sometimes done in investigations). Another potential conundrum is determining an association between (likely) inhaled contaminants and adverse health. Individuals inhale and absorb variable concentrations of various contaminants throughout life, and few produce acute effects. Long-term effects may also be confounded by an individual's exposure to other contaminants that may have the same outcome. Investigators are thus reduced to large-scale epidemiologic studies attempting to understand the relationship between a particular contaminant and its health risks, and such studies have been done as part of determining allowable pollutant levels. For this reason we are unable to provide statistics showing the nature and extent of disease or illness caused by individual contaminants. What we can show is how levels of these contaminants have decreased over time because of the effects of regulations.

Air pollution was first addressed through federal legislation in 1955 with the Air Pollution Control Act, providing funding for research into air pollution. This was followed in 1963 by the Clean Air Act (CAA) that authorized both the study and control of air pollution. It allowed for expanded studies of pollutant emission inventories, ambient air monitoring methods, and various control techniques. In 1970, the next CAA was passed and the EPA (through the National Environmental Policy Act) was created. This legislation authorized the development of federal and state legislation to regulate emissions of air pollutants from both stationary and mobile sources. Four major regulatory programs were established: state implementation plans (SIPs), new source performance standards (NSPSs), national emission standards for hazardous air pollutants (NESHAPs), and national ambient air quality standards (NAAQSs). The Act was expanded and amended in 1990 to increase the authority and responsibility of the government in these and new air quality concerns, including the newly authorized control of acid deposition (acid rain).

The EPA's role is to set limits on certain air pollutants and limit emissions from different sources such as cars and factories. The EPA has oversight of state plans to reduce air pollution. It also assists state, tribal, and local agencies in their efforts by providing research, studies, and engineering designs to reduce air pollution. It is the responsibility of the states to monitor air quality and enforce CAA regulations. This regulatory monitoring of air pollutants is not commonly performed in public health laboratories (PHLs; only four states and DC list the state health department as the contact for air quality). Most often these duties fall to the respective state's department of environmental management/conservation/quality or other, such as natural resources. However, some laboratories can perform some of these analyses upon request and/or may be part of a state's combined laboratory where such work is being done.

The EPA set NAAQSs that identified six *criteria pollutants* to regulate.[2] The choices were based on their ubiquitous nature in the United States and their ability to harm health, the environment, and property. They are termed *criteria* as their permissible levels are determined by scientifically based estimates of their impact on human health (primary standards) and/or the environment (secondary standards). Primary standards protect public health (including those who may be especially vulnerable) and secondary standards protect public welfare (visibility and damage to vegetation and buildings). A geographic area whose air quality exceeds the primary standards is called an attainment area. The six criteria pollutants are particulate matter, nitrogen dioxide (NO_2), sulfur dioxide (SO_2), carbon monoxide (CO), ozone, and lead. Their regulated limits are shown in Table 8-1.

Air sample collection and analysis were discussed briefly in Chapter 3. The analysis of air samples for noncriteria chemical components is similar, though the range of preparation, separation, and detection techniques is expanded. Of significant note as compared to other analyses discussed in other chapters, the analysis of criteria pollutants is only allowed by EPA-approved methods that are largely developed by instrument vendors. Thus, we find that the allowable methods are both associated with and titled by the instrument vendors. So while a water analysis may be done by EPA Method 508.1 using any suitable gas chromatograph set to the analyst's determination, an air analysis for SO_2 by *Monitor Labs/Lear Siegler Model 8850 SO_2 Analyzer* must use that vender's instrument and specified conditions and reagents. Because the conditions for analysis are so vender-specific, we will not describe individual methods to the level of detail seen in other chapters.

Particulate Matter

Particulate matter (PM) refers to liquid droplets and aggregates of compounds and inorganic or organic material such that they reach a size that is directly measurable and has adverse health effects. These particles may be liquid or solid and vary in size and composition. They come from a wide variety of sources, including

Table 8-1 Criteria Air Pollutants and EPA-Regulated Limits[3]

Pollutant	Primary Standard		Secondary Standard	
	Level	Averaging Time	Level	Averaging Time
PM2.5	15.0 μg/m^3	Annual	Same as primary	
	35 μg/m^3	24 hours		
PM10	150 μg/m^3	24 hours	Same as primary	
NO_2	0.053 ppm (100 μg/m^3)	Annual	Same as primary	
SO_2	0.03 ppm	Annual	0.5 ppm (1300 μg/m^3)	3 hours
	0.14 ppm	24 hours		
CO	9 ppm (10 mg/m^3)	8 hours	None	
	35 ppm (40 mg/m^3)	1 hour		
Ozone	0.075 ppm	8 hours	Same as primary	
Lead	0.15 μg/m^3	Rolling 3-month average	Same as primary	
	1.5 μg/m^3	Quarterly average		

combustion, unpaved roads, crushing and grinding operations, dirt blown into the air, and construction activities. Sulfur and nitrogen compounds released from motor vehicles may even react with sunlight and water vapor to form particles. All these particles have the potential to reduce visibility (haze), cause property damage, and impact health.

Some particles are fine enough that they are easily inhaled and, the smaller the size, the farther into the lungs they may travel. There are, therefore, two measurement criteria most often used: particles up to 2.5 µm in diameter (PM2.5) and up to 10 µm (PM10). Figure 8-1 shows the relative sizes of PM and human hair. Particles with a size greater than 10 are unlikely to reach the lungs, though they may cause irritation to the eyes, nose, and throat.[4] Measurements for PM refer to the mass of all PM of a specified size within a volume of air. Thus, a PM10 of 10 mg/m^3 indicates that there is a total of 10 mg of particles with a size between 2.5 and 10 µm in 1 m^3 of air.

PM2.5 (fine particles) particles are 2.5 µm and smaller and are found in smoke and haze. These comprise the majority of *secondary* particles that are formed by the reactions of released chemicals, water vapor, and sunlight. The major constituents of PM2.5 include elemental and organic carbons, nitrates, sulfates, ammonium, and metals. Limits for PM2.5 were published in 1997. National monitoring began in 2000, and Figure 8-2 shows how levels of PM2.5 have decreased since then.

PM10 (course particles) particles are between 2.5 and 10 µm in diameter and are often found near roads and industries that produce dust. These comprise the majority of *primary* particles that are directly emitted from a source.[5] Seven PM10 particles placed next to each other would have a width approximately equal to that of a human hair.[4] Limits on PM10 were set before the 1990 CAA amendments. Figure 8-3 shows how levels of PM10 have decreased since 1990.

The current primary standard for PM2.5 is 15 µg/m^3 annually (3-year average of weighted annual mean) or 35 µg/m^3 for 24 hours (3-year average of 98th percentile of 24-hour concentrations at each monitor must not exceed 15 µg/m^3).The current primary standard for PM10 is 150 µg/m^3 for 24 hours (not to be exceeded more than once per year over 3 years).[6] The secondary standard for each is the same as the primary.[3] There are currently more than 18 EPA-approved

Figure 8-1 Size comparison of a human hair to PM2.5 and PM10. (Courtesy of EPA/ Office of Research and Development.)

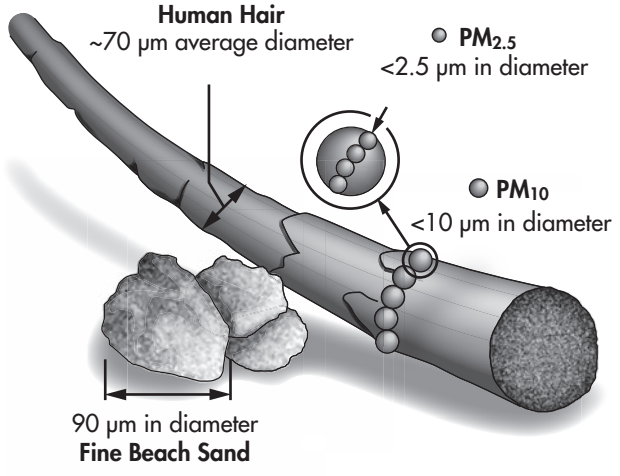


Figure 8-2 Chart showing 19% decrease in national average PM2.5 concentration from 2000 to 2008.

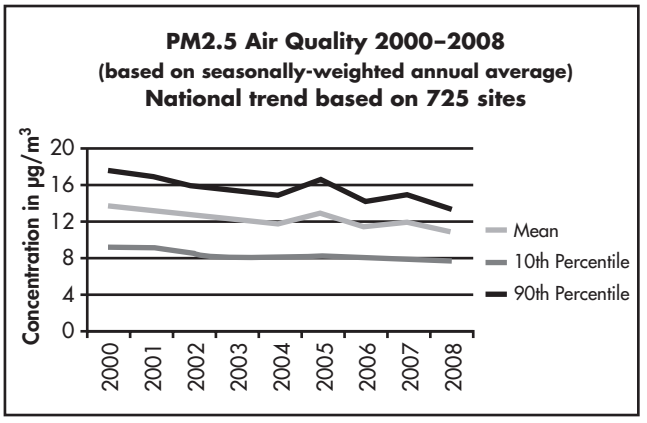
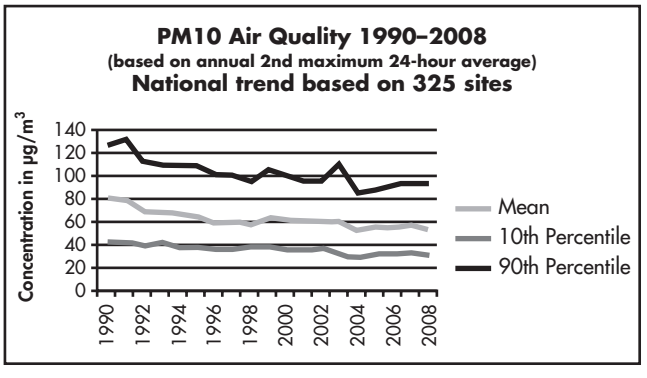


Figure 8-3 Chart showing 31% decrease in national average PM10 concentration from 1990 to 2008.



reference and equivalent methods for the analysis of PM2.5 in air samples, and 22 methods for PM10.[7] Examples include:

- For PM2.5: *BGI Inc. Models PQ200* or *PQ200A PM2.5 Ambient Fine Particle Sampler*, *Met One BAM-1020 Monitor*, *PM2.5 FEM Configuration*, and *Rupprecht & Patashnick Partisol-Plus Model 2025 Sequential Air Sampler*
- For PM10: *Andersen Model RAAS10-100 PM10 Single Channel PM10 Sampler*, *Ecotech Model 3000 PM10 High Volume Air Sampler*, *Met One or Sibata Models BAM/GBAM 1020*, *BAM/GBAM 1020-1*, and *Thermo Scientific* or *Rupprecht & Patashnick Partisol Model 2000 Air Sampler*

Sidebar 8-1 PM2.5 Levels Before and After an Indoor Air Law Implementation[8]

Secondhand smoke (SHS) contains 50+ known carcinogens and even short-term exposures can increase the risk of an acute cardiovascular event. Respirable suspended particles (RSP) are used as a marker for SHS, and levels of PM2.5 were measured before and after an indoor smoking ban in three New York counties in 2003. Particles of this size are released in substantial numbers in cigarettes and baseline levels were measured in 14 bars and restaurants and 4 large recreation areas. Two months later and after the indoor smoking ban took effect, the same venues were measured again to determine the effect of the ban on SHS levels. An air monitor was used to take PM2.5 measurements in each venue on a continuous basis and averaged for the duration. In the 14 bars and restaurants where smoking occurred, the mean PM2.5 reduction was 90% (from 412 to 27 μg/m^3). The amount of decline varied from 98.1 to 73.1%. Two restaurants where smoking was already banned experienced mean reductions of 0% and 2.4%. This case highlights the effectiveness of regulations to decrease health risks to both employees and customers.

NO_X

NO_X primarily refers to the highly reactive compounds nitric oxide (NO) and NO_2 that are produced during high-temperature combustion, such as in internal combustion engines. Many of the compounds are both odorless and colorless, but NO_2 can be seen with air particles as a reddish-brown layer in many urban areas. NO_X compounds are a major source of air pollutants and of special concern in urban environments with high volumes of motor vehicle traffic. NO converts to NO_2 in the presence of oxygen (O_2). Both forms react with volatile organic compounds (VOCs) and sunlight to form photochemical smog (including ozone), which in turn causes respiratory health effects. They also form nitric acid, which is a component of acid rain, and react with ammonia, moisture, and other particles to form nitric acid and chemically related particles.[9] Figure 8-4 illustrates how acid rained is formed from NO_X (and SO_2).[10] Figure 8-5 shows how NO_2 levels have decreased since 1980.

The main adverse health effects associated with inhaling NO_X and subsequent compounds is irritation of the respiratory tract, reduction in lung function, damage to lung tissue, aggravated bronchitis, and emphysema. Nitrous oxide (N_2O) is also considered a greenhouse gas.

The current primary standard for NO_2 is 0.053 parts per million (ppm; 100 μg/m^3) annually (arithmetic mean). The secondary standard is the same.[3] There are currently over 28 EPA-approved reference and equivalent methods for the analysis of NO_2 in air samples.[7] Examples include *Sodium Arsenite Method for NO_2*, *Bendix Model 8101-B Oxides of Nitrogen Analyzer*, *Dasibi Model 2108 Oxides of Nitrogen Analyzer*, *Environnement S. A. Model AC32M NO2 Analyzer*, and *Philips Model PW9762/02 NO/NO2/NOx Analyzer*.

SO_X

SO_X refers to the compounds SO_2 and sulfur trioxide (SO_3) which are produced in volcanoes, industrial processes, and the combustion of sulfur-containing coal and petroleum. Figure 8-6 shows how levels of SO_2 have decreased since 1980. They act as an acid and form tiny sulfate particles in the air, which may be inhaled causing respiratory problems. They dissolve in water to form acids that become a primary component of acid rain. They may also react with other gasses and particles to form sulfates and other harmful products.

The main adverse health effects come from inhaling SO_2, which contributes to respiratory illness and may aggravate existing heart and lung diseases. Those who are active outdoors, children, and the elderly are at increased risk of adverse health effects. Short-term elevated levels

Figure 8-4 Illustration of how NO_X and SO_2 contribute to acid rain formation. (Courtesy of EPA.)

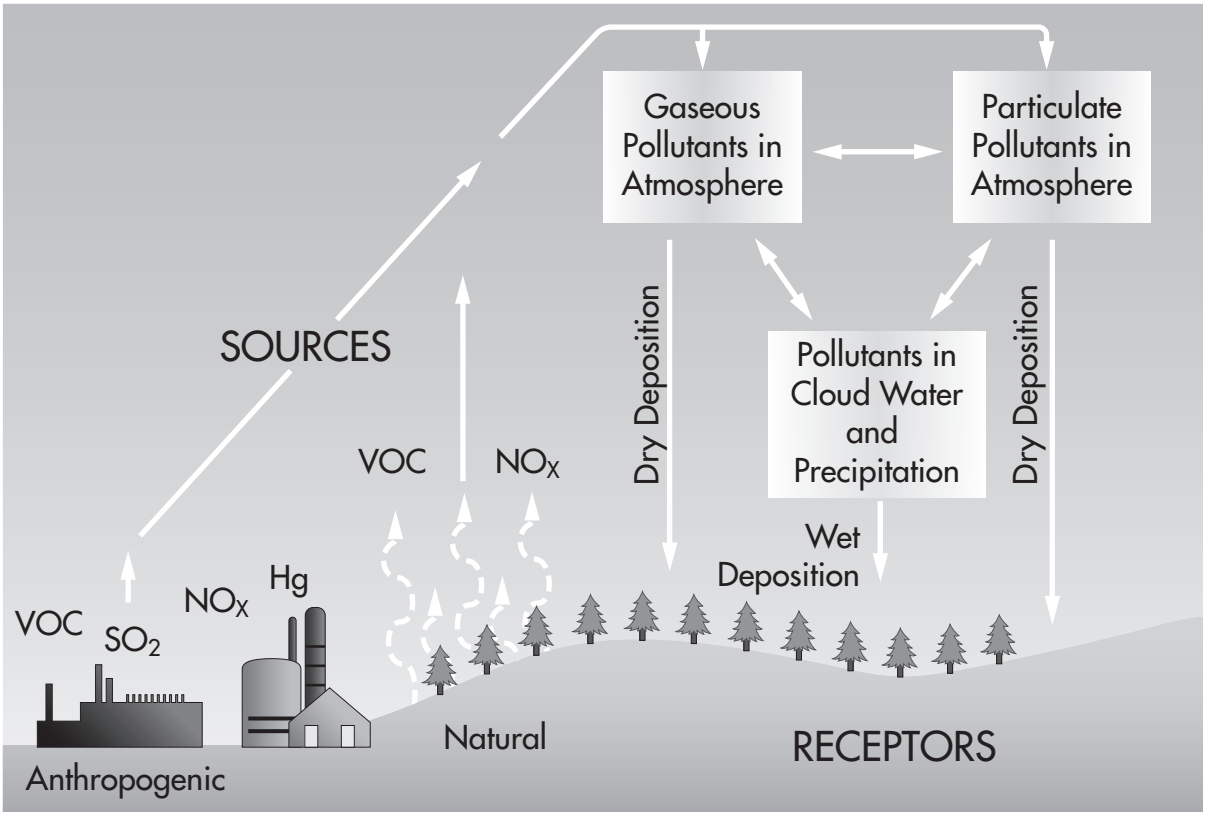


Figure 8-5 Chart showing 46% decrease in national average NO_2 concentration from 1980 to 2008.

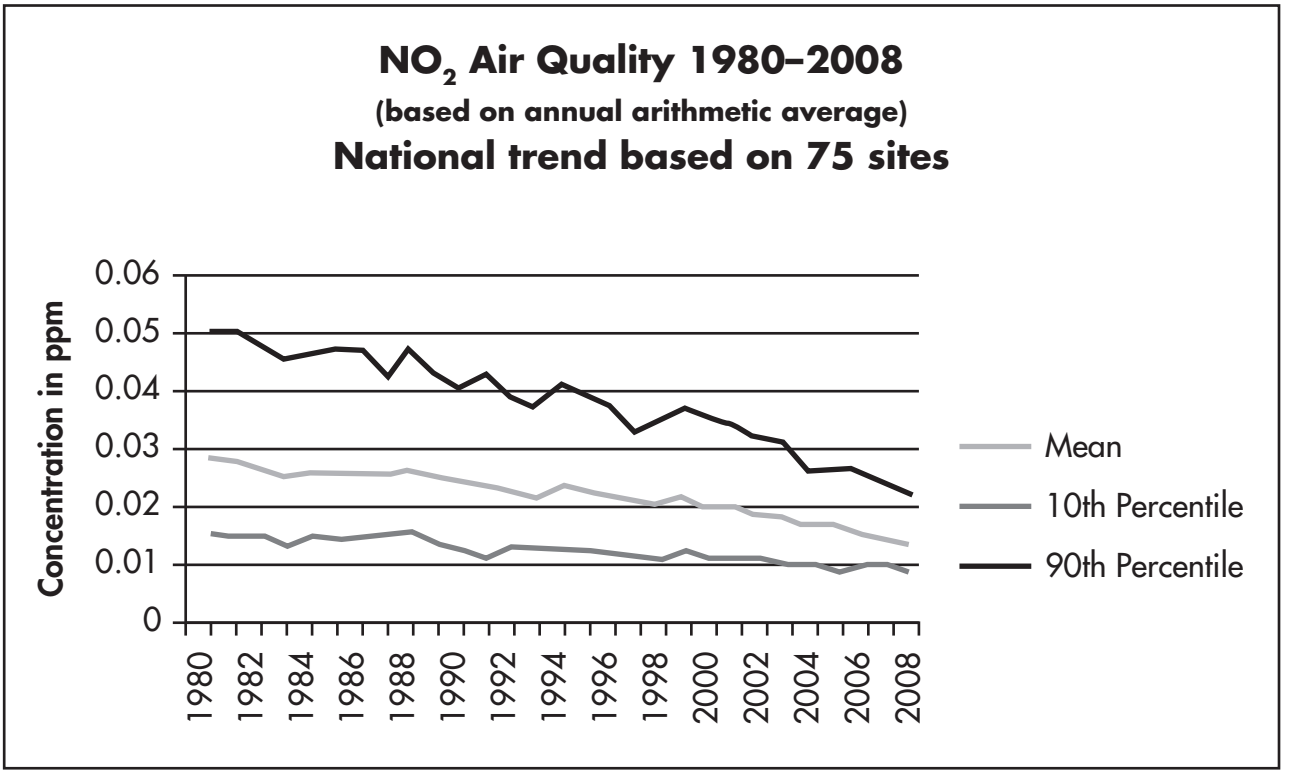


Figure 8-6 Chart showing 71% decrease in national average SO_2 concentration from 1980 to 2008.

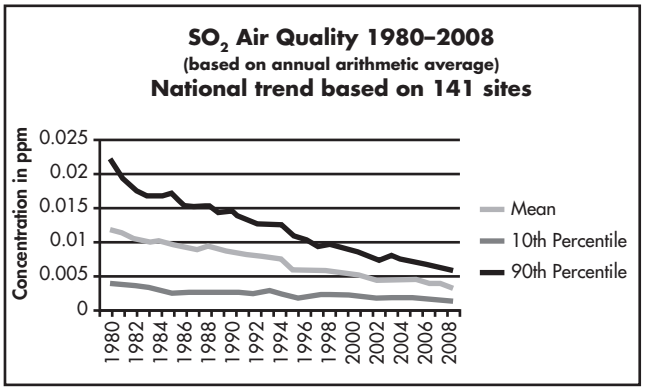
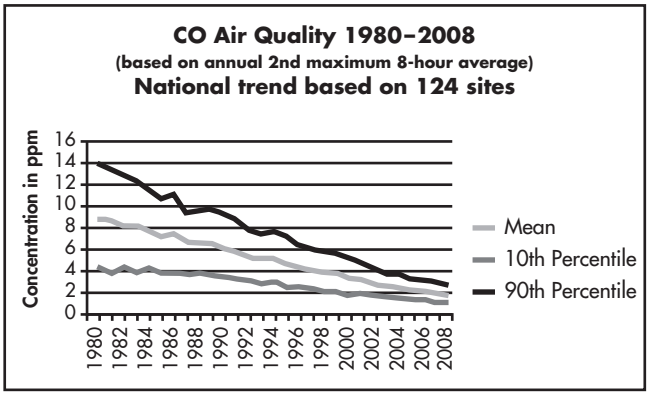


Figure 8-7 Chart showing 79% decrease in national average CO concentration from 1980 to 2008.



of SO_2 may also cause temporary breathing problems for those with asthma.[11]

The current primary standard for SO_2 is 0.03 ppm annually (arithmetic mean) and 0.14 ppm for 24 hours (not to be exceeded more than once each year).[3] SO_2 is the only criteria air pollutant for which the secondary standard has a different value. CO has no secondary standard and the primary and secondary standards are identical for the other pollutants. The secondary standard for SO_2 is 0.5 ppm (1300 µg/m^3) over 3 hours.[3] There are currently over 33 EPA-approved reference and equivalent methods for the analysis of SO_2 in air samples.[7] Examples include *Pararosaniline Method for SO2 Technicon I*, *ASARCO Model 500 SO2 Monitor*, *Dasibi Model 4108 U.V. Fluorescence SO2 Analyzer*, *Environnement S.A. SANOA Multigas Longpath Monitoring System*, and *Monitor Labs/Lear Siegler Model 8850 SO2 Analyzer*.

Carbon Monoxide

Carbon monoxide (CO) is a colorless, tasteless, highly toxic gas produced during internal combustion. Motor vehicle exhaust contributes approximately 56% of all CO emissions nationally, accounting for 85 to 95% of emissions in larger cities, with another 22% nationally from nonroad engines and vehicles. Though often attributed to combustion, the presence of CO in the atmosphere is also caused by natural fires and volcanic activity. While atmospheric CO levels are ~1 ppm, the CO concentration in undiluted cigarette smoke is ~30,000 ppm.[12] CO binds with hemoglobin in blood, leading to toxic effects by reducing the delivery of oxygen to internal organs. Figure 8-7 shows how levels of CO have decreased since 1980.

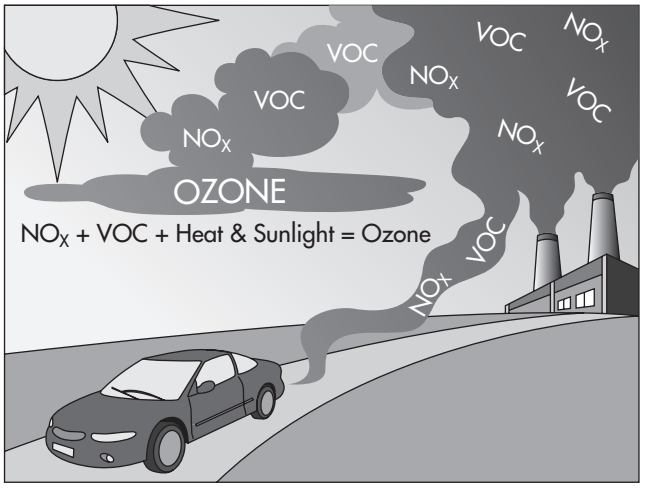
The current primary standard for CO is 9 ppm (10 mg/m^3) over 8 hours (not to be exceeded more than once per year) and 35 ppm (40 mg/m^3) for 1 hour (not to be exceeded more than once each year). CO is the only criteria air pollutant that has no secondary standard.[3] There are currently over 18 EPA-approved reference and equivalent methods for the analysis of CO in air samples.[7] Examples include *Beckman Model 866 CO Monitoring System*, *Dasibi Model 3008 CO Analyzer*, *Horiba Models APMA-360 or APMA-360-CE CO Monitor*, and *SIR S.A. Model S-5006 CO Analyzer*.

Ozone

The well-known ozone layer in the stratosphere (10–30 miles high) is composed of the same molecule (O_3) as ground level ozone. However, while the high layer is extremely beneficial, ground level ozone presents serious risks to health. A small amount of ground level ozone is released from industrial processes but the majority comes from the reaction of NO_X with VOCs and sunlight (illustrated in Figure 8-8). It forms the primary component of smog and is often thought of as a summer pollutant because of its increased concentrations in hot weather.[13]

Breathing ozone may cause a variety of health problems. Immediate symptoms may include throat irritation and coughing, chest pain, and congestion. Exposure may also trigger an asthma attack and aggravate existing bronchitis and emphysema. Repeated/prolonged exposure may cause inflammation in the lung lining and

Figure 8-8 Illustration of how ground-level ozone is formed. (Courtesy of EPA.)



scar lung tissue. External exposure may also cause a sunburnlike skin inflammation.[12]

The current (effective 2008) primary standard for ozone is 0.075 ppm for an 8-hour average time and is not to be exceeded more than once per year. The secondary standard is the same.[3] Figure 8-9 shows how levels of ozone have decreased since 1990. There are currently over 26 EPA-approved reference and equivalent methods for the analysis of ozone in air samples.[7] Examples include *Beckman Model 950A Ozone Analyzer*, *DKK-TOA Corp. Model GUX-113E Ozone Analyzer*, *Environment S.A. Model O342M UV Ozone Analyzer*, *Meloy Model OA325-2R Ozone Analyzer*, and *Seres Model OZ 2000 G Ozone Analyzer*.

Figure 8-9 Chart showing 14% decrease in national average ozone concentration from 1990 to 2008.

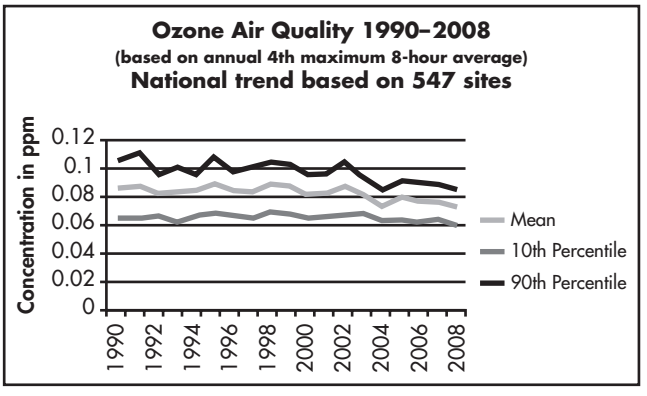
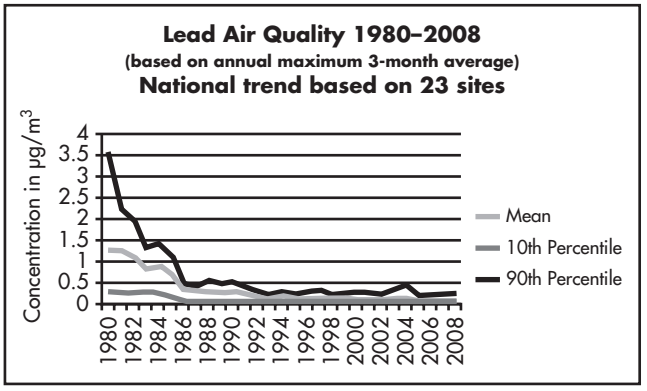


Figure 8-10 Chart showing 91% decrease in national average lead concentration from 1980 to 2008.



Lead

Lead is a naturally occurring element found worldwide. Historically, the major sources of air-borne lead have been emissions from industry and vehicles. While not a normal component of gasoline, it was added for a number of years to reduce engine "knocking." The EPA's regulation of lead resulted in its removal from gasoline, and emissions of lead from the transportation sector have dropped by > 95% since 1980 (corresponding to a > 94% drop in air lead levels for the same time frame).[14] Figure 8-10 shows how levels of lead have decreased since 1980. Remaining sources of air-borne lead exposure include metals processors, waste incineration, and battery manufacturers. Currently, the most common sources of child lead exposure are paint (in older homes) and contaminated soil, water, and food.

Inhaled or ingested lead is absorbed by the blood and accumulates in the bones. Lead has the potential to affect the nervous, cardiovascular, reproductive, immune, and renal systems. Children are at particular risk for adverse effects, as even low levels of lead exposure may cause significant cognitive development delays, behavioral problems, and learning deficits.[14]

The current primary standard for lead is 0.15 µg/m^3 for a rolling 3-month average (effective 2008) or 1.5 µg/m^3 for a quarterly average. The secondary standard is the same.[3] There are currently over 21 EPA-approved reference and equivalent methods for the analysis of lead in air samples.[7] Examples include *Energy-Dispersive X-Ray Fluorescence Spectrometry (TNRCC)*, *Flameless Atomic Absorption Spectrometry (Omaha)*, *Inductively Coupled Argon Plasma-Optical Emission Spectrometry (IL)*, and *Wavelength Dispersive X-Ray Fluorescence Spectrometry (CA)*.

Hazardous Air Pollutants and Indoor Air Quality

Hazardous air pollutants are also known as air toxics or toxic air pollutants. They are compounds that have the potential to cause cancer, reproductive effects and birth defects, and other serious health problems. There are currently 187 hazardous air pollutants over which the EPA has regulatory control and examples include benzene, hexane, and styrene. The majority of these compounds is manmade and come from either mobile (e.g., cars) or stationary (e.g., factories) sources. Stationary sources are further divided into *major*, which release 10+ tons of a pollutant each year or 25+ tons of a combination, and *area*, which release less than 10 tons per year.[15]

The effects of exposure to these compounds vary by the compound and the nature, duration, and extent of exposure. As mentioned, some compounds are known to cause cancer and others may cause adverse health effects to various organs and biological systems. They may also persist in the environment and become incorporated into body tissue, resulting in the potential for long-term exposure. Unlike the criteria pollutants, the EPA does not regulate exposure limits for these compounds. Instead, they regulate the amounts released from sources. The EPA and other state and federal agencies are actively researching the health effects of the individual compounds and may release exposure limits at a future date.

Hazardous air pollutants may or may not be a contributor to sick building syndrome (SBS). This is an acute health or personal comfort effect thought to derive from time spent in a building, but not linked to any specific cause. The EPA cites four factors as being causes or contributors to SBS: inadequate ventilation, chemical contaminants from indoor sources or outdoor sources, and biological contaminants.[16] Indoor air quality investigations are characterized by a walkthrough of the facility, interviewing the occupants, and forming a hypothesis. Actual air testing is not recommended unless substantiated by significant data. This is because air contaminant levels rarely exceed applicable limits or guidelines, and the results may actually be misleading. However, there are some situations where air sampling and testing may be warranted.

There are multiple methods available for the analysis of air for different chemical classes considered hazardous pollutants. Many have been published in the EPA's *Compendium of Methods for the Determination of Toxic Organic Compounds in Ambient Air, Second Edition* (EPA/625/R-96/010b). The current list of methods includes TO-1 through TO-17 and the specifics cover the spectrum of previously discussed procedures (discussed in Chapter 3). Different air sampling techniques are used as well as both liquid and gas chromatography followed by detection and identification by ultraviolet/visible spectrometry (UV/VIS), electron capture detector (ECD), and mass spectrometry (MS).[17]

There are relatively few PHLs that perform testing for the criteria pollutants, but there are more that perform specific testing methods as part of SBS investigations or to investigate other complaints. The specific types and volumes of testing are essentially unique for each PHL and change over time because of need and expense. The IDPH laboratory in Springfield performed occasional analyses for aromatic hydrocarbons in air samples in the late 1990s but does not now have that capability. Though air testing is not widely performed in PHLs, it is still an area of public health concern and research. State health departments and PHLs are often asked questions about air quality, and the knowledge of the basics of air quality regulations and what types of testing are available, is useful in retaining the public's confidence in the PHL's expertise.

Discussion Questions

1. Why may the EPA have chosen the six criteria pollutants for initial review and regulation?
2. Which criteria pollutant levels might be most altered by the widespread adoption of hybrid cars and trucks?
3. Name a federal agency besides the EPA that regulates air quality and describe its functions.
4. What are some air-borne contaminants one might find related to metal or oil refining? What might be their effects?
5. Go on the Internet and find two good sources for detailed information air safety and/or air-borne contaminants. They cannot be government-sponsored (.gov) or listed under Additional Resources. Briefly describe what they provide and why they are good sources of reliable information.
6. If you were to investigate the impact of a specific pollutant upon animal life, how might you design a surveillance plan? What would it include?

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Additional Resources

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9

Terrorism Preparedness and Response

Introduction

This chapter will explore potential agents of biological, chemical, and radiological terrorism and how public health laboratories (PHLs) plan to respond to their use. The nature of the response varies by the agent used, just as individual agent properties and effects vary. In general, though, preparedness activities, and the skills and facilities needed for their response, are readily categorized according to the agents involved. Radiological responses will be only briefly discussed in this chapter, for several reasons. First is the fact that relatively few PHLs have the capacity for any radiological analyses at all, much less at the depth and scale to respond to a dirty bomb or other mass radiological attack. Second is the relative paucity and standardization of analytical methods available. As discussed in Chapter 4, most of the current methods are drawn from Occupational Safety and Health Administration (OSHA), US Environmental Protection Agency (EPA), and other federal agencies with the purpose of workplace and environmental monitoring. They are not necessarily designed for the purpose of clinical sample analysis. Response to this type of threat by PHLs is underdeveloped in comparison to biological or chemical agents.

Before we begin discussing the different agents, we need to mention that much of the information concerning the actual analysis methods is not publically available, although their use in clinical testing has been reviewed and approved through the Clinical Laboratory Improvement Amendments (CLIA). In general, the methods, techniques, and instrumentation described in previous chapters for the analysis of biological, chemical, and radiological agents are used here. However, we are unable to provide specific references to methods, controls, and instrumentation. Some of the main differences involve an increased level of safety (often utilizing an enhanced biosafety Level 2 [BSL-2] or BSL-3 environment) and increased attention to legal aspects such as chain of custody. One of the greater concerns for state health department laboratories involves the analysis of environmental samples for chemical and/or radiological analyses. These samples have the potential to be contaminated with lethal quantities of agent, and few laboratories have the necessary facilities to handle them safely. Screening samples for these agents is often done in the field, and potential positives referred to other agencies for response.

Biological Agents

People have been involved in conflict with their fellow humans since the beginning of recorded history. The pursuit of war has usually been accompanied by technological innovation with the aim of acquiring an advantage. The potential use of biological entities has been long recognized, even if their mechanism of action was not understood. Sidebar 9-1 contains a brief description of some of the uses of biological agents throughout history.

Sidebar 9-1 Selected Use of Biological Agents Throughout History[1,2]

- 6th century BC: During a siege, Solon of Athens poisons the water supply of the city of Krissa with the purgative hellebore.
- 190 BC: The forces of Carthaginian general Hannibal hurl earthen jars containing venomous snakes onto the decks of the enemy fleet of King Eumenes of Pergamon.
- 1346: During the siege of the Kaffa by the Tartars, the besiegers catapult the bodies of plague victims over the city walls to introduce the disease.
- 1650: Polish General Siemenowics places saliva from rabies-infected dogs into artillery shells.
- 1710: Plague victims hurled over the Swedish-held city of Reval, Estonia, during the Russo-Swedish war.
- 1763: Native Americans are given smallpox-contaminated blankets by Colonel Bouquet during Pontiac's Rebellion.
- 1863: Retreating Confederate troops in Mississippi leave dead animals in water sources.
- 1932: Japanese form Unit 731, a large, 150-building biological warfare experimentation complex where an estimated 3000 to 9000 prisoners died in the camp (many from aerosolized anthrax), and another 10,000+ in field tests. Approximately 150 million plague-infected fleas mixed with grain are air dropped over Chinese cities to draw rats. Cholera and anthrax were also extensively tested.
- 1942: Before the battle of Stalingrad on the German–Soviet front, there is a large outbreak of tularemia. Thousands of soldiers are ill and it is determined that 70% have pneumatic tularemia.
- 1979: Accidental release of aerosolized anthrax from Compound 19 in Sverdlovsk. Downwind human deaths are usually estimated at 40, though some report as many as 1000.
- 1984: In order to influence a local election, members of the Rajneeshee cult contaminate salad bars in Dallas, Oregon, with *Salmonella typhimurium*. There are ~750 individuals poisoned with 40 hospitalized and but no fatalities.
- 1991: Iraq admitted to research into the use of *Bacillus anthracis*, botulinum toxins, and *C. perfringens*. It is later discovered to have also worked on aflatoxins and ricin.
- 1995: It is reported that the cult Aum Shinrikyo has attempted to disperse anthrax, botulinum toxin, Q fever, and Ebola virus in Japan. There are no reported infections.
- 2001: Anthrax spores mailed in the United States resulted in 22 cases with 5 fatalities.

In 1969, President Nixon signed an executive order prohibiting the use of biological weapons under any circumstances. The Biological and Toxin Weapons Convention (BWC) was signed by many world powers (including the United States and former USSR) in 1972 and prohibited the development, production, and stockpiling of biological weapons. The Convention has been broken many times, most notably by Russia, whose biological weapon research activities only ceased with the dissolution of the Union in 1991. Other counties such as Iraq, and private groups such as the Aum Shinrikyo cult, are also known to have developed and/or used biological weapons. Thus, the potential for the purposeful release of a biological agent to cause illness still exists and is of significant concern. The text of the BWC may be found on the United Nations Office at Geneva Web site at http://www.unog.ch/.

Coordinated Federal and State Responses

The Laboratory Response Network (LRN) was established by the Centers for Disease Control and Prevention (CDC) in accordance with Presidential Decision Directive 39 that specifies antiterrorism policies and specific missions to federal agencies.[3] It is a collaborative effort with the Federal Bureau of Investigation (FBI) and the Association of Public Health Laboratories (APHL) and became operational in 1999. The primary, and original, objective of the LRN is to coordinate and improve the country's PHL infrastructure so that it is better able to respond to acts of bioterrorism. It has an established, secure Web site and coordinates the activities of local, state, federal, international, and military laboratories. The LRN, in conjunction with CDC funding through the Cooperative Agreement for Public Health Emergency Preparedness (Preparedness grant), has accomplished much to expand and improve PHLs nationwide.

Suitable Laboratory Environment

In a 1998 APHL survey of state PHLs, 12 of 38 responding states reported having BSL-3 capability. BSL-3

laboratories have the structural capabilities necessary to test for infectious agents that could cause serious or potentially lethal diseases. It has been a goal of the LRN to increase BSL-3 capability to at least one BSL-3 laboratory in each state. By 2009, there are 46 states with at least one LRN-member PHL with BSL-3 capability.[4]

Standardized Instrumentation

One of the larger barriers to a consistently robust laboratory response across all states had been the availability of modern equipment. While some states had newer instrumentation and sufficient supplies and reagents, many others had budgets insufficient for their provision. There was therefore a great variability in which states could test for which agents, and the number of tests able to be performed in a given time frame. The Preparedness grant mandated the use of specific equipment for the analysis of bioterrorism (BT) agents, and provided the funding for their purchase and maintenance. A good example of how this worked was the requirement for each state laboratory to have the ability to analyze BT samples using real-time polymerase chain reaction (rRT-PCR). Many states did not have the instrumentation required for this analysis, and those who did used a variety of vendors with varying capabilities. The grant specified a limited number of instrument options and provided the funding for their purchase. By requiring the ability to provide these analyses, limiting the options available, and providing the needed funding, the CDC and LRN were able to ensure that every state laboratory has equivalent equipment that is modern, has similar characteristics, and produces results that are directly comparable.

Standardized Methodologies, Reagents, and Standards

The CDC and LRN continued this standardization by restricting the availability of analysis methods, reagents, and standards to the secure LRN Web site. The CDC would promulgate an analysis method through the LRN, and state laboratories would be required to use that method for sample analysis. In addition, key reagents and controls (e.g., selected primers and positive control strains) for these methods were only available through the same Web site. A laboratory has to officially request access to individual methodologies, reagents, and controls and verify they have the facilities and personnel sufficient to perform the methods as written. They also have to validate each method and return the data to the CDC. By requiring that all state laboratories obtain their methodologies, reagents, and standards through the LRN, the CDC was able to achieve a level of quality control and standardization not previously attained.

Standardized Reporting

The LRN Web site also provides a standardized template for result reporting. Gone are the days when laboratories sent in sample result and method validation data to the CDC in a variety of formats, via a variety of methods, all of which required human actions for receipt, storage, and analysis. While the human factor is not eliminated, the LRN has caused all sample result and method validation data to be reported electronically, through the Internet-based LRN Results Messenger. This uniform method of data collection ensures that the data are received and collected in a uniform fashion, which is of tremendous assistance. The LRN, and the data which is sent through it, also allows for much more rapid outbreak analysis and cluster investigations as all data associated with the sample and its analysis and results are electronically received, compared to other data, and subjected to further analyses.

The Federal Bureau of Investigation and Legal Aspects

The chain of custody deserves some explanation. The vast majority of samples handled by a PHL each year are essentially innocuous. That is, there are few, if any, legal ramifications behind the samples' analyses or results. This is not true for samples submitted for the analysis of a BT agent. By definition, these are considered to potentially be part of a terrorist action and are therefore evidence in a legal case. In fact, the Illinois Department of Public Health (IDPH) requires that all samples be screened through the FBI *prior* to receipt by the laboratory. This is done for two important reasons. First, the FBI decides if there is "just cause" for the analysis. That is, if there is any reasonable chance the sample is criminal in nature, and not just spilled confectioners' sugar or talc (which has occurred in the past). Second, for those samples given an FBI case number, the FBI ensures that they do not contain hazardous chemicals, explosives, or radiologicals. The IDPH laboratories, like other PHLs, are not equipped to deal with these dangerous substances, and such screening reduces risk to lab personnel.

BioSense

BioSense is a disease surveillance system designed to provide early recognition of a potential biological terrorism attack or naturally occurring infectious disease outbreak.

The hope is to identify and address the disease before it becomes epidemic and thereby limit its scope and cost. As of 2003, the program received real-time data from 350 hospitals, 466 Department of Defense (DOD), and 863 Department of Veterans Affairs (VA) healthcare facilities.[5] These data are used to monitor the disease events, their size, location, and rate of spread. Data and results are shared with national partners and state and local health departments to coordinate disease response activities.

BioWatch

The BioWatch program began in February 2003 and is an early BT detection system that tests the air for the presence of biological agents. Currently, there are over 30 cities participating in the program. Each city has a number of outdoor stations, maintained by the EPA, that continuously collect air samples. These samples are collected on a daily basis and tested at a local LRN laboratory via rapid methods (PCR) for the presence of a number of pathogenic organisms. This important collaboration among CDC, EPA, and the Department of Homeland Security (DHS) may help our nation detect a bioterrorist attack more quickly. To date, there have been no samples confirmed positive for an agent of bioterrorism origin.[4,5]

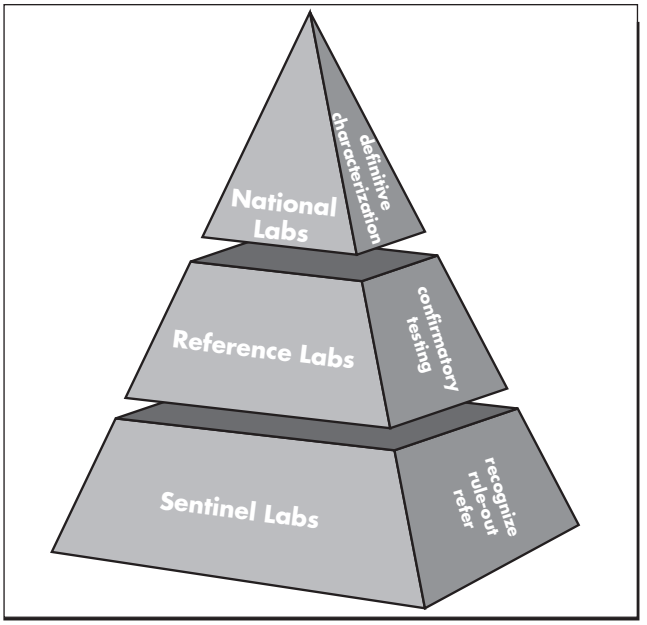
Laboratory Organization and Preparedness

Not all laboratories are created equal. Indeed, they often have separate missions and so do not need to have redundant capabilities. The CDC and LRN recognize this and have based laboratory preparedness plans on a three-tier/pyramid-shaped system (Figure 9-1).[4] The purpose of this strategy is to both use the skills and structure already in place in clinical labs nationwide and define responsibilities and expectations.

Sentinel Laboratories

These are in the great majority numberwise and consist of private and commercial labs at hospitals, clinics, and independent commercial sites. There are an estimated 25,000 such labs in the United States. They perform the initial testing for the majority of clinical samples. Samples that they suspect of containing a Category A agent but cannot rule out are forwarded to a reference laboratory for confirmatory/rule out testing. For example, a commercial lab may identify *Bacillus* spp. in a sample, but be unable to conclude that it is not *B. anthracis*. The sample would be sent to the state lab that can provide that confirmatory determination.

Figure 9-1 Pyramid structure of the Laboratory Response Network for bioterrorism response. (Courtesy of Centers for Disease Control and Prevention [CDC]).



Reference Laboratories

These are the state, local, and territorial health department; military; and other laboratories with significant analytical capabilities. State labs in particular are directly funded through the Preparedness grant and perform confirmatory testing, when possible, on those samples forwarded from the sentinel laboratories. Some organisms such as smallpox may only be cultured and confirmed at the CDC. Select samples received from sentinel laboratories may, in turn, be forwarded to the CDC or another national-level lab for further typing or characterization. They are also responsible for training sentinel laboratory personnel in their jurisdiction on identifying, packaging, and shipping procedures. In all, there are in excess of 100 labs worldwide classified as reference laboratories.

National Laboratories

These are highly specialized and well-equipped laboratories and include those at the CDC, the US Army Medical Research Institute for Infectious Diseases (USAMRIID), and the Naval Medical Research Center. They are responsible for the handling and analysis of highly pathogenic organisms (Ebola, smallpox), research into select agents, agent identification, subtyping and characterization, and bioforensics.

Potential Bioterrorism Agents

Category A Agents

According to the CDC, Category A agents are those that "pose a risk to national security because they can be easily disseminated or transmitted from person to person; result in high mortality rates and have the potential for major public health impact; might cause public panic and social disruption; and require special action for public health preparedness."[6] The following agents are considered in this category.

B. anthracis is the causative agent of anthrax and is a large, aerobic, Gram-positive, spore-forming rod (Figure 9-2). It has a tendency to form long chains and in culture it is nonmotile and nonhemolytic. *B. anthracis* also produces a complex of proteins that exhibit a variety of toxic effects based on their configuration.[7] It is endemic in many vertebrates such farm animals and in the soil. The spores are considered quite hardy and may survive in the ground for extended time periods. Figure 9-3 shows spores from the Sterne strain. Infections naturally occur because of contact with infected animal products, inhaling spores, or consuming undercooked meat. There is approximately one case per year in the United States. The great majority (> 95%) are cutaneous infections, though gastrointestinal and inhalation illness may also occur. Symptoms of infection can appear within 7 days. Cutaneous anthrax presents as a painless ulcer with a black necrotic center. Figure 9-4 shows an example of cutaneous anthrax in a middle-aged woman. Deaths are rare when treated with antibiotics. Inhalation anthrax presents with flulike symptoms; and, while not contagious, it is usually fatal. In the 2001 anthrax attacks in the United States, 45% were fatal even with aggressive treatment and supportive care. Gastrointestinal anthrax presents with symptoms of inflammation of the intestinal tract and may include nausea and vomiting, abdominal pain, fever, and severe diarrhea. The fatality rate is 25 to 60%. There are effective antibiotics to treat anthrax, including ciprofloxacin, levofloxacin, doxycycline, and penicillin. There is also an anthrax vaccine. Anthrax is considered one of the most likely choices for biowarfare.[8]

Figure 9-2 **Transmission electron micrograph of *Bacillus anthracis*. (Courtesy of CDC/ Dr. Sherif Zaki; Elizabeth White.)**

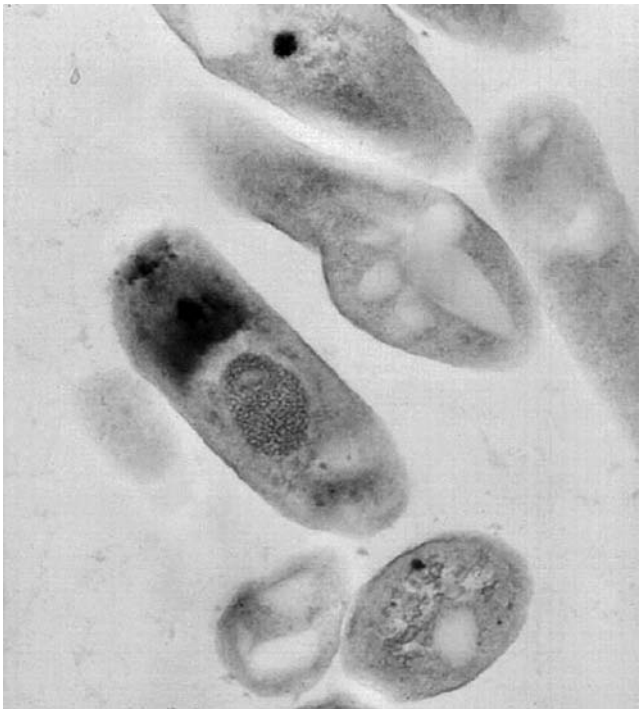


Figure 9-3 **Scanning electron micrograph of *Bacillus anthracis* spores (12,483×). (Courtesy of CDC/Laura Rose, Photographer: Janice Carr.)**

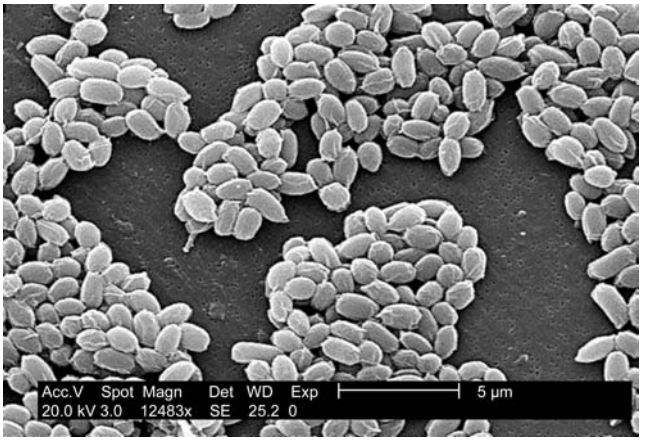
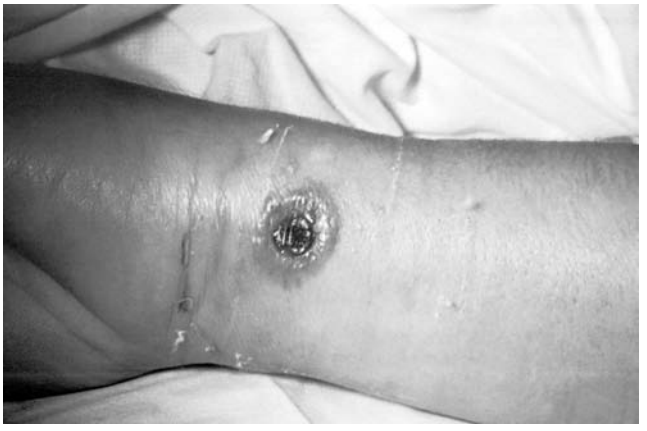


Figure 9-4 **Cutaneous anthrax lesion. (Courtesy of CDC/Dr. Philip S. Brachman.)**

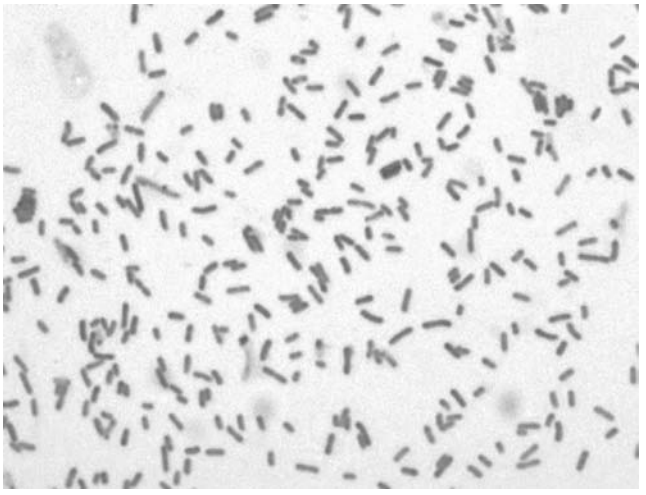


Sidebar 9-2 2001 Anthrax Mail Attack[9]

The reader is likely aware of the anthrax attacks, which occurred in the United States in the fall of 2001. What is not widely known is the large number of threats that were made before that time, beginning in 1997. By 1998, these threats were an almost daily occurrence and many consisted of envelopes mailed to government offices and various clinics. In 2001, the threat was carried out and four letters containing anthrax (Ames strain) spores were recovered. They were addressed to Senators Tom Daschle and Patrick Leahy in Washington, DC; an NBC newscaster; and an editor of *The New York Post*, both in New York. There are also indications that at least three other letters were mailed but subsequently lost and not found. Cases and environmental samples positive for anthrax were found at two other New York television companies and one in Florida. In all, there were 22 confirmed cases of anthrax, 20 of which were associated with workplace exposure, the other two unknown. Eleven of the cases were inhalational with five fatalities, and the others were cutaneous with victims all recovering.

Most people have little idea of the sheer volume of testing that occurred as a result of these letters. For example, while three of the letters were recovered from their addressee, the one to Senator Leahy was misdirected to the State Department. It was discovered after a search of unopened mail from the US Capitol, which was collected into 635 garbage bags. Each of these was tested, and 62 found to be contaminated with anthrax spores. The Leahy letter was then found by individually examining and testing the letters in those 62 bags. In addition to these letters, there were near innumerable analyses performed on suspicious objects, powders, and environmental samples of all kinds associated with determining the extent of the attack and contamination. The attack also spurred many subsequent hoaxes, many of which had to be treated more seriously than perhaps before. During the acute phase of this outbreak (October through December), LRN laboratories tested in excess of 120,000 clinical and environmental samples for the presence of anthrax. This level of coordinated response was in large part directly a result of the LRN's coordination and training activities and the PHL infrastructure improvements realized through the Preparedness grant.

Figure 9-5 Magnification 1000× of *Yersinia pestis*. (Courtesy of CDC/Larry Stauffer, Oregon State Public Health Laboratory.)



Y. pestis is the causative agent of plague and is a Gram-negative, non–acid-fast, nonmotile, nonsporulating coccobacillus. Figure 9-5 shows *Y. pestis*. Infections occur naturally worldwide, with 1000 to 3000 reported annually by the World Health Organization (WHO). There are 5 to 15 cases annually in the United States, usually in the western United States and rural/semirural areas. Infections are usually caused by the bite of an infected flea. Figure 9-6 shows a common carrier flea infected with plague. Symptoms of infection can appear within 1 to 7 days. *Y. pestis* initially infects the lymph nodes, causing inflammation and swelling (termed buboes) accompanied by pain,

Figure 9-6 Plague-infected male *Xenopsylla cheopis* 4 weeks after a blood meal. (Courtesy of CDC/Dr. Pratt.)

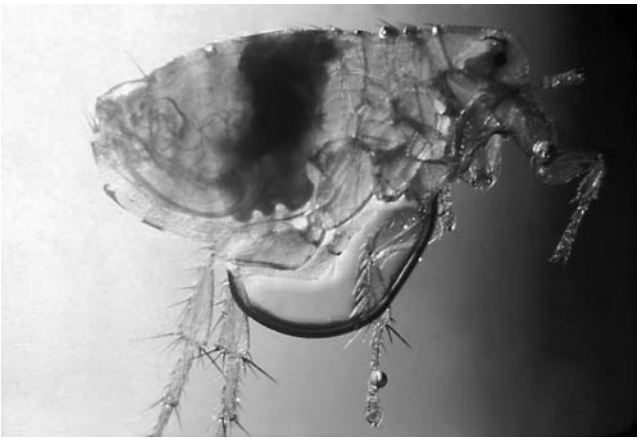
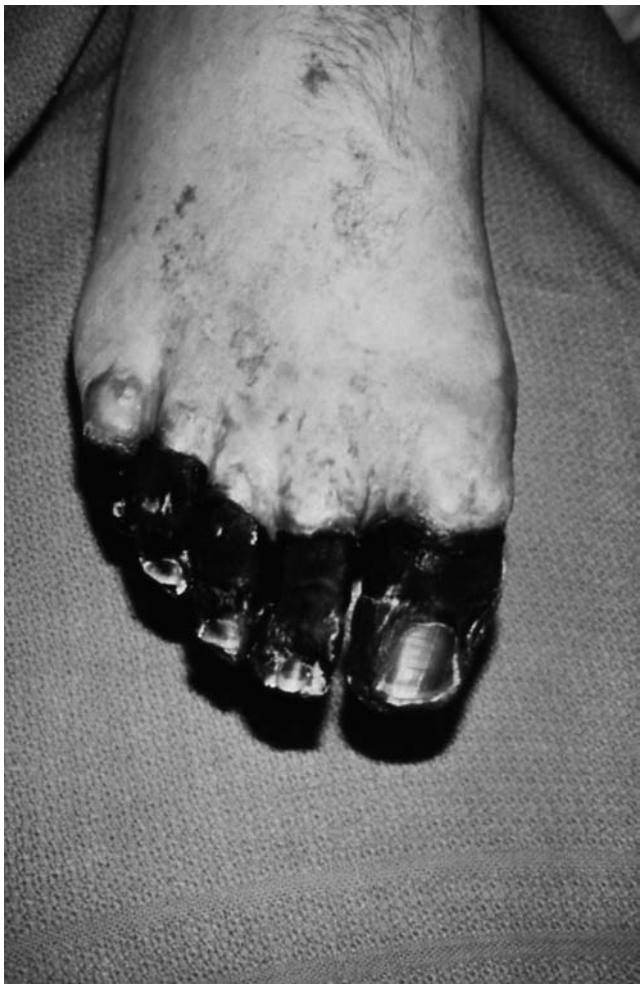


Figure 9-7 Toe necrosis in patient with disseminated (septicemia) plague. (Courtesy of CDC/William Archibald.)



fever, chills, headache, and exhaustion. Untreated infections may overwhelm the immune system and enter the bloodstream, leading to septicemic plague (which may in turn cause tissue necrosis; seen in Figure 9-7) and infection of the lungs. Lung infection is termed pneumonic plague and has the potential to spread to others by aerosolization of the bacteria via sneezing and/or coughing. The overall plague fatality rate is ~14% in the United States. Untreated bubonic plague is fatal 50 to 60% and untreated septicemic and pneumonic plague fatality rates are substantially higher. There are effective antibiotics to treat plague, including streptomycin, gentamicin, tetracyclines, and chloramphenicol.[10] In the 14th century, pneumatic plague is estimated to have killed one fourth of the population of Europe, and recent research suggests that a single point mutation in *Y. pestis*, enabling it to survive in the midgut of the rat flea, set the stage for it evolving from an organism causing mild human stomach illness acquired via contaminated food or water to the flea-borne agent of the "Black Death."[11]

Francisella tularensis is the causative agent of the disease tularemia (also known as "rabbit fever"). Of the two predominant subspecies, *F. tularensis tularensis* (Type A) is considered more virulent than *F. tularensis palaearctica* (Type B).[9] The bacterium is a Gram-negative, nonmotile, obligate aerobic coccobacillus found in rodents, rabbits, and hares in the United States. It is shown in Figure 9-8. There are ~200 annual cases of tularemia in the United States. Individuals become infected through the bites of infected insects (e.g., ticks), handling sick or dead infected animals, consuming contaminated food or water, or inhaling air-borne bacteria. Symptoms of infection usually appear within 3 to 5 days, with a range of 1 to 14 days. Symptoms vary by route of exposure. External or ingested exposure may lead to skin ulcers (Figure 9-9) and mouth sores, inflamed eyes and sore throat, swollen and painful lymph glands, and diarrhea and pneumonia. Inhalational exposure may lead to flulike symptoms including fever, chills, headache, muscle and joint pain, cough, and weakness. Pneumonia, while not contagious, may progress to chest pain, difficulty breathing, and respiratory failure. There are effective antibiotics to treat tularemia, including tetracyclines, fluoroquinolones, and streptomycin. Tularemia is considered a potential bioweapon because of its high level of infectivity. It is estimated that the inhalation of only 10 to 50 organisms is sufficient to cause infection.[12]

Figure 9-8 Stained *Francisella tularensis*. (Courtesy of CDC/Larry Stauffer, Oregon State Public Health Laboratory.)

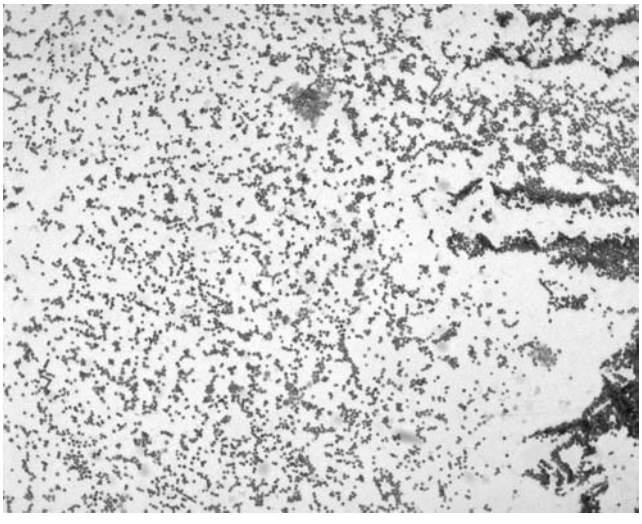


Figure 9-9 Skin ulcer caused by tularemia infection. (Courtesy of CDC/ Emory University; Dr. Sellers.)

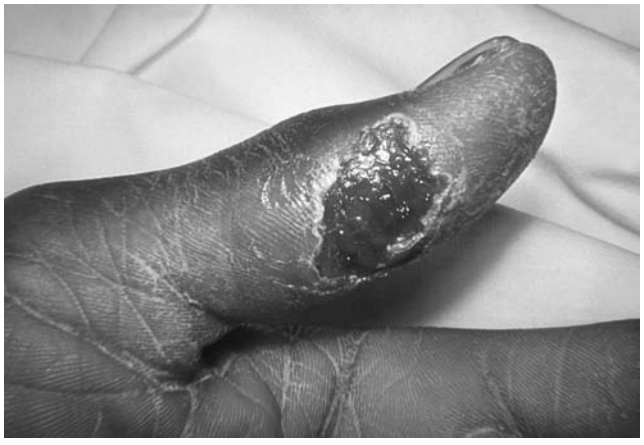
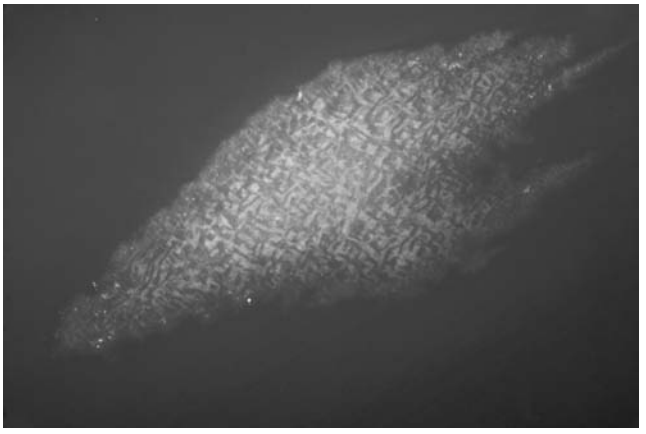


Figure 9-10 *Clostridium botulinum* type A colony on blood agar (5×). (Courtesy of CDC/Dr. Holdeman.)

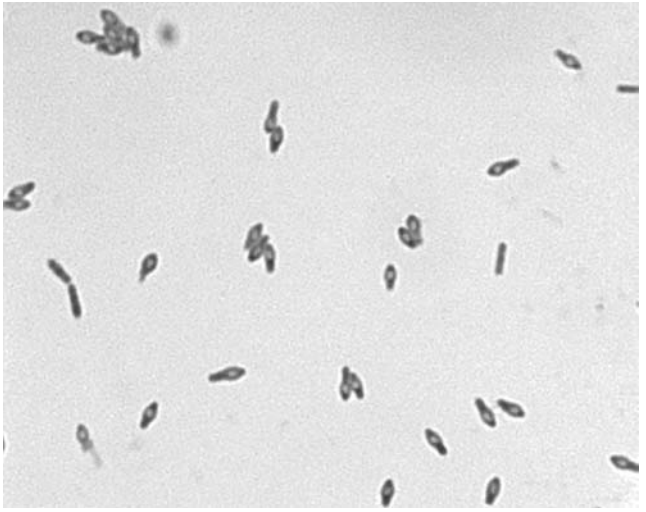


Sidebar 9-3 Naturally Occurring Tularemia in Missouri[13]

Upward of 40% of cases of tularemia in the United States each year occur in Arkansas, Oklahoma, and Missouri. The Missouri Department of Health and Senior Services undertook a retrospective analysis of 190 cases of tularemia in Missouri for the time period 2000 to 2007 with the intent of establishing its epidemiologic and clinical characteristics. Of the 190 cases, 87 were confirmed and the others classified as probable. They also found that three quarters of cases were contracted during the summer months, two thirds were contracted by males, and children were twice as likely as adults to be diagnosed with glandular tularemia, while adults were 10 times more likely than children to be diagnoses with the pneumatic form. Fifteen patients required surgery to incise and drain suppurated lymph nodes, and two others required thoracotomy and decortication. One individual (adult) died as a result of the illness, seven adults developed a more severe secondary form of disease, and six adults experienced severe organ dysfunction. Of importance to laboratories, 33 of the 39 culture-confirmed cases were identified by the laboratory without prior suspicion of tularemia presence. This underscores the importance of consistent and good laboratory practice at all times, and the adoption and use of measures appropriate for testing samples for tularemia in areas where it is endemic.

C. botulinum is the *Clostridium* species that produces the toxin botulinum, a potent neurotoxin. The bacteria are Gram-positive, rod shaped, anaerobic, motile, and found worldwide in soil. A culture of *C. botulinum* is seen in Figure 9-10, and spores are observed in Figure 9-11. The seven subtypes are distinguished by their respective neurotoxins (A to G). These toxins

Figure 9-11 *Clostridium botulinum* spores stained with malachite green. Image bank of full-color photos available online at http://www.jbpub.com/catalog/9780763771027/. (Courtesy of CDC/Larry Stauffer, Oregon State Public Health Laboratory.)



are the most acutely lethal of all known toxic natural substances.[9] Human LD_{50} (dose required to kill 50% of the test population) values ranging from 0.0004 (D) to 0.0025 (F) μg/kg.[14] Human cases are usually caused by strains A, B, and E (and rarely F), and there are ~145 reported cases in the United States annually. There are three main types of illness associated with infection. Food-borne botulism is caused by consuming food contaminated with the toxin. Wound botulism is caused by the infection of a wound with *C. botulinum*. Infant botulism is caused in infants by the consumption of spores, which subsequently grow in the intestine. Symptoms may occur within 6 hours to 10 days, varying in part by route and extent of exposure. Symptoms are associated with flaccid muscle paralysis caused by the toxin and include blurred vision and drooping eyelids, muscle weakness, slurred speech, and difficulty swallowing. These may progress to paralysis of the arms, legs, and respiratory muscles and death. Equine antitoxin is somewhat effective in removing blood-circulating toxin if administered early. Further treatment is palliative and may include the use of mechanical ventilators. Recovery can take multiple weeks, though the death rate has fallen to 3 to 5%.[15]

Smallpox is caused by infection with the variola virus. This is an orthopoxvirus, visible by light microscopy, and larger than some bacteria (seen in Figure 9-12). There are two forms of smallpox: variola major and minor. Variola major is the more frequently occurring and severe form with extensive rash and higher fever. There are four types of variola major:

- *Ordinary* accounts for > 90% of cases
- *Modified* is a milder version occurring in previously vaccinated individuals
- *Flat* is characterized by flattened pustules
- *Hemorrhagic* is characterized by confluent pustules

The historical fatality rate for all variola major is ~30%, but rates for both flat and hemorrhagic are in excess of 90%. Variola minor is a milder form of the disease, is relatively uncommon, and has a fatality rate of ~1%. Smallpox is spread by face-to-face contact, or contact with infected items such as body fluids or clothing. Symptoms may appear 7 to 17 days after exposure and follow a well-described course:

- The *prodrome phase* includes fever, malaise, and head and body aches. Duration is 2 to 4 days.
- This is followed by the *early rash* beginning in the mouth and progressing to the face and the extremities. The rash develops into bumps filled with opaque fluid. Duration is ~4 days.
- The bumps now develop into a *pustular rash*. The pustules are raised and sharply defined, usually round and quite firm to the touch (seen in Figure 9-13). Duration is ~5 days and the pustules begin to scab over.
- The scabs fall away leaving pitted scars. Duration is ~6 days.

Figure 9-12 **Transmission electron micrograph of a single smallpox virion (310,000×). (Courtesy of CDC/ J. Nakano.)**

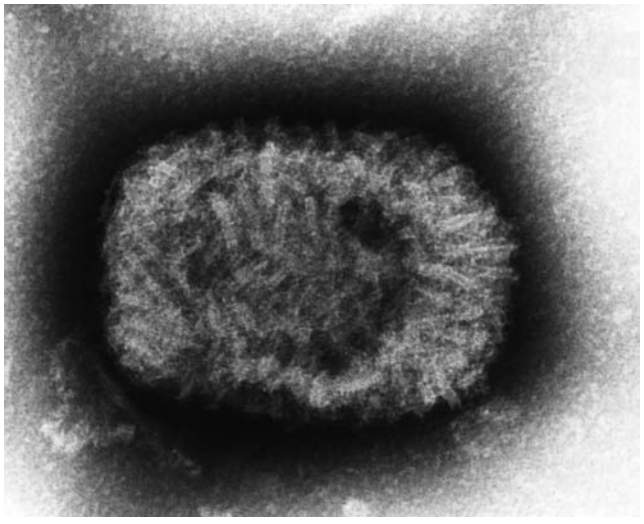


Figure 9-13 **Arm of an individual infected with smallpox during the late pustular stage. (Courtesy of CDC/Dr. John Noble, Jr.)**

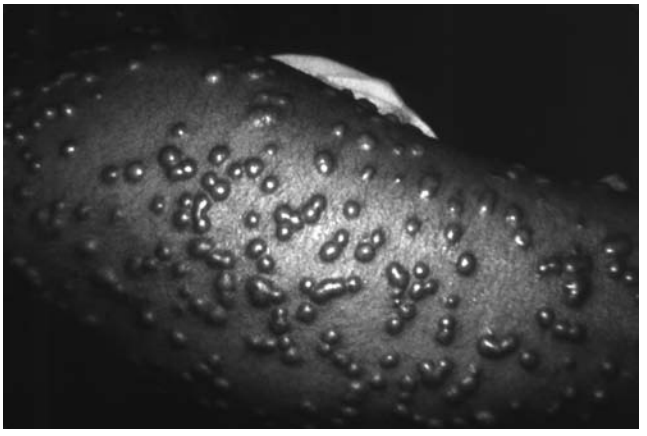


Figure 9-14 Scanning electron micrograph of the tip of the distinctive bifurcated smallpox inoculation needle. (Courtesy of CDC/Janice Carr; Photographer: Janice Carr.)

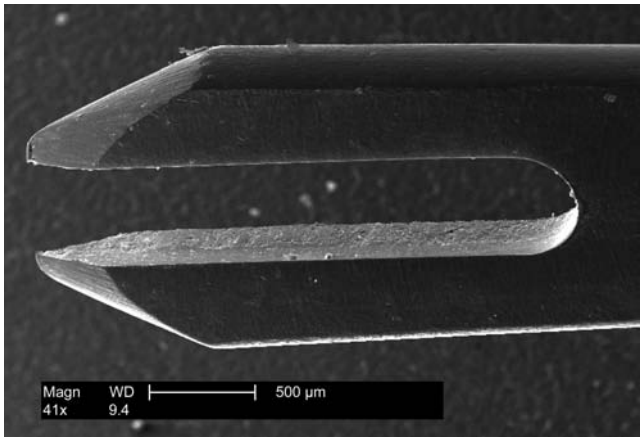
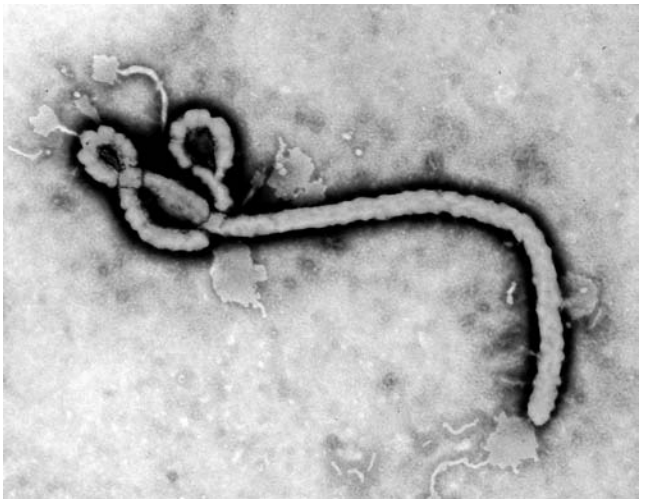


Figure 9-15 Transmission electron micrograph of an Ebola virion. (Courtesy of CDC/Frederick A. Murphy.)



There is no effective treatment for smallpox, though vaccination is effective both as a preventive measure and within 3 days of exposure. Vaccination against smallpox has a long history, dating back to the 18th century. A bifurcated needle used for smallpox vaccination is shown in Figure 9-14. Smallpox was the target of a worldwide vaccination program and declared eradicated by WHO in 1980. However, frozen stocks of the virus are reportedly maintained by the United States and Russia, and it is for this reason that variola major is still considered a potential bioweapon. The last naturally occurring case was in Somalia in 1977.[16]

Viral hemorrhagic fevers are a group of four virus families that cause multisystem illness in humans. Extensive vascular damage is characteristic of these fevers, resulting in substantial hemorrhage. The four virus families in this group are arenaviruses, filoviruses, bunyaviruses, and flaviviruses. These viruses are RNA-based, require an animal host for survival, and are geographically restricted to where the host animals live (absent rapid air travel for an infected individual). Human cases and outbreaks are irregular and difficult to predict, and there are no effective vaccines or treatments (with a few exceptions). Transmission usually occurs through close contact with infected host animals, though some (e.g., Ebola) are able to be transmitted person-to-person from the index case: directly through close contact with the person or indirectly through contact with contaminated items.

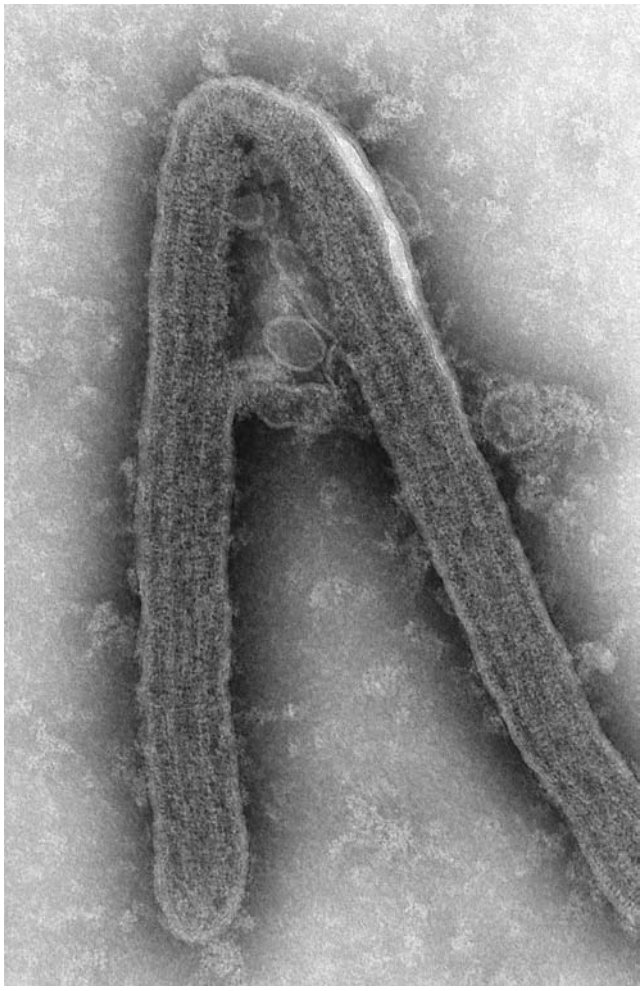
Symptoms may occur within 1 to 2 days of exposure or much later, and vary somewhat by virus. They generally include severe flulike symptoms such as high fever, fatigue, dizziness, and muscle aches. Severe cases show bleeding under the skin, in internal organs, and/or from body orifices (though blood loss is rarely the cause of death). Further symptoms include shock, nervous system malfunction, delirium, seizures, coma, and death. Treatment is generally palliative as there are no known effective treatments or vaccines for the majority of viruses. Fatality rates for some viruses (especially filoviruses such as Ebola and Marburg) regularly exceed 50% of cases. There are treatments for Lassa fever and Argentine hemorrhagic fevers. Hemorrhagic fever viruses as a group are of concern because of their high fatality rate and lack of treatment options.[17] Figures 9-15 and 9-16 show the well-known shapes of Ebola and Marburg viruses.

Category B Agents

Category B agents are the second highest level of threat and are "moderately easy to disseminate; result in moderate morbidity rates and low mortality rates; and require specific enhancements of CDC's diagnostic capacity and enhanced disease surveillance."[6] Many of them already naturally occur in varying circumstances. The following agents are considered in this category.

Brucellosis is the illness caused by *Brucella* spp. The bacterium occurs naturally in the United States in many farm and wild animals, and there are 100 to 200 human cases annually in the United States. Humans become infected when in contact with infected animals or their products, and person-to-person infection is rare. There are effective antibiotics for treatment, though recovery can take weeks to months.[18]

Figure 9-16 Transmission electron micrograph of a Marburg virion. (Courtesy of CDC/Frederick A. Murphy.)



Glanders is the illness caused by *Burkholderia mallei*. The bacterium primarily infects horses and there has not been a naturally occurring case in the United States since 1945. Humans become infected with direct contact with infected animals and the organism passes through the skin or mucous membranes. While bloodstream infections are usually fatal, there is evidence to suggest that multiple antibiotics may be effective for treatment for other sites of infection.[19]

Melioidosis (Whitmore disease) is the illness caused by *Burkholderia pseudomallei* and very similar to glanders. The difference is mainly in geographical extent and source of infection. Melioidosis is primarily tropical and humans are infected through contact with contaminated water or soil, though it does occasionally occur in the United States (fewer than five cases annually). Similar to glanders, bloodstream infections are often fatal though there are effective antibiotic treatments for other sites of infection.[20]

Psittacosis is the illness caused by *Chlamydia psittaci*. Since 1996, there are < 50 cases annually in the United States. All birds are susceptible hosts and transmission to humans is via inhalation of dried bird secretions. Symptoms usually appear in 5 to 19 days and antibiotics are effective for treatment though there are occasional fatalities.[21]

Q fever is the illness caused by *Coxiella burnetii*, which is found worldwide in many common farm animals (e.g., goats and sheep). Human infection usually occurs through inhalation of contaminated farmyard dust, though transmission through consumption of contaminated milk has also been reported. Acute infections may progress to chronic (illness exceeding 6 months). Antibiotics are effective for both presentations, though chronic infections are more difficult to resolve.[22]

Typhus fever is the illness caused by *Rickettsia prowazekii*. It is relatively rare with only 39 reported cases in the United States during 1976 to 2001. Transmission usually occurs through person-to-person spread of body lice or contact/association with flying squirrels (only known vertebrate reservoir).[23]

Epsilon toxin is produced by *Clostridium perfringens*, which is widespread in the environment and human digestive tracts, though the strains that produce epsilon toxin (B and D) do not usually infect humans. The bacterium must be consumed in large numbers to produce illness, and may be eliminated by properly cooking and handling food. The toxin may be isolated from cultured *C. perfringens*. There is no antidote for epsilon toxin poisoning, and treatment is supportive. Deaths are rare.[24]

Viral encephalitis is the inflammation of nervous tissue, specifically the brain and spinal cord. There are many viruses that cause encephalitis (e.g., measles, varicella zoster virus, and herpes simplex virus I) and most illnesses are mild. However, some have greater potential to cause serious illness or death, and these include some of the mosquito-borne alphaviruses such as Venezuelan equine encephalitis and eastern and western equine encephalitis. These are discussed in more detail in Chapter 5.

Ricin toxin is naturally occurring in castor beans and is extremely toxic by inhibiting protein synthesis (and is also often listed as a chemical agent). The toxin may be isolated from whole beans or waste from castor oil processing. The consumption of beans may release the toxin, and the isolated or purified toxin may be ingested or inhaled. Symptoms may appear within 4 hours of exposure. There is no antidote for ricin and treatment is supportive. Death may result from a lethal dose within 36 to 72 hours. Ricin was the agent used to fatally poison the Bulgarian writer

Georgi Markov in 1978. An umbrella was used to inject a small pellet containing ricin under his skin.[25]

Food safety threats is a blanket category that includes any organisms that may be purposefully introduced into food products. Such organisms include *Salmonella* species, *Escherichia coli* O157:H7, *Shigella*, and others, some of which are discussed in Chapter 7.

Water safety threats is a blanket category that includes any organisms that may be purposefully introduced into drinking or recreational water. Such organisms include *Vibrio cholerae*, *Cryptosporidium parvum*, and others, some of which are discussed in Chapter 6.

Category C Agents

Category C agents are the final level and include "include emerging pathogens that could be engineered for mass dissemination in the future because of availability; ease of production and dissemination; and potential for high morbidity and mortality rates and major health impact."[6] Some, such as hantavirus, already appear at irregular intervals. The other agent specifically listed in this category is Nipah virus. The list is not longer as it is not known which organisms may be changed, by either purpose or through natural mutation, from one possessing benign characteristics to those that are pathogenic to humans. The emergence of new and pathogenic agents, to which humans have not been previously exposed and have no natural immunity, underscores the need for continuous surveillance.

Chemical Agents

Chemical agents, and PHLs' responses to them, are much more complex and expensive than for their bacterial counterparts. This is because of the inherent differences between chemical compounds and bacteria/viruses/toxins and how they behave. For example, latex gloves are effective in protecting analysts from exposure to almost any biological agent, but may be quite permeable to many chemical agents. Some chemical agents (such as hydrofluoric acid) may also actively penetrate such defenses. Second, many compounds are carcinogenic, mutagenic, or outright lethal at microgram quantities of exposure and may have few, if any, effective exposure treatments. Finally, analyses are much more varied and complex. Whereas most microorganisms may be identified through varied protocols involving selective growth media, differential staining, immunoassays, and PCR, different classes of chemical compounds require substantially different methods of preparation and analyses. These are often quite costly as well. An rRT-PCR instrument may cost ~$35,000 (2008) and be able to provide analyses for any number of organisms. On the other hand, a liquid chromatograph–mass spectrometer may run in excess of $200,000 and be useful for only a handful of compounds.

The prohibition against the development, stockpiling, and use of chemical weapons is described by the Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on their Destruction (CWC), which is administered by the Organization for the Prohibition of Chemical Weapons (OPCW). The text of the CWC can be found on the OPCW Web site at http://www.opcw.org/. The CWC was approved by the United Nations in 1992, and opened for signature in 1993. At least 188 states have signed the Convention. However, there are four countries, including Russia and the United States, that still maintain stockpiles of chemical weapons (though there are elimination efforts currently underway). The Convention controls three classes of chemical substances. Schedule 1 compounds are those with no legitimate use outside of chemical weapons, while Schedule 2 and 3 compounds have some small- or large-scale legitimate uses.[26]

Unfortunately, the outright ban of all potential agents is not possible for a couple reasons. The first reason is that many of these compounds might be synthesized from readily available precursors with minimal equipment. Those individuals with training in organic chemistry may be able to determine the required steps to turn an innocuous industrial compound into a lethal agent. Secondly, many potential agents have significant and/or widespread industrial use. The World War I gas phosgene is used in industry, and fentanyl and botulinum toxin are used in medicine. Finally, many compounds used in industry are simply inherently toxic and their release into an unprepared population would cause significant injury or death. Some compounds that may be considered high-hazard by the North Atlantic Treaty Organization (NATO) include ammonia, carbon disulfide, formaldehyde, hydrogen bromide, fuming nitric acid, and sulfuric acid.[9]

Sidebar 9-4 Bhopal Disaster[27,28]

On December 3, 1984, the Union Carbide pesticide plant in Bhopal, India, accidentally released an estimated 42 tones of methyl isocyanate (MIC) gas. This disaster is considered one of the world's worst industrial accidents and > 500,000 people were exposed. It is estimated that 8000 people died within the first 2 weeks, with another 8000+ succumbing later from gas-related illness. An additional

100,000+ are estimated to have sustained permanent injuries. MIC is an intermediate compound used in the production of the pesticide carbaryl. MIC itself is produced from phosgene and other compounds. Factors contributing to the magnitude of the disaster were the use of these compounds in large scale, the storage of MIC in a few large tanks rather than several smaller ones, and the location of the plant quite close to a densely populated area (Bhopal). The absolute extent of exposure and subsequent injury is a matter of considerable dispute, with legal actions continuing into 2008. Web sites stating the positions and claims of both the Indian government and Union Carbide Corporation have been set up and are maintained. They may be found at:

- Bhopal Gas Tragedy and Relief and Rehabilitation Department (Government of Madhya Pradesh) at http://www.mp.gov.in/bgtrrdmp/
- Bhopal Information Center (Union Carbide Corporation) at: http://www.bhopal.com/

The use of chemicals as warfare agents has not been as extensively recorded as for biologicals. In fact, there are few recorded uses of any specific compounds before the Renaissance. This may be in part because of reluctance on the part of military personnel to pursue these types of weapons as they were considered "inhumane" by many. Sidebar 9-5 contains a brief list of the purposeful use of chemicals in warfare throughout history.

Sidebar 9-5 Selected Use of Chemical Agents Throughout History[1]

- 1000 BC: Chinese use arsenical smokes in battle
- 429 and 424 BC: Spartans use noxious smoke during the Peloponnesian war
- 200 BC: Carthaginians spike wine with mandrake root to sedate the enemy
- 1618–1648: Toxic smoke projectiles used during the Thirty Years war
- 1864: Proposed use of hydrochloric and sulfuric acids to defeat the Confederates at Petersburg, VA (plus other proposals; none used)
- 1914–1918: Extensive use of phosgene and other gases during World War I

Chemical agents saw their most widespread use during World War I where phosgene, chlorine, and other gasses were dispersed by both Allied (Entente) and Central Powers. During the course of the war, more than 110,000 tons were used, causing at least 1.3 million casualties and 90,000 deaths.[9] They have not been widely used in warfare since. A more recent known use of chemical weapons during war occurred during the 1980–1988 Iran–Iraq war, where it was concluded that Iraq used mustard and nerve agents on both Iranian military and civilian populations, causing in excess of 100,000 Iranian military and civilian personnel to seek treatment and > 25,000 deaths.[9] Reports of the use of such weapons by Iran were not fully investigated. Other reports of use came in association with the Libyan invasion of Chad and by the Cuban-backed government forces during the Angolan civil war.[1]

Of more potential importance for PHLs is the use of these and other agents by nonmilitary groups for the purpose of chemical terrorism (CT). The cult Aum Shinrikyo has used both chemical and biological agents in the past 20 years. In 1994, they released sarin in the city of Matsumoto resulting in 200 overnight hospitalizations and 7 deaths. In 1995, they placed five sarin-filled packages in five different subway trains in Tokyo. The result was in excess of 1000 hospitalized and 12 deaths. The cult also experimented with botulinum toxin, anthrax, cholera, and Q fever. A 1990 release of botulinum toxin was a failure and did not cause illness.[29]

Coordination of Federal and State Responses

The chemical side of the LRN is devoted to the promulgation and coordination of chemical terrorism preparedness activities. It is organized much the same way as the biological side and performs many of the same functions. On the Web site, laboratory staff will find sampling and testing methodologies, be able to order reagents, and participate in and report the results of performance tests. Also like the biological side, laboratory capabilities are stratified into three different levels.

As mentioned in the previous section on biological agents, one of the great lessons to be learned from the anthrax attacks in 2001, and subsequent "white powder" samples that flooded the CDC and state laboratories, was the value of a well-equipped and distributed system of analytical assets. Between October and December 2001, LRN laboratories performed in excess of 1 million analytical tests on 125,000 samples submitted from postal service facilities, newsrooms, and government offices.[4] This explicitly demonstrated that it is not a good idea for the CDC to be the sole entity with a specific

analytical skill set, which may be needed on a large scale with short notice. This has been addressed in biological analysis by the expansion and augmentation of biological agent analysis capability in all state PHLs. They now have the capacity to do much of what is reasonably expected, freeing the CDC and other federal resources for more specific investigative and diagnostic services.

The same lesson holds true for chemical analyses, though there are some significant caveats. First is expense. Biological analyses, even sophisticated ones using rRT-PCR in a BSL-2/3 setting, are relatively inexpensive to set up and maintain. For $100,000 a laboratory can purchase two rRT-PCR instruments and reagents sufficient for the analysis of hundreds of samples a day for most biological agents. For an initial outlay of $200,000 to $400,000, a laboratory can purchase essentially any and all equipment required for the analysis of almost any sample type for almost any biological agent. Chemical analyses are much more expensive in part because their instrumentation is more expensive, and more of it is required. A single ICP-MS for the analysis of metals in clinical samples may itself cost in excess of $100,000. The addition of LC-MS, GC-MS, and sample preparation devices can exceed $1 million. However, even this will not allow the same flexibility of analysis (any sample for any analyte); sample analysis is slower and maintenance is generally more expensive. Expense is not the sole difficulty though.

One of the great strength of biological agent analysis augmentation is the idea of dual use. That is, the PCR instrument purchased for anthrax analysis may be used for other purposes. And why not? Once the proficiencies have been performed, it is always better to keep an instrument in use as opposed to shutting it off for months at a time. Laboratories often use these instruments for the analysis of other clinical samples for other agents, such as influenza or pertussis. Many of the methods are identical to those for threat agents, and it is simply a matter of switching reagents and probes. Likewise, the analytes themselves are quite similar. Those who are trained in biological agent response are likely those who regularly perform clinical specimen analysis for these other organisms. The same cannot be said for chemical analyses. Many state laboratories perform few, if any, chemical methods. They therefore have no use for chemical analysis instrumentation and they remain unused outside of quarterly proficiencies. Personnel are also not so easily interchangeable, and it is expensive for the laboratory to maintain chemists for the sole purpose of maintaining chemical response equipment and performing proficiencies. There is frequently little else for them to do as they are not trained in microbiological or molecular analyses. In theory, they could be used to perform biomonitoring analyses (analyzing clinical samples for chemical compounds), but these activities are difficult to fund.

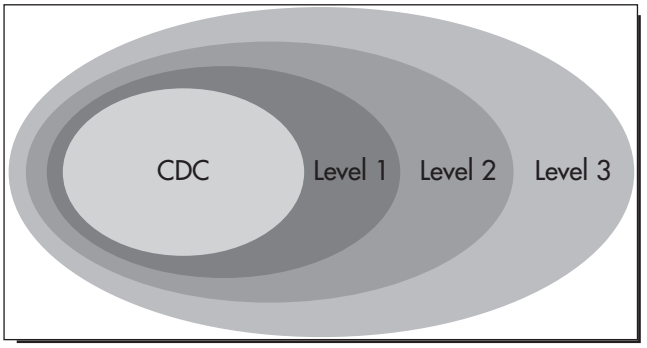
Laboratory Organization and Preparedness

For these reasons, and others, the national plan of response for chemical agents (as it applies for sample analysis) is different than for biological agents. Rather than augmenting previously existing capabilities in every state laboratory, the CDC has developed three levels of chemical response laboratory, illustrated in Figure 9-17. The most basic, Level 3, is required of every state PHL and has no analytic responsibilities. Level 2 retains all Level 3 requirements (see later), but is also augmented with some equipment (and personnel, if needed) to perform some limited analyses. Level 2 is not required, but each state is generally encouraged to have one. Finally, Level 1 laboratories receive a considerable investment in funding for instrumentation, personnel, and facilities. They are capable of performing analyses for almost any chemical agent in clinical samples and have significant depth and flexibility. They are also equipped to analyze large number of specimens.

Level 3 Activities and Capabilities

Level 3 activities are relatively few in number, complex in execution, and very time-consuming. Every state PHL is expected to participate in these activities, and in 2005 there were $>$ 60 PHL participants.[4] Just as the Preparedness grant requires minimum competencies and resources for biological analyses, there are minimum requirements for chemical agent response. Of significant difference is the lack of actual ability for any type of sample analysis by the laboratory. Whereas PHLs are expected to be able to analyze specific biological agents via specific methods and instrumentation, this is not a universal expectation in the case of

Figure 9-17 Illustration of the Laboratory Response Network structure for chemical emergency response. (Courtesy of CDC.)



CT preparedness. Level 3 responsibilities essentially revolve around the ability to instruct clinicians to collect, package, and ship samples from those potentially exposed; to handle and manage clinical samples received from clinicians; and to coordinate laboratory response planning. To explore these in more detail, we will consider the following discrete activities associated with clinical samples: training in collection and shipment, accept, store, package, and ship.

Training

One responsibility of Level 3 laboratories is the training of clinical workers statewide to perform the tasks of sample collection, packaging, and shipping to the state laboratory. This may be done by the chemical terrorism coordinator and/or the bioterrorism coordinator (who may also fulfill the role of state laboratory trainer). The chain of custody and legal requirements begin when the sample is first collected, and local hospital and clinic staff are those most likely to be collecting the samples. They therefore need to be trained in the process of collecting the samples (which types are needed and in what order they are collected) and sending them to the state lab (packaging, shipping, and form completion). Flowcharts created by the CDC are provided for training and detail the requirements for sample collection (Figure 9-18) and shipping (blood, Figure 9-19; urine, Figure 9-20). State laboratory training personnel spend a considerable amount of time providing such training sessions throughout the year.

Figure 9-18 Flowchart describing how blood and urine samples should be collected from those potentially exposed to a chemical agent. (Courtesy of CDC.)

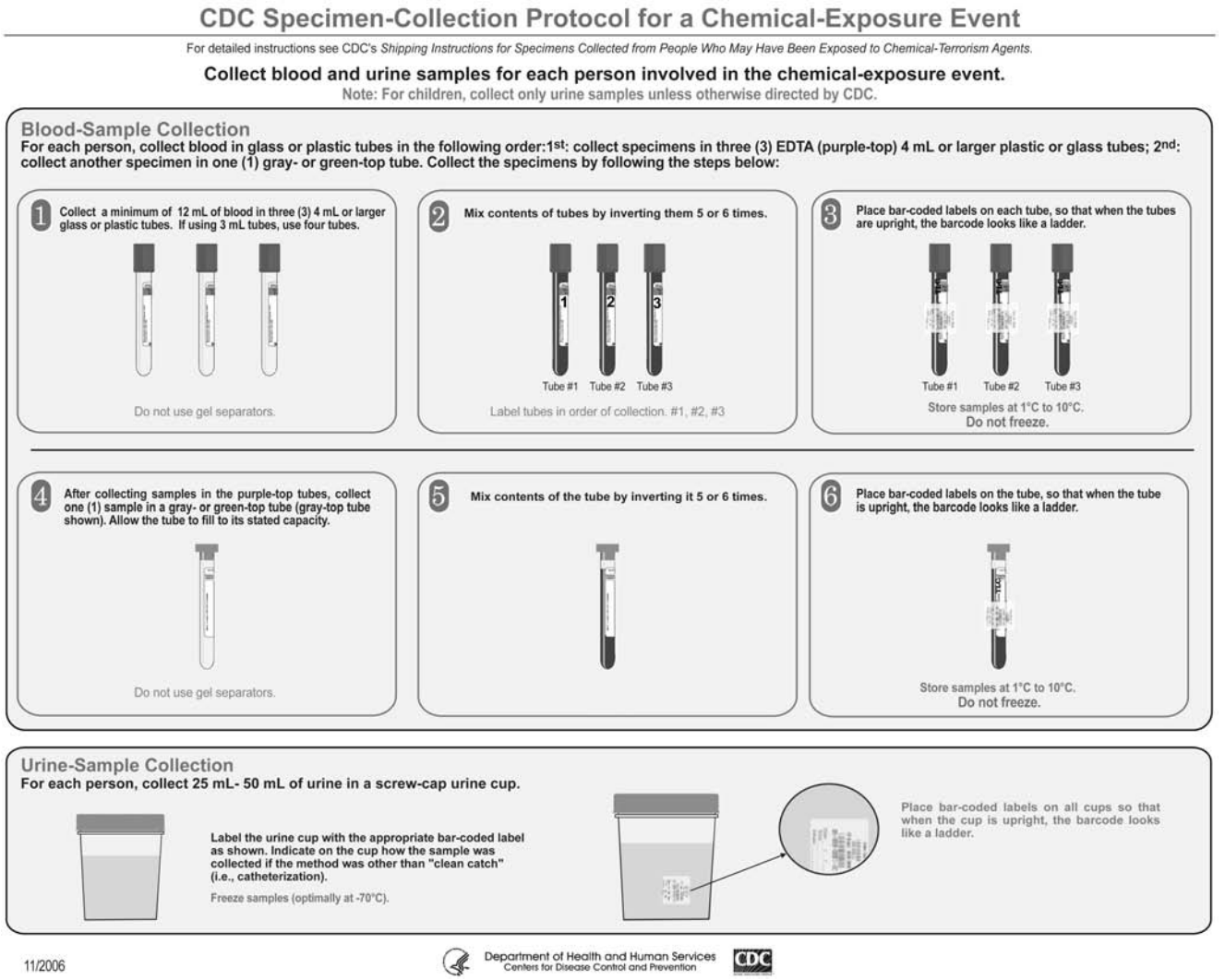
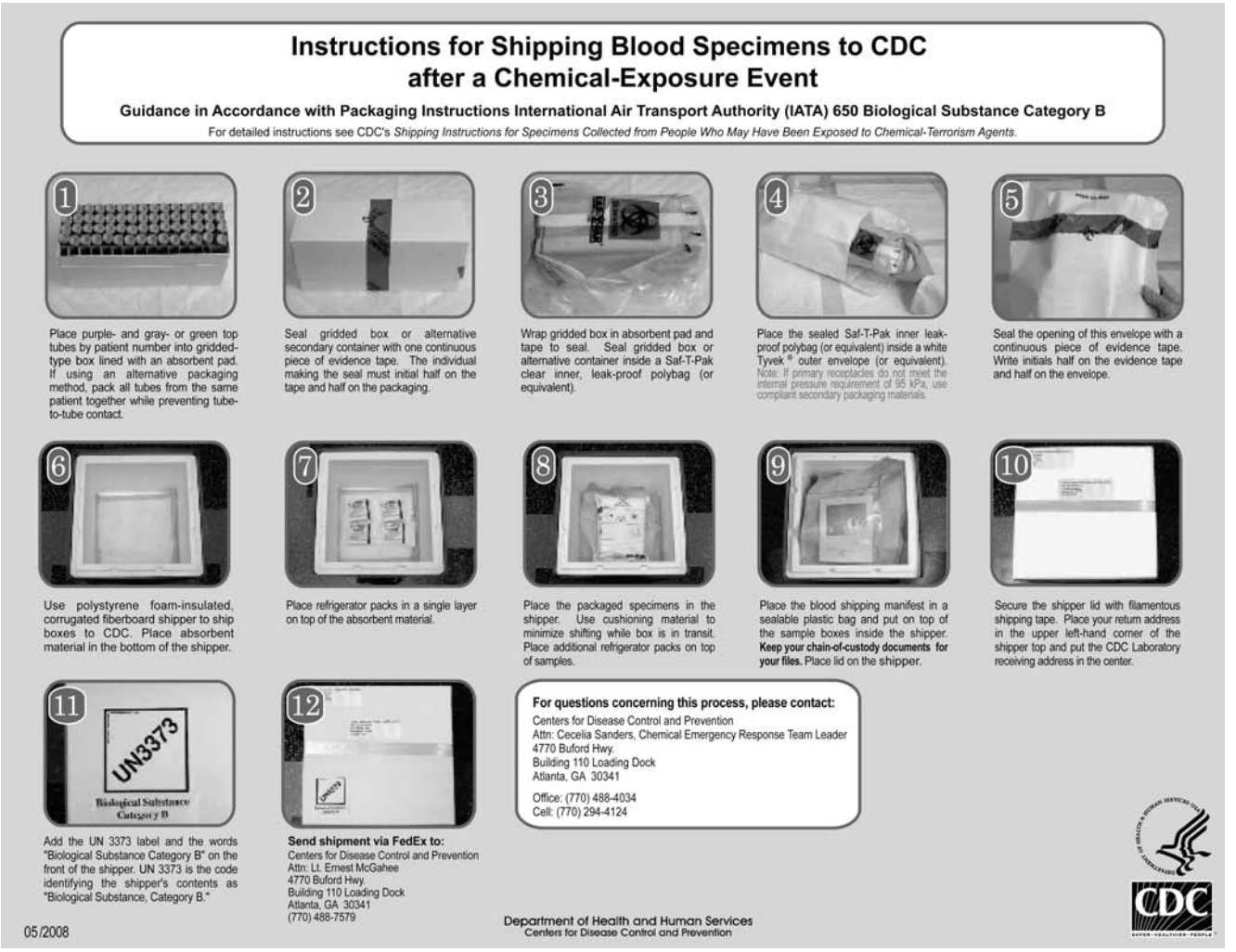


Figure 9-19 Flowchart describing how blood samples should be packed and shipped to the laboratory. (Courtesy of CDC.)



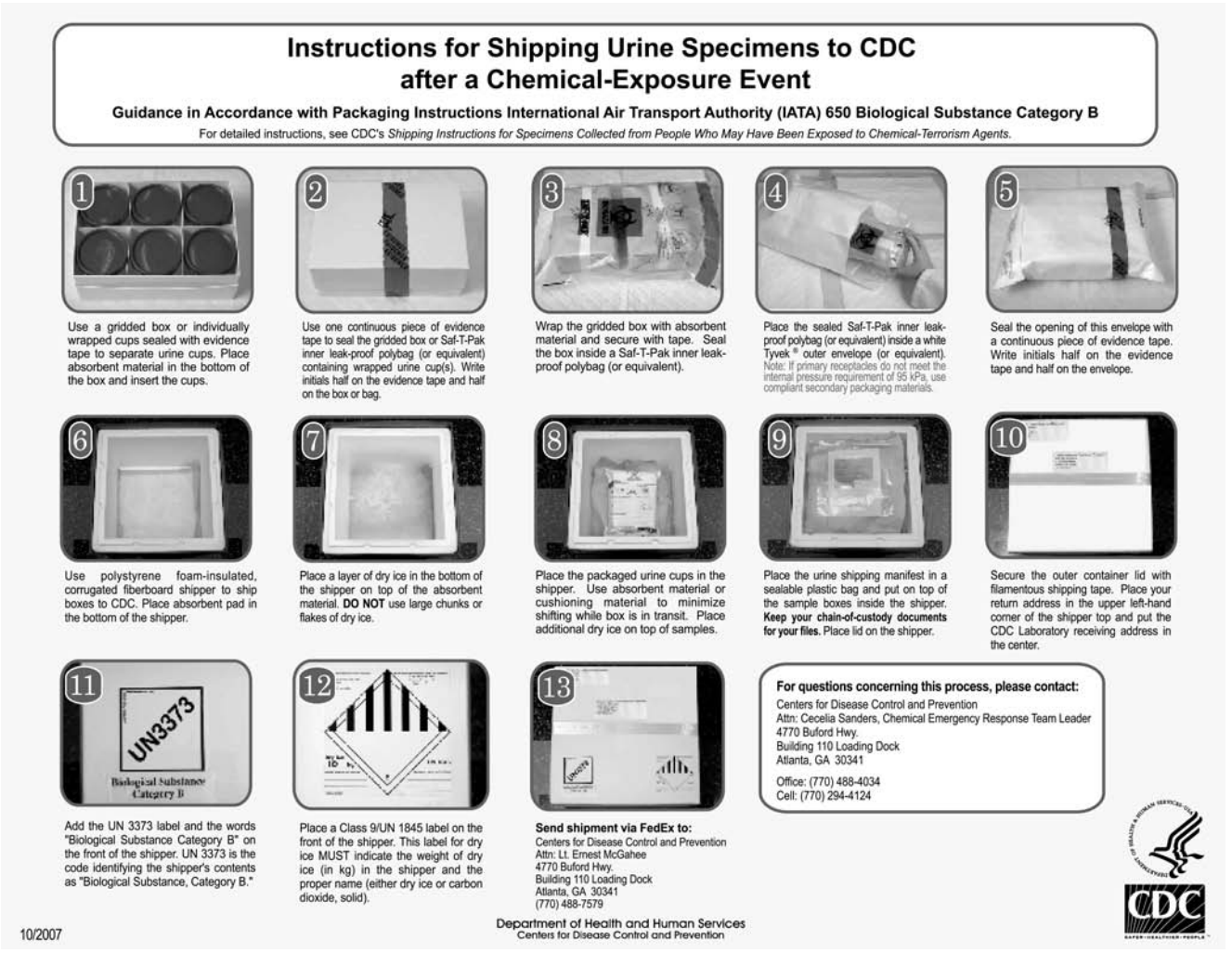
Accept

Most laboratories already have space set aside for opening, logging in, and distributing samples. This space is likely suitable for CT purposes as well. The differences come with the logging in of the samples. These are all, by their very nature, potential pieces of evidence to be used in future legal proceedings. They must therefore be accepted and handled with a legally robust chain of custody procedure. They must also be logged and documented according to CDC-specified protocols so that their handling and analytical fate may be coordinated at the national level.

Store

Sample storage may seem like a foregone conclusion and too basic to be tested. However, the issue becomes clearer when one examines the potential number of samples that may be submitted within a short time, and the amount of space available in currently existing refrigerators and freezers. Consider, for example, the blood samples drawn from those potentially exposed to a chemical agent. If we assume that this is a mass-casualty event, there could realistically be hundreds to thousands of people exposed. With four blood tubes collected from each individual, the laboratory is facing the need for storage space for literally thousands of samples. This may require one or more refrigerators just for these samples. The refrigerators must also be of a type, and in a position within the facility, to maintain adequate chain of custody for all these samples. Where these refrigerators will be obtained, and what will become of their previous contents, needs to be determined during annual evaluations and planning.

Figure 9-20 Flowchart describing how urine samples should be packed and shipped to the laboratory. (Courtesy of CDC.)



Package

Unless the PHL is Level 1 (or possibly Level 2), it is most likely that received samples will be subsequently sent elsewhere for analysis. This is certainly true for the first set of 40 samples sent to CDC for screening and preliminary agent identification. The host laboratory must therefore be able to properly package its acquired samples for shipment to another facility. While a fairly straightforward task, there are multiple steps that must each be performed correctly. This turns out to be quite labor intensive. Noncompliance with packaging instructions could result in anything from nondelivery to the intended recipient to loss of chain of custody. To ensure that laboratory staff are well trained, they should sign up for packaging and shipping proficiencies through the LRN.

Ship

The process of shipping these samples is a bit more involved than for more routine clinical samples. First, the chain of custody must be preserved and this requires the use of quite specific packaging, forms, and evidence tape to prevent/reveal sample tampering. Second, the sample temperature must be tightly controlled. The blood samples must be shipped at ~4°C (simple ice pack) and the urine at ~−70°C (dry ice required). They therefore cannot be shipped together, and the laboratory must maintain supplies of various shipping materials and dry ice and complete substantial shipping manifests and forms associated with the samples. This process of packaging for shipment, for just the 40 sample initial set, can be expected to take several hours.

Level 2 Activities and Capabilities

Level 2 activities are much more technical than Level 3 in the sense that they involve actual sample analyses, as well as maintaining all Level 3 activities. These laboratories have acquired the personnel, equipment, and training required to analyze clinical samples for selected chemical agents or their metabolites (e.g., cyanide, nerve agents, and metals). In 2005, there were 37 laboratories designated Level 2.[4] The actual analyses developed and promulgated by the CDC are constantly evolving and are not disclosed to the general public. While states are not required to apply for and maintain a Level 2 laboratory, they are encouraged to do so. The long-term problem for states with a Level 2 facility is continued funding for its activities and staff. Unlike personnel and equipment involved with microbiological analyses, the skills and equipment used for chemical analyses are not readily useable for other. In the event of declining grant funding, states must determine how to pay for people and equipment whose most frequent activities are quality assurance and performance evaluations.

Level 1 Activities and Capabilities

Level 1 activities are the most comprehensive and expensive to maintain. These laboratories serve as surge capacity for the CDC, are able to perform all Level 3 and 2 activities as well as analyze clinical samples for a much wider range of compounds, such as mustard and nerve agents and industrial compounds. As is the case with Level 2 laboratories, the actual analyses are not disclosed to the general public. In 2005, there were 10 laboratories at Level 1.[4]

Chemical Event Response

From what has been described previously, we find that responding to a chemical event is quite different than for a biological one. Many state laboratories lack the capacity for any sample analysis for chemical agents, while some are augmented with limited analytical ability. The CDC has developed a plan for responding to a chemical event so that the agent may be identified and the samples sent to the proper laboratory for analysis.

Should there be a chemical event/terrorism with an unknown agent released, the state may request assistance from the CDC. The CDC will then dispatch their Rapid Response Team to the site to help coordinate event response and sample collection. In addition, the first 40 sample sets (urine and blood) will be sent to the CDC for their rapid toxin screen. By utilizing the extensive resources at the CDC laboratories, they will quickly identify the released agent. At that point, the LRN will be used to coordinate the sending of further samples to members that have the analytical ability for the identified compound(s). Analysis results are then sent back to the CDC through the LRN and from there to the sending state.[4] In this way, the brunt of sample analysis is shifted from the CDC's laboratories to the states and they remain free for further response activities and are less likely to become overwhelmed.

Potential Chemical Agents

Defining what chemical compounds may or may not be potential threat agents is both difficult and somewhat arbitrary. Water, for example, can be quite toxic in large doses, or even small doses in certain circumstances. However, in order to prepare for adequate laboratory response, the list of all potentially harmful substances must be limited to those with the greatest potential for use and/or harm. The CDC maintains a listing of these compounds of current concern. The agents described in this section are grouped in order of either their source or effects on the human body. As a matter of interest, we will also show the chemical structure of many of the agents. The reader may find the complexity of many biotoxins interesting in comparison to the often simple structure of manufactured warfare agents.

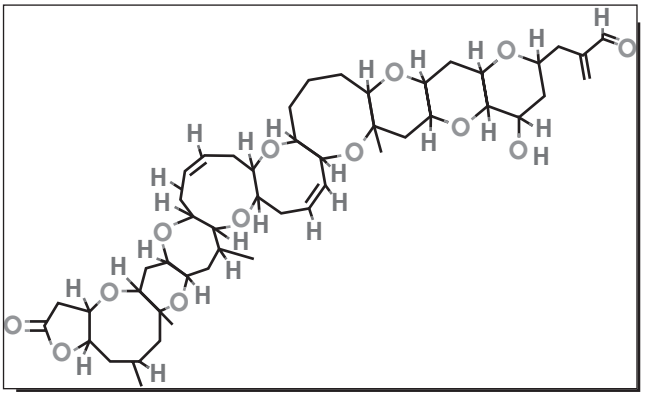
Biotoxins

Biotoxins are poisons that come from plants or animals. The toxins themselves are not alive in any sense, but are products of cellular activity. Because of their source, there is some inconsistency in their placement on the lists of biological or chemical agents. The toxin botulinum, for example, is produced by *C. botulinum* and is the causative agent for botulism poisoning. Though a toxin, it is often listed as a biologic agent. Biotoxins may be simple or complex chemical compounds, or proteins or enzymes. The latter are analogous to venoms made by poisonous snakes, though the effects are different. Some are toxic enough that the consumption of their source plant or seed is sufficient to induce illness. Others need to be extracted and concentrated to have significant effects. There are no known antidotes to any of the following biotoxins and the only available treatment is supportive care.

Brevetoxin

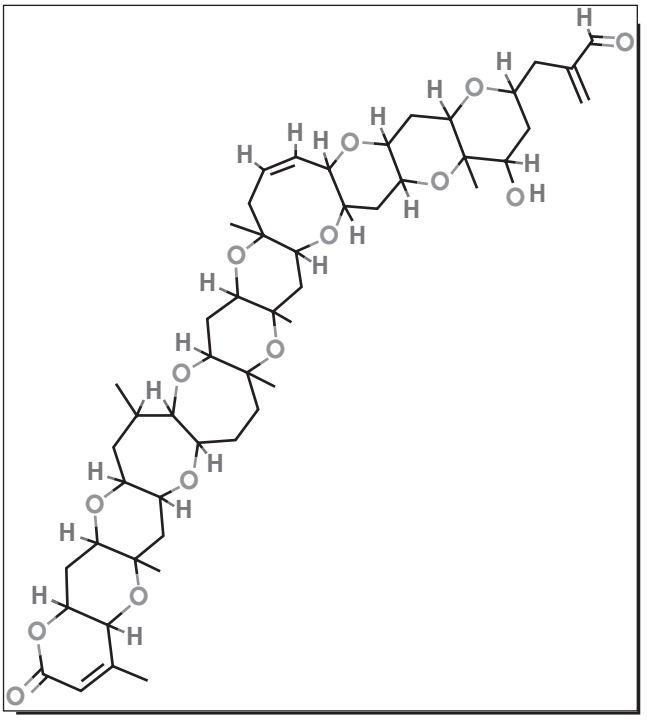
Brevetoxins are produced by a species of dinoflagellate known as *Karenia brevis* (*Gymnodinium breve* and *Ptychodiscus breve* prior to 2000) that are associated with

Figure 9-21 Structure of brevetoxin A. (Courtesy of National Center for Biotechnology Information [NCBI].)



"red tides." Shellfish consume these organisms but are not affected by the toxins. Human consumption of these shellfish, or the inhalation of contaminated sea spray, may result in neurotoxic shellfish poisoning (NSP). Brevetoxins may also be inhaled via sea spray exposure. There are two main chemical types of brevetoxin (A and B), with 10 identified subtypes.[30] The mice LD_{50} is estimated at 94 to 6600 mg/kg. Symptoms may begin within 15 minutes, or delayed up to 18 hours, depending on route and amount of exposure. Symptoms may include abdominal pain, diarrhea, vomiting, paresthesias, vertigo, and ataxia for gastrointestinal exposure and cough, dyspnea, and bronchospasms if inhaled.[31] The structures of brevetoxins A and B are shown in Figures 9-21 and 9-22.

Figure 9-22 Structure of brevetoxin B. (Courtesy of NCBI.)



Colchicine

Colchicine is the major medicinally active alkaloid of *Colchicum autumnale* L (and other species of the Liliaceae family). Purified, it is a pale yellow amorphous or crystalline powder. It is acutely toxic, a teratogen, and LD_{50} estimations vary widely with species tested (from 0.5 to 470 mg/kg intraperitoneal).[32] Symptoms of exposure include profuse vomiting and diarrhea. This may be followed within 24 to 72 hours by hypovolemic shock and organ failure. Finally, some succumb to coma, convulsions, and sudden death. Longer term complications may include bone marrow suppression, leukopenia, thrombocytopenia, and sepsis.[33] The structure of colchicine is shown in Figure 9-23.

Digitalis

Digitalis (digoxin; digitoxin) is obtained from foxglove, *Digitalis purpurea*. It has some cardiovascular medical uses such as preventing arrhythmia and heart failure. Symptoms of acute poisoning via ingestion include

Figure 9-23 Structure of colchicine. (Courtesy of NCBI.)

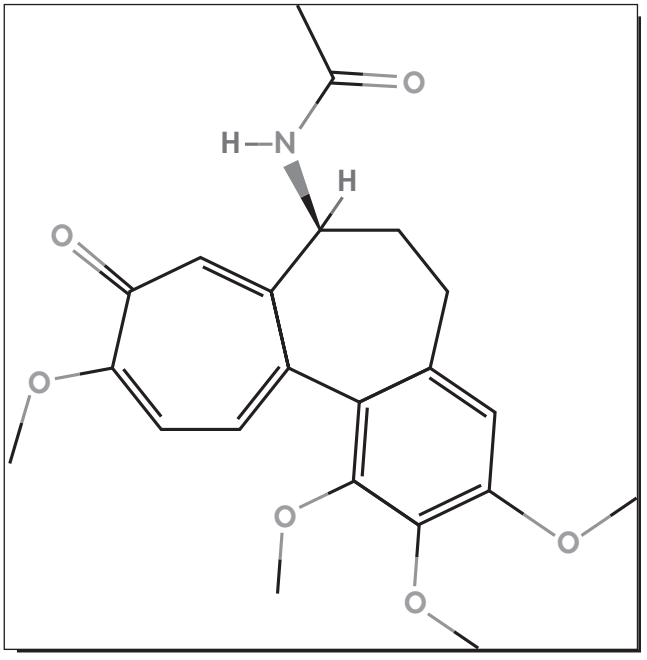
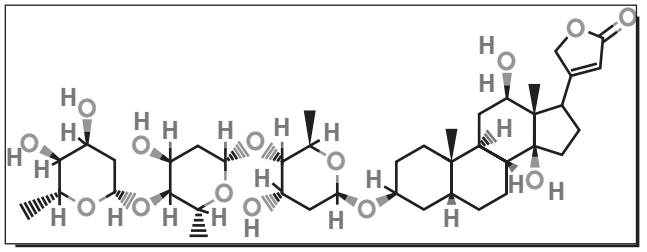


Figure 9-24 Structure of digoxin. (Courtesy of NCBI.)



nausea and vomiting, hyperkalemia, and cardiovascular effects.[34] It was recently featured in the popular media in the film "Casino Royale" where it was used to poison James Bond's martini. The structure of digitalis is shown in Figure 9-24.

Nicotine

The most common source of exposure to nicotine is via tobacco smoking, though it may be extracted, concentrated, and purified for other uses. As an individual compound, it is a highly toxic alkaloid. Ingestion symptoms include excessive oral secretions, vomiting and diarrhea, abdominal cramping, bronchorrhea, diaphoresis, mental confusion, and potentially convulsions. Heart rate and blood pressure may either increase or decrease depending on exposure level.[35] The structure of nicotine is shown in Figure 9-25.

Saxitoxin

Saxitoxin is the causative agent for PSP and there are 20 known chemical derivatives.[24] Exposures are associated with mussels, clams, cockles, and scallops. Strong control programs such as in the United States are important

Figure 9-25 Structure of nicotine. (Courtesy of NCBI.)

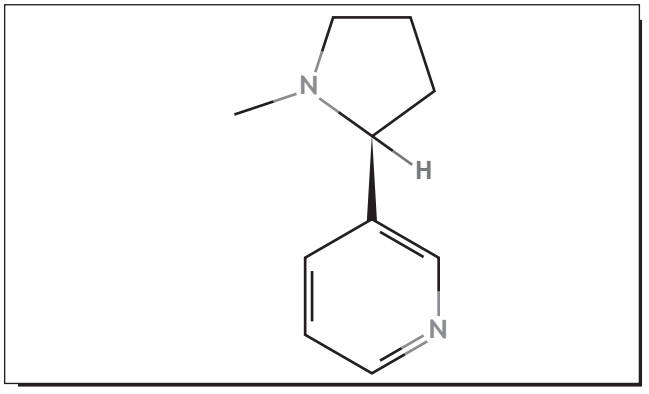
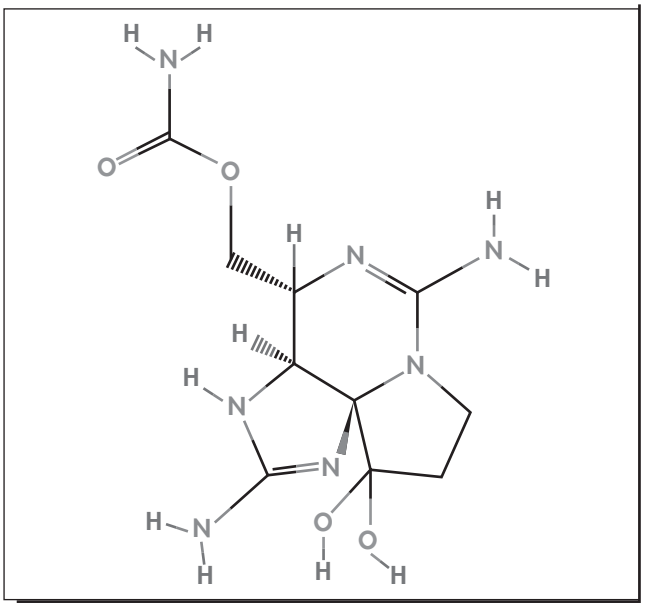


Figure 9-26 Structure of saxitoxin. (Courtesy of NCBI.)



to limit potential exposures. The symptoms associated with poisoning include tingling, burning, numbness, incoherent speech, drowsiness, and respiratory paralysis. Death may occur without respiratory support. Provision of such support within 12 hours usually ensures complete recovery. Diagnosis is based on presenting symptoms and recent food intake.[36] The structure of saxitoxin is shown in Figure 9-26.

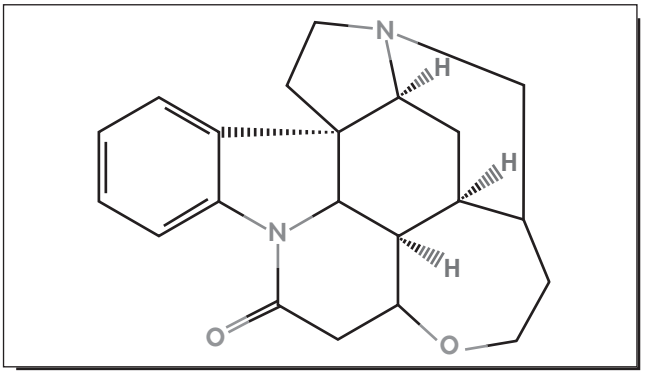
Strychnine

Strychnine is found in the plant *Strychnos nux-vomica* and is primarily used as a pesticide (specifically for rats). It is a white, odorless, bitter powder. In past centuries, it was used medicinally. Exposure can be from inhalation, ingestion, or injection and the time for symptom presentation varies by route. All exposure routes can result in agitation, apprehension, restlessness, muscle spasms, muscle pains and tightness in different areas (e.g., spine, jaw), difficulty breathing, and dark urine, leading to potential respiratory failure and death.[37] The structure of strychnine is shown in Figure 9-27.

Tetrodotoxin

Tetrodotoxin is the causative agent for puffer fish poisoning and is one of the most violent intoxications from a marine animal source.[24] The source of the toxin is not definitively known, but recent reports suggest it is the metabolic product of common marine bacterium (e.g., Vibrionaceae and

Figure 9-27 Structure of strychnine. (Courtesy of NCBI.)



Pseudomonas spp.). Reported poisonings are almost exclusively associated with the consumption of puffer fish from the Indo-Pacific area. Initial symptoms include numbness of the lips and tongue, followed by increasing paresthesia in the face/extremities, headache, nausea/vomiting, and diarrhea. Continuing symptoms include increasing paralysis, dysrhythmias, hypotension, and death. Death usually occurs within 4 to 6 hours after exposure.[38] The structure of tetrodotoxin is shown in Figure 9-28.

Trichothecene

Trichothecene is found in various fungi, including *Fusarium*, *Trichoderma*, *Myrothecium*, and *Stachybotrys*. There are in excess of 60 known related toxins. Symptoms are initially route specific. Dermal exposure may cause burning pain, redness, and blisters. Oral exposure may cause vomiting and diarrhea. Ocular exposure may cause blurred vision and inhalational exposure may cause

Figure 9-28 Structure of tetrodotoxin. (Courtesy of NCBI.)

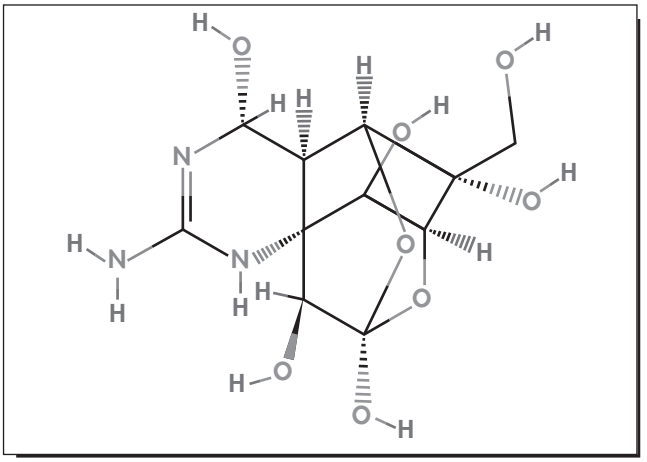
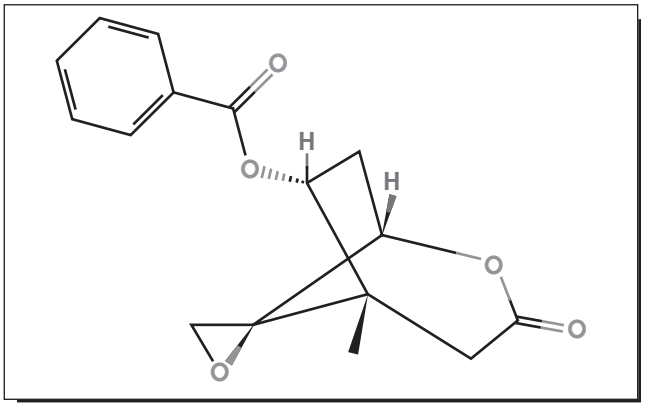


Figure 9-29 Structure of trichothecene. (Courtesy of NCBI.)



cough and nasal irritation. Systemic symptoms developing from each route are similar and include weakness, hypotension, ataxia, coagulopathy, and death.[39] The structure of trichothecene is shown in Figure 9-29.

Abrin

Abrin is a poisonous protein found in the seeds of a plant called rosary or jequirity pea. The seeds are red with a black spot. The seeds are sometimes used as herbal remedies and in jewelry. Powdered abrin is yellowish-white in color. Abrin is not known to have been weaponized, and there are no known instances of its use as a terrorism weapon. There are five known toxic proteins identified in abrin and human LD_{50} is estimated at 0.007 mg/kg.[14] Symptoms vary by route of exposure. Inhalation may cause respiratory distress, cough, nausea, and fever within 8 hours. Ingestion causes vomiting, bloody diarrhea, seizures, hallucinations, but is usually delayed 1 to 3 days.[40]

Ricin

Ricin is a poison protein found in castor beans (*Ricinus communis*). The toxin is released when the beans are eaten, and there is a known case where the consumption of a single bean resulted in death. Ricin can be separated from castor bean oil processing waste. The LD_{50} values in mice and rats for various exposure sites ranges from 2.8 to 8 μg/kg and lethal doses are reported to be ~1 μg/kg body weight for mouse, rat, and dog.[41] Symptoms of poisoning vary by exposure route and usually begin within 6 to 8 hours. Inhalation exposure may cause respiratory distress, tightness of the chest, cough, nausea, and fever, which may progress to low blood pressure, respiratory failure, and death. Ingestion exposure causes vomiting and potentially bloody diarrhea. This may be followed

by dehydration, low blood pressure, hallucinations, and seizures. Select organ failure and death may then occur.[42]

Blister Agents and Vesicants

These are chemical warfare agents that severely blister the eyes, respiratory tract, and skin on contact. They are alkylating agents that affect cellular DNA, suppress the immune system, and may enter the body via inhalation, ingestion, or skin absorption. Mustard agents, which are so named because of their color, particularly had widespread use in World War I and during the Iran–Iraq war. Experience with the ~400,000 soldiers exposed to mustard agent in WWI shows that the fatality rate is 2 to 3%.[9] The mustard agents have similar structures. The Lewisites are distinguishable by their arsenic component, and phosgene gained notoriety in WWI for use in trench warfare in Europe (and is used in large quantities in industry). The following agents are of concern to the CDC because of their extreme toxicity and history of use. Very few laboratories of any kind in the United States have facilities for the safe handling of environmental samples that may contain these agents. Provisional toxicity levels for these compounds place the LD_{50} at 1400 mg/70-kg man and the LCt_{50} (concentration in air required to kill 50% of the test population) at 1000 mg-min/m^3.[43]

Distilled Mustard

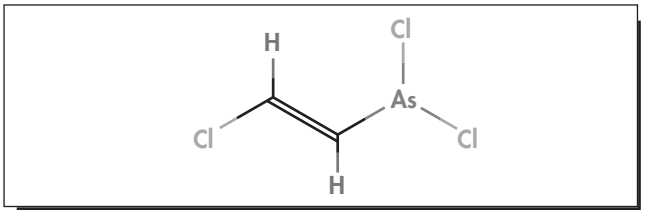
Distilled mustard (HD) is a pale yellow to brown liquid with a garlic odor. The eyes are more quickly affected than other exposed areas such as the respiratory tract. Symptom presentation may be delayed many hours postexposure and include tearing, burning, runny nose, sneezing, and hacking cough. This may progress to lid edema, corneal damage, ocular pain, productive cough, dyspnea, and vesication (blistering). Mustard gas (H; Levinstein; sulfur mustard) is the gas of WWI vintage and has much the same properties as HD. Symptoms and dosages are also essentially the same as HD. The structure of sulfur mustard is shown in Figure 9-30.

Nitrogen Mustards

These are colorless liquids with a soapy and/or fishlike odor (HN-1, HN-2, and HN-3). Nitrogen mustards are not known to have been used in warfare and their properties are quite similar to their sulfur cousins. They are more toxic than sulfur compounds, but the actual onset of pain may still be delayed by many hours. Most damage occurs to the eyes, skin, and respiratory tract. Exposure to high levels may be fatal.

Figure 9-30 Structure of sulfur mustard. (Courtesy of NCBI.)

Figure 9-31 Structure of Lewisite. (Courtesy of NCBI.)



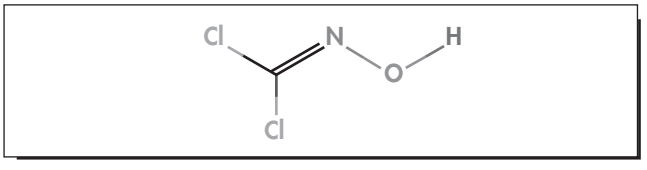
Lewisites/Chloroarsine Agents

These include Lewisite (L) and mustard/Lewisite (HL). L is an arsenical compound producing respiratory tract irritation and lung injury. Exposure may also cause eye lesions and dermal vesication. Sufficient dermal exposure may cause systemic intoxication or death. Lewisite is a brown liquid causing immediate tearing and skin pain. There are no verified warfare uses of L alone, but it has been used as a freezing point depressant for H.[9] HL is a liquid mixture of the two compounds (e.g., 37% HD) and has a garlic odor. HL is considered equally potent to H alone, but less potent than L alone. The structure of Lewisite is shown in Figure 9-31.

Phosgene Oxime

Phosgene oxime (CX) is a colorless, crystalline deliquescent solid with an irritating odor resembling new-mown hay at low concentrations. Exposure results in almost immediate pain; irritation and severe tissue damage on skin; and pain, conjunctivitis, and corneal inflammation in the eye. Unlike the mustard agents, the effects are not delayed. The structure of phosgene oxime is shown in Figure 9-32.

Figure 9-32 Structure of phosgene oxime. (Courtesy of NCBI.)



Sidebar 9-6 Disposal of WWII Chemical Agents in the North Sea[44,45]

During World War II, Nazi Germany and some Allies amassed vast quantities of chemical weapons that were not used in combat (though the agent Zyklon B was used in some Nazi concentration camps). At the end of the war, large volumes of these compounds were captured by the Allies. The question then became one of what to do with these weapons. Common methods of disposal in the 1940s included burying, open pit burning, detonation (with subsequent dispersal/dilution in the air), and sea burial. In the 1940s, the United States dumped 32,000 tons of captured German chemical weapons at sea and the British another 100,000 tons (plus an additional 75,000 tons of their own compounds). Much of this was placed in decrepit ships that were taken to the Baltic Sea and scuttled.

The emerging problem in the Baltic and North Sea areas is that these munitions are starting to leak. After years of immersion in salt water, the shell casings of these chemical weapons are corroding and becoming brittle. It was thought at the time that any leaking chemicals would be neutralized by the water, but it is relatively common for fisherman to find clumps of mustard agent clinging to their nets. Because of fish stock depletion, fishermen are now pursuing fish into riskier areas (nearer to where the ships and munitions were dumped) and using tackle that drags the bottom of the sea. They occasionally bring up entire chemical bombs that are unstable and likely to leak after 60 years under water.

To combat this problem, many ships now carry heavy waterproof gloves, masks, and special medications and powders to treat those exposed. The Danish government has also established a protocol whereby their Navy is called if vintage munitions are recovered. The ship is decontaminated, the catch destroyed, and the ship reimbursed for the loss of the catch. In this way, they hope to better safeguard the heath of the crew and potential consumers. Other methods of addressing this problem, such as finding and retrieving these weapons or encapsulating them in place with concrete, are impractical because of the high cost and unknown authority/responsibility for such projects.

Blood Agents

Blood agents are poisons that affect the body by being absorbed into the blood. They prevent cell respiration and the transfer of oxygen from blood to tissues. The cyanogen agents inactivate the cytochrome oxidase system, preventing cell respiration and the transfer of oxygen from blood to tissue. Lethal concentrations for hydrogen cyanide and arsine gas are 2860 and 7500 mg-min/m^3, respectively.[43]

Arsine (SA): SA is a colorless gas with an odor like garlic. Exposure may cause abdominal pain, confusion, headache and dizziness, nausea and vomiting, and weakness.

Carbon monoxide is a colorless, odorless, tasteless, and nonirritating gas produced by the combustion of organic matter. Exposure may cause headache, nausea and fatigue, followed by tachycardia, hypertension, dizziness, respiratory distress, and death.

Cyanide compounds: There are different cyanide compounds of concern. Most important is hydrogen cyanide (AC), a volatile, colorless liquid with a faint odor of bitter almonds. Exposure causes giddiness, headache, difficulty breathing, and chest pain. Greater exposure causes deeper breathing that may be followed by respiratory arrest and death. Cyanogen chloride (CK) is similar to AC with the hydrogen atom replaced with chlorine. At high concentrations, the effects of CK are similar to AC. It is also distinguishable by its lacrimatory effects and irritating odor. Also of concern are the potassium and sodium cyanide salts KCN and NaCN.

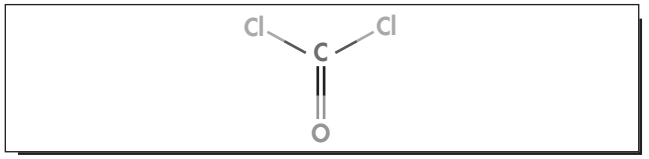
Choking Agents

These are chemicals that cause severe irritation or swelling of the respiratory tract (lining of the nose, throat, and lungs). They also cause tearing, coughing, tightness of chest, nausea, vomiting, and headache. In cases of higher exposure, the membranes may swell and the lungs fill with fluid. Death can result from "choking" but is relatively rare except in circumstances of extensive/prolonged exposure.[43]

Chlorine (CL): CL is a greenish yellow gas (Cl_2). Exposure produces immediate symptoms such as larynx muscle spasms; burning of the eyes, nose, and throat; bronchitis; and asphyxiation.

Phosgene (CG, DP): Phosgene may come in two forms, was widely used in WWI, and is commonly

Figure 9-33 Structure of phosgene. (Courtesy of NCBI.)



used in industry today with ~1 million tons produced annually in the United States.[9] It is a colorless gas that is readily liquefied and has a smell similar to musty hay or rotting fruit. The full physiological effects of exposure may be delayed up to 72 hours. Exposure is via inhalation/ocular and LCt_{50} is estimated at 1500 mg-min/m^3.[43] Phosgene DP is similar to CG, but exposure produces a heightened lacrimatory effect. The compound is a colorless liquid with a musty hay odor. The toxicity is approximately the same as for CG. The structure of phosgene is shown in Figure 9-33. Other chemical compounds that are potential choking agents and are of concern include hydrogen chloride, ammonia, bromine (CA), methyl bromide, methyl isocyanate, and osmium tetroxide.

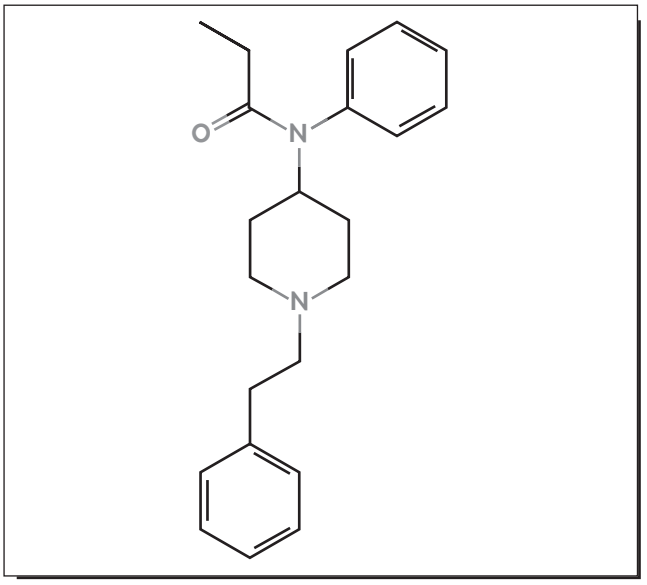
Incapacitating Agents

These are drugs that make people unable to think clearly or that cause an altered state of consciousness (possibly unconsciousness). Incapacitating agents are distinguishable from other agents in that their lethal dose is many times higher than the effective dose, they do not seriously endanger life, and they do not produce permanent injury. Conceptually, the drugs whose primary effects are behavior/psychological may be classified into four categories: deliriants, stimulants, depressants, and psychedelics.

BZ: BZ is a deliriant that produces its effects at very low dosage. It is a white crystalline solid and exposure is primarily by inhalation.

Opioids: Fentanyl and other opioids are drugs that have effects similar to morphine and work by depressing a bodily functional activity or instinctive desire as well as pain reduction. Fentanyl specifically depresses heart rate and respiration and causes lethargy and immobilization. The effects are near identical to heroin, but the drug is hundreds of times more potent. A fentanyl derivative was used by Russian Special Forces to rescue hostages taken by Chechen

Figure 9-34 Structure of fentanyl. (Courtesy of NCBI.)



rebels in the Moscow opera theater in 2002.[43] The structure of fentanyl is shown in Figure 9-34.

Nerve Agents

Nerve agents are highly poisonous chemicals that work by preventing the nervous system from working properly. They are all liquid at room temperature, though their respective volatilities vary. They are divided into the G and V agents, with the V agents having higher boiling points and lower volatility. All produce the same physiologic effects by interfering with the enzyme acetylcholinesterase (AChE). AChE works by neutralizing the activity of the neurotransmitter acetylcholine (ACh). In the absence of this neutralization, ACh accumulates and causes continuous nervous activity. This causes the symptoms of headache, blurry vision/difficulty seeing, runny nose, excessive flow of saliva, tightness of chest/difficulty breathing, nausea, and muscular twitching around areas of exposed skin.[43] Nerve agents are also one of the few compounds/classes of compounds for which there are effective antidotes. The military has developed a binary antidote consisting of atropine and 2 PAM Cl. Atropine competitively binds to nerve receptor sites, blocking the activity of excess ACh. 2 PAM Cl reactivates the neutralizing enzyme AChE.

The following agents are of concern to the CDC because of their extreme toxicity and history of use by terrorists (Aum Shinriko in Tokyo). Very few laboratories of any kind in the United States have facilities for the safe

Table 9-1 Lethal Doses and Concentrations of Nerve Agents of Concern[43]

Agent	LD_{50} (mg/70-kg man)	LCt_{50} (mg-min/m^3)
Tabun	1500	70
Sarin	1700	35
Soman	350	35
VX	5	15

handling of environmental samples that may contain these agents. Because of the swift effects of these agents, and the time required for sample collection and analysis, their detection in clinical samples is more useful for determining who/extent of exposure and coordinating longer term care and follow up rather than guiding treatment. Toxicity levels for these compounds are provided in Table 9-1.

G Agents

Tabun (GA) was the first agent developed, originally as a pesticide, and primary exposure is via inhalation. It is a colorless to brown liquid with no odor when pure. *Sarin (GB)* was developed shortly after tabun and shares many of its physical properties (though is more volatile). *Soman (GD)* has similar chemical properties to tabun and sarin, but is significantly more toxic via dermal exposure. In addition, part of the standard antidote for nerve agent poisoning (2 PAM Cl) is not as effective for soman as it "ages" quickly upon uptake. The structure of sarin is shown in Figure 9-35.

V Agents

VX is colorless and odorless when pure and significantly less volatile than the G agents. It is also much more toxic, and its lowered volatility results in increased persistence in the environment. The structure of VX is shown in Figure 9-36. *VR* agent has actually been produced in higher quantities than VX, but little information has been published on this compound. Even less is known about a new type of agent termed *novichok*, which is thought to be significantly more toxic than VX or VR.[9]

Figure 9-35 Structure of sarin. (Courtesy of NCBI.)

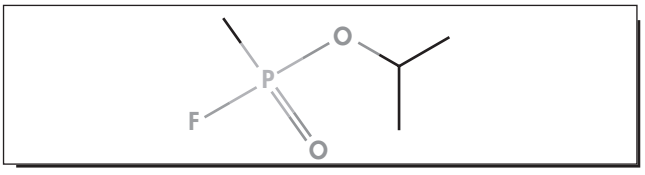
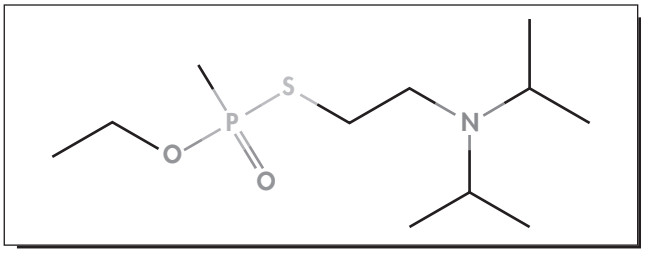


Figure 9-36 Structure of VX. (Courtesy of NCBI.)



Riot Control Agents/Tear Gas

These are highly irritating agents normally used by law enforcement for crowd control or by individuals for protection. They produce sensory irritation or disabling physical effects (e.g., nausea) within seconds to minutes after exposure. Fortunately, the effects are transitory and usually resolve within 15 to 30 minutes after exposure is terminated. *Chloroacetophenone (CN)* is a now-obsolete military agent and was replaced by *O-Chlorobenzylidene malononitrile (CS)* in the United States in1959 and has similar properties. CN is more toxic and less potent/effective than CS. CS is a white crystalline powder often dispersed as a gas. Exposure results in intense burning in the eyes, nose, and mucous membranes resulting in tearing and runny nose. In addition, there is a tightness of the chest, a feeling of unable to breath, and skin irritation. *Dibenzoxazepine (CR)* is less toxic and more potent than CS and has similar effects. Other agents of concern include *chloropicrin (PS)* and *bromobenzylcyanide (CA)*.[43] The structure of CS is shown in Figure 9-37.

Figure 9-37 Structure of CS. (Courtesy of NCBI.)

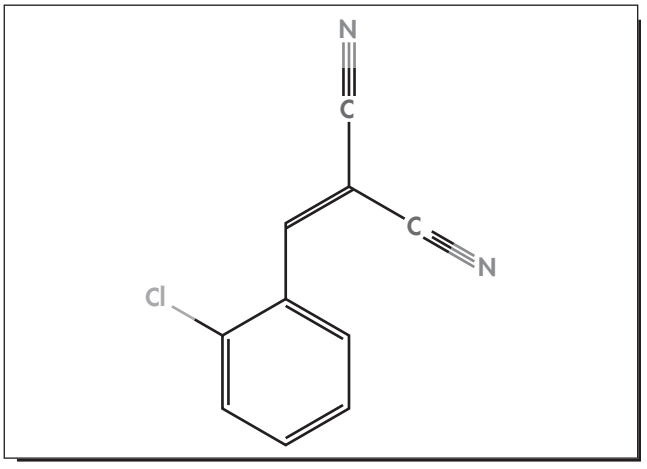
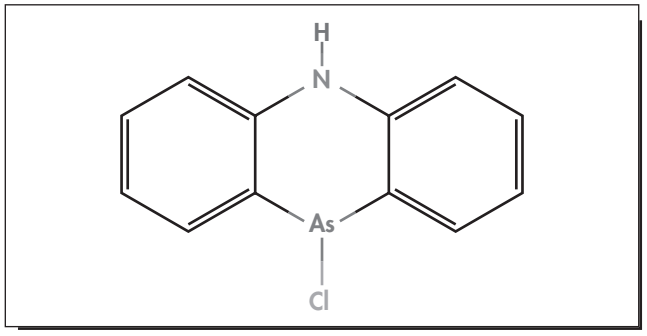


Figure 9-38 Structure of adamsite. (Courtesy of NCBI.)



Vomiting Agents

Members of this class of compounds generally produce vomiting upon exposure. However, some might be more properly called respiratory irritants as that is their primary action. The one compound in this class of CDC concern is adamsite (DM). Symptoms are often delayed by several minutes after exposure and include burning in the nose, throat and chest, rasping cough, and acute general depression. DM has been associated with human deaths, though its provisional lethal concentration is relatively high (LCt_{50} = 11,000 mg-min/m^3).[43] The structure of adamsite is shown in Figure 9-38.

Other Chemical Agent Categories

There are other agent categories that either have few compounds or that are very broad in scope. Many of these are used in industry in very large scales and the potential for a large release is not trivial. Others are not used in such large quantities, but are readily available and quite poisonous:

- *Caustics (acids)* are chemicals that burn or corrode people's skin, eyes, and mucus membranes on contact. The compound if highest concern is hydrofluoric acid (HF; hydrogen fluoride). Many are used in industry.
- *Long-acting anticoagulants* are compounds that prevent blood from clotting properly, which can lead to uncontrolled bleeding. Warfarin was developed as a rodenticide and is also used in medicine. *Super warfarin (brodifacoum)* is a weaponized version that has greater effects at lower doses.
- *Metals* are agents that consist of metallic poisons. Many are used in industry, but are also acutely toxic (e.g., methyl mercury). Toxic metal compounds of interest include those containing arsenic, barium, mercury, thallium, and other heavy metals. Light metals such as lithium are not generally considered as toxic.
- *Organic solvents* are compounds that damage the tissues of living things by dissolving fats and oils. An example is the industrial solvent benzene.
- *Toxic alcohols* are poisonous alcohols that can damage the heart, kidneys, and nervous system. An example is ethylene glycol that is widely used as an engine coolant/antifreeze and is acutely toxic.

Radiological Agents

The nation's coordinated response to terrorism and other emergencies is constantly evolving. Preparedness for biological agents and their release/spread was largely an augmentation of already existing abilities in state PHLs. That is, most states had some level of working infrastructure for the collection, transport, and analysis of clinical samples for different biological agents. The CDC and LRN standardized many of these methods and provided needed updates to equipment, personnel, training, and communications. Preparedness for responding to chemical emergencies is more difficult and expensive. Many state PHLs had minimal, if any, chemical analysis ability. While funding has allowed increased communication and training through the LRN for Level 3 activities, not all are able to actually analyze samples for chemical agents. Those that are able can do so for a limited number of compounds.

In much the same manner, radiological analysis response is evolving. The CDC is the chief public health entity to respond to a radiological event, but the role of the state PHLs is still in the process of being defined. To date, there are no publicly promulgated roles for PHLs in response to radiological events, or methods for analysis. We are not implying that sufficient analytical capability does not exist, just that it resides in places other than PHLs. This is likely because of a number of reasons.

Existing Capacity

There are relatively few PHLs equipped to perform radiological analyses. As was discussed in Chapter 4, radiological analyses can be fairly complex and require instrumentation that cannot be used for nonradiological purposes. These instruments are more likely to be found in state's departments of nuclear safety or EPA. Equipping a substantial number of state PHLs to perform these

analyses would be quite expensive. The CDC therefore currently relies on existing partnerships with other Federal agencies for proper response to this type of event.

Lack of Methods

A quick search of various Web sites (e.g., National Environmental Methods Index [NEMI], EPA) will show that there are many methods available for the analysis of radiological agents in environmental samples such as water and soil. Complementary methods for the same radionuclides in clinical samples are lacking. For example, we know from Chapter 4 that alpha particles are easily blocked by the sample matrix. Common methods to solve this problem include evaporation, precipitation, and scintillation. How would these be accomplished with a blood sample? It is likely that in a crisis situation an experienced analyst could create a method for emergency use, but the ongoing need is for standardized methods with associated quality control that can be promulgated through the LRN.

Is There a Need?

Biological agents have the ability to multiply, may require as few as 1 to 10 copies to cause illness, and may be transferable from person to person. Identification and treatment are critical for infection control. Chemical agents may be delivered in large quantities, cause acute illness by touch alone, and require identification for proper treatment. One may argue that radiologicals do not pose the same level of threat to health. Of the three discussed radiological decay products (alpha, beta, and gamma), all may be washed from an individual and only strong (highly energetic) beta particles and gamma rays are able to penetrate the skin and potentially cause damage. Alpha and beta are much more of a concern if there is an internal exposure. None are likely to cause acute illness unless there is a large and/or concentrated source to which a person is directly exposed.

The point to take from this is that the measurement and identification of radiologicals in clinical samples may not have the same urgency for treatment seen in biological and chemical samples. Part of the CDC's response to a radiological event includes the establishment of an Exposure Registry. This is used to identify those who were potentially exposed and perform dose reconstructions to determine to what levels as well as track exposures for those involved in the response. This registry will then be used to determine the long-term medical follow up that may be required.[46] The future role of PHLs in this area of response is still under development.

The past decade has seen tremendous growth in ability of PHLs to respond to a terrorist attack or other emergency. The Preparedness grant has done a great deal to properly equip PHLs nationwide to respond both locally within their jurisdiction and also serve as surge capacity for their partners. While not discussed, this has been observed in the response to hurricane Katrina (where newborn screening samples were shipped out-of-state for analysis when the Louisiana PHL was evacuated) and in the emergence of influenza H1N1 in 2009 where laboratories were inundated with samples and exercised their surge capacity. The CDC, APHL, and other organizations have also developed networks of information dissemination, laboratory training, results communication, and national surveillance, which allow the United States to be better prepared to respond to an event, and also more likely to detect a new event at an earlier stage.

Discussion Questions

1. What are some characteristics of biological agents that make them attractive to terrorists?
2. How has the CDC bolstered the ability of PHLs to respond to terrorist and other emergency events? What more can/should be done?
3. Outside of the immediate testing and decontamination, what has been the longer term impact of the anthrax attacks on the US mail system?
4. How does terrorism preparedness on the part of PHLs translate into preparedness for other events?
5. In preparing your laboratory to respond to an act of potential biological terrorism, which of the following would be of greatest concern, and why: the ability of staff to safely handle samples, the ability of staff to identify likely agents, the ability of staff to handle large numbers of samples?
6. The last known case of smallpox occurred approximately 30 years ago. Why is it still a concern?
7. How might public health officials determine that a case of anthrax is a result of natural exposure and not bioterrorists?
8. Creating and maintaining a national ability for biological agent surveillance is expensive and time consuming. Given that many of the Category A agents may be effectively treated with antibiotics, what arguments can you offer to justify the expense

of surveillance, as opposed to simply treating cases when they might appear?

9. We find many more examples of the use of biological agents through history than we do for chemical agents. Why might that be?

10. What are some characteristics of chemical agents that make them attractive to terrorists?

11. In preparing your laboratory to respond to an act of potential chemical terrorism, which of the following would be of greatest concern, and why: the ability of staff to safely handle samples, the ability of staff to identify likely agents, the ability of staff to handle large numbers of samples?

12. Few laboratories are prepared to handle environmental samples potentially contaminated with lethal chemical agents. What might they do to become prepared and would it be worth the expense? Why or why not?

13. Go to the APHL's Web site Emergency Preparedness and Response at http://www.aphl.org/aphlprograms/ep/Pages/default.aspx. Describe some of the different activities in which laboratory staff may participate to increase their preparedness. Which specific activities might be most beneficial for a laboratory?

14. Exercises are often key components of preparedness planning. How might laboratories participate in preparedness exercises and what might they test in addition to actual sample analysis ability?

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10

Operations and Management

Bernard Johnson

Introduction

The management of a public health laboratory (PHL) is dependent on its operations, which are program dependent, and the structure of its relationships with public health programs. PHLs are structured in a variety of ways: as a division within a public health agency, as part of a state university, or even as a separate laboratory agency. The pros and cons of these arrangements are well described in Novick and Mays' *Public Health Administration: Principles for Population-Based Management*.[1] While change is unlikely to occur within the PHL system with which one interacts, it is useful for planning purposes to understand the weaknesses and the strengths of the system.

On the surface, PHLs are similar to commercial laboratories. PHLs, hospital laboratories, and commercial clinical laboratories generally provide similar diagnostic testing; PHLs and commercial environmental laboratories provide the same testing on water, air, food, etc. There are two obvious distinctions of PHLs that set them apart in terms of management: (1) the mission of a PHL is not profit based; and (2) the mission of a PHL is to provide laboratory data to public health programs. The former distinction does not relieve a manager of fiscal constraints of course, as the bottom line is as challenging at "zero" as it is at a "positive net balance" for private laboratories. The not-for-profit approach of a PHL is consistent with testing operations used to document regulatory compliance (e.g., in the milk industry) and reference testing (e.g., identification of difficult organisms for a hospital laboratory; testing of dairy samples). The second distinction, collecting laboratory data, is critical to understanding what sets a PHL apart. The data generated by a PHL includes not just test results on patients to aid in diagnoses or on environmental samples to meet regulatory compliance, but also demographic data. Analysis of this data is used by public health programs to recognize developing or evolving trends in disease outbreaks, for targeted interventions, and as the basis for public health policy.

Personnel

Personnel rules for a PHL follow those of other organizations. However, the laboratories often provide testing that is federally regulated, which, in turn, impacts personnel roles and responsibilities.

Testing of clinical specimens impacts patient care and thus is regulated under the US Department of Health and Human Services (DHHS), Centers for Medicare and Medicaid Services (CMS), Clinical Laboratory Improvement Amendments (CLIA) of 1988, and CLIA regulations (42 CFR Part 493). Testing of environmental samples falls under the requirements of the US Environmental Protection Agency (EPA). Testing of dairy products is regulated by the US Public Health Service/US Food and Drug Administration (FDA) *Grade "A" Pasteurized Milk Ordinance*. These and other regulations and a number of certification programs in which PHLs participate overlay on to the normal personnel organization chart of the laboratory.

Under CLIA, for example, the clinical laboratory has a laboratory director who may or may not be the laboratory manager of all operations. The CLIA

laboratory director is charged with meeting the requirements of the regulations, and to do so in a complex laboratory environment, they must delegate roles and responsibilities. These roles, as defined under CLIA, include testing personnel, general supervisors for day-to-day benchwork, and technical supervisors who bear the responsibility of providing competent, knowledge-based guidance in selecting test methods, ensuring adequate quality controls, and interpreting results. Because a PHL is part of a political structure, the roles under CLIA may or may not match well with an individual employee's skill set, experience, and label. For example, a bench supervisor in the laboratory's organizational chart may be both CLIA testing personnel and a technical supervisor. A technologist may have the skill set to be the CLIA general supervisor in a test area, and thus acts as the supervisor of fellow technologists, and yet not be an administrative supervisor of those technologists. This can be a challenge in a union environment, which structures roles and responsibilities without regard to the regulatory requirements that certify the laboratory to perform testing.

Whatever the titles of various roles of laboratory personnel, all certification programs have similar requirements for the supervision, review, and quality of internal operations. Environmental laboratories, with the support of the EPA, have worked together to standardize individual state accreditation programs. The result has been the National Environmental Laboratory Accreditation Program (NELAP), which has made it possible for a laboratory certified by a participating state to be certified by reciprocating states. This is an important step forward in sharing resources between PHLs for those states that inspect and certify private environmental laboratories.

Facility Design

Throughout this book, we have discussed different types of tests and the organisms, compounds, and radionuclides for which they look. We have yet to discuss the physical environment in which this testing occurs, and will do so here. Though infrequently considered by the general public, the design of laboratories is critically important to the ability of laboratory staff members to accurately and safely analyze the samples submitted. Poor procedures and poor design can easily compromise the health of laboratory personnel and the accuracy of test results. This section will discuss the different types of facility design and practices associated with the analysis of biological, chemical, and radiological agents.

Biological Analyses

It has long been recognized that those who manipulate biologic agents have the potential for acquiring infection under study. There has therefore been a steady development of administrative, procedural, and engineering controls to limit the potential for infection. The current state-of-the-art for safe practices and design in biological analyses can be found in the Centers for Disease Control and Prevention's (CDC) *Biosafety in Microbiological and Biomedical Laboratories* (BMBL). This book outlines a progressive system of laboratory safety using laboratory technique, safety equipment, and facility design to provide increasing levels of primary and secondary containment. *Primary* containment refers to the protection of personnel and the immediate laboratory. *Secondary* containment refers to protection of the environment surrounding the laboratory. Laboratory techniques such as mechanical pipetting and personal protective equipment (PPE) use, and the use of safety equipment such as safety cabinets are the main contributors to primary containment. Facility design such as single-pass air and the use of double-door entry contribute to secondary containment. The BMBL outlines four biosafety levels (BSLs 1 to 4), with each level building on the one before and retaining all previous features.[2]

Biosafety Level 1

This is the simplest level and is exemplified by high school and undergraduate laboratories. It is appropriate for working with organisms not known to cause disease in healthy people. The BMBL lists organisms suitable for analysis at this level (and the others) and examples include *Bacillus subtilis* and *Naegleria gruberi*. There is a door to limit foot traffic, appropriate hazard signs are posted on the door, and waste is segregated (e.g., chemical, radiological). Work is performed on an open bench and a sink for hand washing is available. The lab should be designed so it can be easily cleaned, but does not need to be isolated from the rest of the building. PPE is employed such as lab coats, gloves, and eye protection. Personnel are prohibited from eating, drinking, and smoking in the lab and must wash their hands when exiting the lab. No specific primary or secondary barriers are recommended. The laboratory is supervised by an individual who has general training in microbiology or a related science and is responsible for providing training and establishing work procedures. An example layout of a BSL-1 laboratory is shown in Figure 10-1.

Figure 10-1 Illustration of a laboratory utilizing BSL-1 features. (Courtesy of CDC/ Office of Health and Safety.)

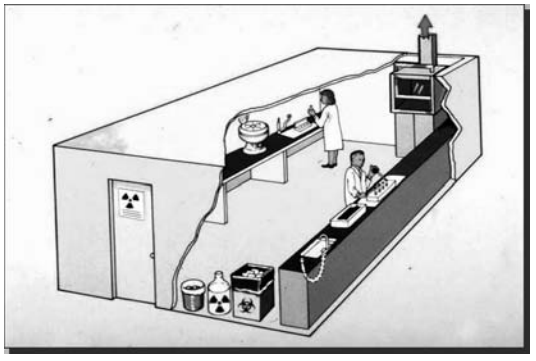
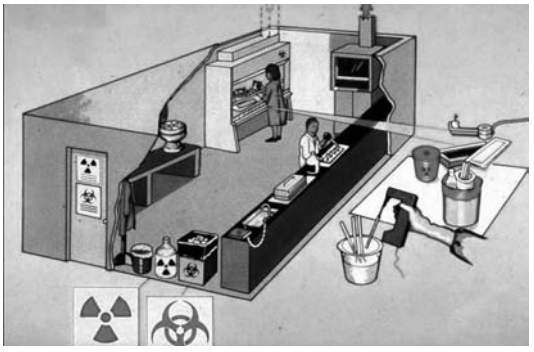


Figure 10-2 Illustration of a laboratory utilizing BSL-2 features (including mechanical pipetting and the use of a biological safety cabinet). (Courtesy of CDC/ Office of Health and Safety.)



Biosafety Level 2

This is the second level of laboratory design and incorporates all BSL-1 concepts plus additional ones. BSL-2 is suitable for handling organisms that have moderate risk to personnel and are not known to be transmitted by aerosol route. Furthermore, there are recommendations for immunizations for those working in BSL-2. Examples of organisms that may be handled in this environment include *Toxoplasma* spp. and hepatitis B virus. In addition to the door, access is restricted by the supervisor to those who have been trained and need to be in the laboratory. Some work may be done on an open bench, but any manipulations that may cause an aerosol or splash of infectious material is done in a biosafety cabinet (BSC). Waste is still segregated, and infectious waste must be mechanically of chemically decontaminated. Special attention is paid to objects that may pierce protective equipment ("sharps" such as needles) and these are often collected in special containers. Other procedures commonly employed include decontaminating work surfaces after use, wiping materials and equipment with 70% ethanol (a disinfectant) before placing them within the BSC, and keeping an accident/incident log. Some laboratories also collect "baseline" serum samples from personnel. These can then be compared to future samples to determine if there has been any immunologic response to agents manipulated by the laboratorian. Primary barriers such as splash shield and face protection should be used as appropriate. Secondary barriers such as hand washing sinks and waste decontamination facilities must be available. The laboratory is supervised by an individual who has a technical understanding of the risks associated with the microbial agents likely to be encountered, and who is responsible for granting laboratory access, providing training and establishing work procedures, and developing the laboratory's biological safety manual. An example layout of a BSL-2 laboratory is shown in Figure 10-2.

BSCs deserve a special description because of their importance. They are cabinets in which work with infectious substances are performed. They are designed such that air is drawn across work surface in a single direction, minimizing flow disruption and cross contamination. They are also equipped with high efficiency particulate air (HEPA) filters that remove in excess of 99.97% of all particles 0.3 microns (μm) or larger. There are different styles of BSC, but the most frequently used is the Class II type A (BSC-IIa). Performing work in these cabinets is likely the single greatest method for reducing personnel infection and laboratory contamination.

Biosafety Level 3

This is the third level of laboratory design and incorporates all BSL-1 and 2 concepts plus additional ones. BSL-3 is suitable for handling organisms that may cause serious or lethal disease as a result of exposure via inhalation. Examples of organisms that may be handled in this environment include *Mycobacterium tuberculosis* and *Coxiella burnetii*. BSL-3 should be located away from high traffic areas and are equipped with a number of physical enhancements, which are the major differences between BSL-2 and BSL-3. Entry is through a double-door system (e.g., with an anteroom before the actual laboratory) that minimizes air disruption from door openings/pressure changes. All air into the laboratory is single-pass and exhausted outside.

Figure 10-3 Illustration of a laboratory utilizing BSL-3 features (including single-pass air and increased use of PPE). (Courtesy of CDC/Office of Health and Safety.)

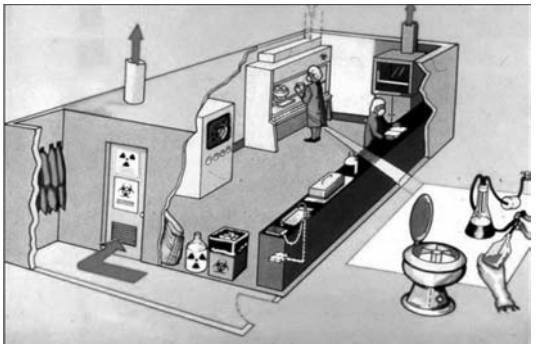
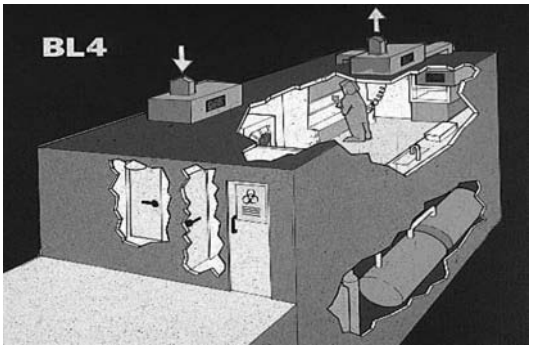


Figure 10-4 Illustration of a laboratory utilizing BSL-4 features (including self-contained protective suits and enhanced waste storage). (Courtesy of CDC/Office of Health and Safety.)



The floor, walls, and ceiling are sealed to keep in aerosols and must be waterproof. The floor itself is monolithic and has moldings extending at least 4 in. up the wall. Acoustic tiles, commonly used in ceilings, are unsuitable. Additional protective measures, such as the use of powered air purifying respirators (PAPR), may be utilized. Finally, all sample/agent manipulations should be done in the BSC. The laboratory is supervised by an individual who has a technical understanding of the risks associated with the microbial agents likely to be encountered, and who is responsible for granting laboratory access, providing training and establishing work procedures, and developing the laboratory's biological safety manual. An example layout of a BSL-3 laboratory is shown in Figure 10-3.

Biosafety Level 4

This is the highest level of laboratory design and incorporates all BSL-1 to -3 concepts plus extensive physical controls. BSL-4 is suitable for handling organisms that may cause serious or lethal disease as a result of exposure via inhalation and for which there is no known vaccine or therapy. Examples of organisms that may be handled in this environment include smallpox and Marburg virus. The laboratory utilizes extensive engineering controls to provide isolation of the analyst from the agent, primarily through the use of BSC Class III and/or positive-pressure isolation suits. Facility designs are extensive as well and include the use of air locks, extensive air and waste management, and laboratory isolation. Often this isolation means the laboratory stands alone and unattached to other parts of a complex. These are very expensive to build and maintain and there are only a handful in existence. BSL-4 facilities exist at the CDC, the United States Army Medical Research Institute of Infectious Disease (USAMRIID), and a few other government and academic institutions. An example layout of a BSL-4 laboratory is shown in Figure 10-4.

Chemical and Radiological Analyses

The requirements for safety during chemical analyses, at least as expected in PHLs, are much less extensive than for biological. For both these classes of parameter, one is usually attempting to identify a trace amount of a parameter, or culturing a sample for biologicals. Whereas disease may be induced by less than 100 bacterium (for some species), such small amounts of chemical (i.e., 100 molecules) is unlikely to have significant effect. The greatest threat to personnel safety is therefore commonly from the reagents used for analysis. Common safety techniques and controls are employed, but not structured into increasing levels as is the case for biologicals. Such techniques include the use of PPE (gloves, eye protection), safe storage (acids and bases stored separately, log book maintained), safe use (small volumes, proper disposal), and engineered items such as chemically resistant counters and fume hoods. All these controls may be specified by the laboratory's chemical hygiene plan.

The chemical fume hood looks quite similar to a BSC, but its mechanism of action is substantially different. Like BSCs, their use is one of the best safety features in a chemical laboratory because they draw chemicals that may be aerosolized or vaporized away from the analyst. However, a fume hood does not typically filter the air and recirculate part/all of it back into the laboratory.

Instead, the air is exhausted to the outside. Some of the operations typically performed inside a fume hood include:

- Liquid/liquid extraction where large volumes of organic solvent are used
- Volume reduction where sample extracts are heated to evaporate the solvent
- Dilution/creation of toxic reagents such as diazomethane

There are other engineering and facility controls that may be employed, such as air scrubbers to clean exhaust air, and more specialized protective equipment and room design. Much more substantial protective gear, engineering control, and facility design must be used for handling many extremely toxic compounds (e.g., chemical warfare agents), even in small amounts. These controls are not typically encountered in a PHL.

Radiological analyses use much of the same precautions and safety features. Differences include specific hood use protocols for any radioisotopes that may be volatile and the use of Plexiglas shielding. This shielding is usually mobile and can be placed around areas where beta-emitters may be present. They may also be placed around waste storage containers. You may recall that alpha particles are stopped by skin and even strong beta particles by 1 cm of Plexiglas. An analyst will thus place a small shield between him- or herself and a beta source being used. Gamma rays require much more significant shielding to be stopped (e.g., lead sheets or concrete blocks). These and other safety controls such as shielded glove boxes are not typically encountered in a PHL.

All Hazards Receipt Facility

So far we have described laboratories as they might be found in typical state health departments. We have also mentioned the relatively basic safety features commonly employed for chemical and radiological analyses. Given the nature of samples submitted to laboratories, these controls and features are usually more than adequate. However, they have serious shortcomings when it comes for the analysis, or even receipt of, samples for chemical or radiological terrorism. Biological terrorism sample handling and analysis is not terribly different, safety-wise, than other infectious disease and the great majority of state health department laboratories are equipped to safely handle them. One may say a *Bacillus* is a *Bacillus* is a *Bacillus* . . . and the difference in handling *subtilus* versus *anthracis* is relatively small. In addition, most biological agents likely to be encountered can be effectively treated with antibiotics and/or therapy.

Chemical warfare agents, on the other hand, are entirely different than the typical sample submitted for chemical analysis. The greatest danger posed by the analysis of a paint chip for lead is probably from the acid used for sample digestion. However, a single drop of nerve agent may kill within minutes. How does a laboratory safely handle such a lethal substance? There is an antidote for nerve agent poisoning, but it must be applied immediately after exposure. There are no effective medications or treatments for many of the other agents. A similar scenario applies for radiologicals. Analysts are equipped and prepared to analyze samples for trace levels of radionuclides, but a dirty bomb may release huge amounts of gamma-emitting radionuclides. And if someone mixed a powerful radionuclide (e.g., cobalt) with an explosive without announcing it, how might the laboratory even know a gamma-emitter was even present in the sample? These types of samples and scenarios require laboratory facilities much more extensive than what is typically available.

One answer to this is the All Hazards Receipt Facility (AHRF). The design of these facilities is a joint effort by the Department of Homeland Security (DHS), EPA, the US Department of Defense (DoD), Federal Bureau of Investigation (FBI), and the Association of Public Health Laboratories (APHL). This is designed to be a screening facility to rapidly, and safely, triage samples that may contain unknown chemical, radiological, and/or explosive hazards. The EPA and DHS have published a screening protocol for the submission and screening of samples.[3] The protocol specifies how samples are submitted, the tests that are performed, and how the results are used. The procedures and equipment utilized are expensive to obtain and maintain and there are therefore few operational AHRFs in existence. A description of an AHRF and its operations is beyond the scope of this text, but the protocol may be found at the EPA/DHS research Web site at http://www.epa.gov/nhsrc/.

Clinical Laboratory Improvement Amendments

All laboratory testing performed on humans in the United States for the purposes of diagnosing, preventing, or treating disease or for the assessment of health is regulated by CMS through the CLIA. These were enacted in 1988 with the intention of ensuring the

accuracy, reliability, and timeliness of clinical test results. The Amendments do not cover tests done for research or as a part of clinical trials. CLIA covers approximately 200,000 facilities, including 382 PHLs.[4] There are three types of facilities under CLIA: waived, moderate, and high complexity.

Waived complexity laboratories are usually in local health departments and physician offices and only perform those tests that are categorized as "simple laboratory examinations and procedures that have an insignificant risk of an erroneous result."[4] Such tests include fecal occult blood and many self-contained kits. Waived laboratories must enroll in CLIA, obtain a certificate of waiver, pay all applicable fees, and follows all kit manufacturer instructions.

Moderate complexity (including provider performed microscopy) and *high complexity* laboratories must obtain a certificate of either compliance (inspected by CLIA) or accreditation (inspected by a separate, approved, entity) and also have the following specified by CLIA: proficiency testing, test management, quality control and assurance, and personnel qualifications. Moderate complexity tests are usually done in local health departments, physician offices, and some hospitals and include the Abbott Aeroset (Kamiya K-Assay) and Siemens ADVIA Centaur XP System.[5] The following personnel are specified for moderate complexity laboratories:

- *Director* may have a medical degree with 1 year of nonwaived supervisory experience, or a bachelor degree with 2 years laboratory experience and 2 years nonwaived supervisory experience. The director is responsible for the overall laboratory management.
- *Testing personnel* must have a high school diploma and adequate training. Personnel are responsible for sample handling, testing, and result reporting.
- *Technical consultant* must have a bachelor degree with 2 years nonwaived experience. The technical consultant is responsible for the technical and scientific oversight of all testing.
- *Clinical consultant* must have a medical degree and be board certified. The clinical consultant is responsible for clinical oversight.[6]

In addition, moderate complexity laboratories must also have substantial documentation about testing and reporting procedures, perform positive and negative controls each day samples are tested, enroll in applicable proficiency testing, and maintain records for a minimum of 2 years.

High-complexity tests are usually done at state health departments, private laboratories, some hospitals, and federal agency laboratories. Most, if not all, state health laboratories are high complexity so we will spend more time discussing these requirements. Examples of tests done in these laboratories include the Becton Dickinson BD Viper (BD ProbeTec Neisseria Gonorrhoeae Qx Amplified DNA Assay) and the Trek Diagnostic Systems Susceptibility Panel.[5] The following personnel are specified for high-complexity laboratories:

- *Director* must have a current license as a laboratory director and possess a medical or other science (e.g., microbiology) doctoral degree with 2 years of nonwaived supervisory experience and be certified as a high complexity laboratory director. The director is responsible for the overall laboratory management, testing, and reporting.
- *Testing personnel* must have a high school diploma and adequate training. Personnel are responsible for sample handling, testing, and result reporting.
- *General supervisor* must have a current license (if required) and either qualify as a laboratory director or technical supervisor or possess a combination of degree level and experience. The supervisor is responsible for providing supervision of all testing personnel and test result reporting and oversight of testing done by personnel.
- *Technical supervisor* must have a medical degree, a doctoral, masters, or bachelor degree with varying lengths of experience (more experience associated with lower degrees). The technical supervisor's responsibilities are identical to those for the technical consultant for moderate complexity laboratories.
- *Clinical consultant* must have a medical degree and be board certified. The clinical consultant's responsibilities are identical to those for the clinical consultant for moderate complexity laboratories.[4]

Outside of the required qualifications for different laboratory positions, the two areas laboratory workers will most notice the impact of CLIA will probably be in the performance of proficiency studies and completing comprehensive documentation of training, testing, quality control and assurance, and reporting.

Under CLIA, all laboratories certified for nonwaived testing must participate in an approved proficiency test (PT) program. CMS annually evaluates PT programs

and publishes a listing of acceptable programs for laboratories. A laboratory must enroll in PTs such that all tests that it performs are "challenged" annually. All documentation must be available to demonstrate that the testing, and any activities related to the testing, was actually performed by the laboratory. CLIA specifically forbids referring a PT to another laboratory, even if that is the normal procedure for testing patients. Any incorrect result for a PT must be followed by a corrective action plan.

When a CLIA surveyor (auditor) determines a deficiency in the laboratory's operation, it is cited as a "standard level deficiency" or "condition level deficiency." Standard level deficiencies are generally quickly noted and corrected. These may include such lapses as failing to record daily refrigerator temperatures or to write down a quality control result properly. A condition level deficiency is of a serious nature; it may or will impact patient care negatively and requires correction before a laboratory can continue testing for the specialty or subspecialty under the CLIA license. Condition level deficiencies are "actionable." Further information concerning surveys and deficiencies are found in Appendix C Survey Procedures and Interpretive guidelines for Laboratories and Laboratory Services.[7] A particularly negative survey result is the determination of "immediate jeopardy." This is defined in 42 CFR Section 493.2 as one or more condition level deficiencies that creates "a situation in which immediate corrective action is necessary because the laboratory's noncompliance with one or more condition level requirements has already caused, is causing, or is likely to cause, at any time, serious injury or harm or death, to individuals served by the laboratory or to the health or safety of the general public."[8] The seriousness of the condition level deficiencies will determine if testing can be continued after immediate correction, suspended, or, if deemed necessary to protect patients or the public health, the license may be revoked.

Health Insurance Portability and Accountability Act

Health Insurance Portability and Accountability Act (HIPAA) was enacted by Congress in 1996 with two main Titles. Title I is named *Health Care Access, Portability, and Renewability* and is designed to protect health insurance for workers and families if they lose or change their jobs. This Title has little impact on PHLs. Title II is named *Preventing Health Care Fraud and Abuse; Administration Simplification; Medical Liability Reform* and has significant impact on how laboratory data is used and shared. This Title requires the creation of rules standardizing the use and distribution of health information.[9] Two of these in particular, the Privacy Rule and the Security Rule, have cause substantive changes in laboratory data management.

The Privacy Rule established rules for the use and disclosure of any information held by a covered entity (one which must comply with HIPAA standards) that concerns health status, provision of health care, and healthcare payment that can be linked to an individual. Termed *protected health information* (PHI), this includes data often associated with laboratory samples and forms, such as name, address, test requested, and the result(s). In simplistic terms, the Rule requires that entities with such information: make the information available to the individual within 30 days of request; disclose the minimum PHI necessary to other authorized entities for their needs (e.g., billing); inform individuals to what use their PHI will be used; and document all uses and disclosures. The most immediate impact on laboratory work is ensuring that sample requests and test results are kept as confidential as possible. This might mean that stacks of sample request forms are not left in a common hallway, but retained in the laboratory where the analyses take place. Likewise, test results are seen by only those with a need, and only shared with appropriately authorized entities such as the submitter and health department program staff.

The Security Rule is a complement to the Privacy Rule but deals specifically with electronic protected health information (EPHI). It specifies security standards for each of the three types of security safeguard. *Administrative safeguards* are those policies and procedures that show how the entity will comply with the Act. It will show management oversight, which employees will have access to which information, the specifics of ongoing training, and other items. *Physical safeguards* are those that control physical access to the PHI and include procedures to retire equipment, access controls to records, hardware and software, and other items. *Technical safeguards* are those protecting the data from being intercepted electronically. They include the use of encryption, data corroboration, authentication of data receivers, and other items. Taken together, these three safeguards ensure that PHI is stored, accessed, and transmitted to and from authorized entities in a fashion that minimizes the opportunity for data loss, theft, or corruption. The most visible impact of this Rule in the laboratory might be the official designation of employees as having access to select information, regular training on PHI use, and data protected by passwords.

A bigger impact of HIPAA is seen in research. It was recognized when HIPAA was being developed that there were uses of PHI for the public good that would be adversely impacted by the Act. There are many investigators who use PHI to perform research into such things as the distribution and spread of infectious disease. Under HIPAA, the health department cannot simply give the researcher all the data they require, no matter how laudable the cause. As an example, we can consider researching chlamydia (CT) in Illinois where the annual reported incidence is regularly in excess of 50,000 cases. The investigator might want to know such things as the age, gender, race, residence, and date of test of each case to see how they vary. Some of these items are considered PHI. How might an independent investigator obtain access to all these data? There are several possible scenarios:

- Request the data from each individual: Each person has the ability to release their information to whomever they wish. The investigator could ask the health department to contact each person and ask for their permission. While theoretically possible, such a solution is a practical impossibility for large sets of data.

- Use deidentified information: This is a data set that has either been statistically aggregated or had 18 personal identifiers removed.[10] Permission from the individuals does not need to be obtained. Identifiers include names, all geographic subdivisions smaller than state, telephone numbers, and Social Security numbers. For the researcher, such data as an individual's gender, age, and race are available, but not the county of residence or date of the test. This is useful for examining the state as a whole, but not too useful for seeing how cases vary by region, or if testing varies by time of year.

- Use a limited data set: This set gives more information than the deidentified set, but is still not directly identifiable. However, a data-use agreement/contract must be established between the entity and researcher before this information may be shared. Permission from the individuals does not need to be obtained. A limited data set may include smaller geographic subdivisions (city, state, and zip code) and elements of dates.[10] Now the researcher knows which cases are from which county, and when the tests were done.

- The researcher could direct the health department staff to conduct the analyses themselves. Specified health department staff already have authorization to the full data set, and some types of analysis would be appropriate for their position.

There is much more detail involved with HIPAA and the appropriate use of PHI. We would like to stress that appropriate and confidential use of PHI is of paramount importance and potential users should always err on the side of caution and NOT use data if there is doubt. For researchers, and even health department staff with authorized access to PHI, we strongly recommend project review by an Institutional Review Board *before* any nonroutine analysis or data sharing.

Laboratory Certification and New Testing

An important aspect of laboratory work is the process of certification. While there are several agencies and entities that provide oversight of analytical activities, the review and audit of records is not always sufficient to show actual proficiency in performing any particular analysis. That is, the laboratory may document a sample's acceptance and handling, the preparation and use of analytical reagents and quality controls, and result oversight and release in an exhaustive manner. Yet this does not indicate that the analyst can correctly determine if a particular bacterium is Gram-negative or -positive or even inoculate an enrichment broth. Often, the best way to show that a laboratory or particular analyst can actually perform an analysis correctly, and not just document it, is to have them analyze a sample known to contain a specific pathogen or chemical compound. This is termed a PT, and the participation in performing these samples as part of a series over time or multiple samples for different analytes is termed a proficiency study. The correct identification of a proficiency study contaminant is a powerful complement to complete documentation in showing that the laboratory or analyst is able to perform the analysis as described and that the results are accurate.

Just as we find that different agencies and entities oversee different types of testing, we find that different certifications may or may not be required. These requirements differ by test method, analyte, and oversight entity. Some methods require participation in a defined proficiency program coupled with substantial documentation and audits. Others require participation in proficiency studies determined by the laboratory. Finally, other types of testing require no proficiency at all.

Clinical testing is regulated by the CMS under CLIA and regulates clinical laboratories through proficiencies studies, documentation, and audits. Under CLIA, a successful PT is required for each analyte or organism for each test method for the laboratory. The laboratory is required to document a personnel assessment for each employee performing each test for each analyte or organism result. This is most often accomplished by providing a proficiency test to each employee for each test, or a specimen as an unknown that was previously tested. *Molecular* analyses of clinical samples are still relatively new in the overall scheme of things and the requirements for proficiency and certification are fairly minimal. The College of American Pathologists is the only CMS-approved provider of molecular proficiency studies for molecular analysis of infectious disease.

An audit is conducted every 2 years to review the documentation of all of these requirements. The most dramatic citation that a laboratory can receive has been for failing to follow the same procedure for PTs as for normal specimens. As an example, an inspector has the authority to close down a laboratory by discontinuing certification if an employee tests a PT multiple times to ensure a correct answer. Since specimens are not tested in this manner (as documented in the Standard Operating Procedure and by interviews with staff), then the CLIA auditor has the option to close the laboratory. The CLIA director, by the way, will not be able to be recertified as a CLIA director for 3 years. PTs are taken quite seriously.

Water analyses done to meet Safe Drinking Water Act (SDWA) requirements are regulated by the EPA and delegated to regional and state agencies. In Illinois, for example, the Illinois EPA (IEPA) regulates laboratories providing these analyses through their successful participation in commercially obtained proficiency studies, documentation, and audits.

Just as there are multiple rules and agencies associated with food testing, there are also multiple requirements for demonstrating laboratory and/or analyst proficiency. For example, the FDA sends out a set of 11 proficiency samples once a year to be tested (often referred to as "food splits"). The set includes both quantitative and qualitative analyses. Quantitative results are submitted for total coliform, fecal coliform, *Bacillus cereus*, *Staphylococcus aureus*, and standard plate count. Qualitative results are submitted for *Salmonella* spp., *E. coli* O157:H7, and *Listeria monocytogenes*. These are to be tested by all analysts that want to be listed as participating in the splits samples. Each individual generates their own numbers from an initial 1:9 dilution of the sample. There is not an official certification generated as a result of successful analyses, but each analyst's results are tallied and statistically compared to the rest of the participants. Both the Laboratory Response Network (LRN) and the Food Emergency Response Network (FERN) periodically and on no specific schedule send out PTs that focus on one particular organism of interest, usually all in a specific matrix of their choice. The results are collected and reported on a laboratory rather than individual analyst. Other proficiency test providers include commercial vendors such as Silliker Laboratories.

Air and *other* sample analyses may be subject to varied or nearly nonexistent certification. There are few PHLs that perform air analyses, and they may not perform regulatory-type testing. Thus, they simply use EPA toxic organic methods and standard documentation practices with no other certification or proficiency requirements. Other laboratories may maintain animals as part of their testing catalog (e.g., mice for botulinum testing) and be required to show safe animal handling procedures. Still others rely on the in-house generation of proficiencies, especially when performing an uncommon analysis for which proficiencies are simply unavailable or developing new analytical methods. The process of developing new methods, especially for the diagnosis of disease in humans, may sometimes be a daunting task. This is frequently undertaken by the FDA so that suitable methods may be examined in a consistent basis and adopted nationally.

When evaluating a new test method, or an alternative method for current testing, a number of factors need to be considered. Environmental methods are often closely described with exact procedures and acceptable equipment; in some cases, to the exact brand and model. Clinical laboratories tend to have more leeway in choosing equipment and test methods. FDA approval is a consideration in selecting equipment. FDA approved equipment has met the requirements claimed by the manufacturer for sensitivity, selectivity, safety, etc. PHLs, however, often do tests that are not commonly done by commercial laboratories and no FDA-approved test procedure may be available because the requirements for approval may be too expensive for a reasonable return on a manufacturer's investment. Many biochemical tests for microbes are so characterized through long use that approval would not offer any significant improvement in confidence in the method. Another area where FDA approved equipment is not developed is in those areas where equipment formerly used for environmental

chemistry is being applied to clinical specimens. This includes measuring enzymes or metabolism markers with mass spectrometric analysis of blood spots, testing for volatile organic compounds (VOCs) in urine and blood, and testing for metals in blood by inductively coupled plasma mass spectroscopy (ICP-MS). In these circumstances, the CLIA director must determine what level of documentation is required to verify a test such that it is sufficiently accurate and sensitive enough to meet the test objectives of the program receiving the data, and the clinician caring for the patient.

Budgeting and Accounting

Short of an endowment, certainly a rare source of PHL funding, there are three basic funding mechanisms for PHLs. These are legislated funding of tax dollars by state general assemblies, federal grants, and fee for services.

PHLs may be considered a political solution to a public health need. As such, they are generally funded through state legislative budgets (indirectly in those cases where the PHL is part of a state university). Funding, then, is subject to the politics of a state, particularly as it reacts to the state's overall economic condition. This can be particularly restrictive when a PHL expands its testing in response to a federal grant, which then experiences dwindling funds over several years. Often, a federal grant is initiated to assist states to improve or expand its testing with the idea that the state will take over the ongoing expenses of the initiative. Federal funding then is a double-edged sword for PHLs and is probably best seen in this light. A new program can provide funding for new equipment, training, initial supplies, and staffing, but then the program needs an ongoing source of revenue if the initiative is to become a continuing Public Health Program support by the PHL.

If the state's laws provide for a laboratory division of a governmental agency to provide a fee for service, then this alternative may provide a mechanism for a PHL to provide expanded support for Public Health Programs. Many PHLs charge a fee for newborn screening, screening of pediatric blood for lead contamination, HIV and sexually transmitted infection (STI) testing for clinics, etc. The characteristics of such programs include one or more of the following: a low fee to recover the laboratory's costs, a beneficial price for the healthcare provider, a requirement to report results to the public health program, an underserved segment of the population, a new test not commonly available commercially, support for a research project, etc. Many states bill for testing services for Medicaid eligible patients. Few states, however, have the resources to bill the many health insurance agencies, and, in fact, patients with such insurance coverage generally receive laboratory testing from commercial laboratories. (A notable exception is anonymous testing for HIV status by public health entities.)

Preparing for the Unexpected

One of the more difficult responsibilities of laboratory management is to determine the vulnerabilities of a laboratory and how to mitigate those in preparation for a manmade or natural event that could interfere with the normal operations of the laboratory. The event may be one in which an outbreak of infectious disease overwhelms the laboratory's ability to respond with appropriate testing, or one in which a critical component of the laboratory is unavailable, such as staffing (widespread influenza, strike in a contentious union environment), supply inventory (national shortage of acetonitrile for newborn screening blood spot extractions because of a recession that interrupted normal chemical production in China), equipment (electrical surge that wiped out much of the building's electronics), or building infrastructure (broken water pipes, electrical blackout, etc.). Checklists are available, and without a doubt, discussions among the staff in the laboratory who best know weaknesses and vulnerabilities can provide much information on what to shore up, repair, or back up. These are opportunities that should not be missed.

There are many resources available that guide one through planning, and many approaches to choose from. Each requires time on the part of all staff to not only obtain training and to develop, but also to exercise the plans. A valuable resource for the PHL is the APHL, which not only offers guidance in the form of a continuity of operations plan (COOP) but also offers a network of PHLs that often assist each other by sharing plans, supplies, methods, and lessons learned. A growing trend, resulting from the interactions of DHS and its recognition of the roles played by PHLs, is the National Incident Management System (NIMS) approach to responding to an event. Staff is assigned roles and work as a coordinated team with an emphasis on objectives, command structure, logistics, safety, and operations. Training can be obtained through the Federal Emergency Management Agency (FEMA).

At the laboratory bench, however, there are laboratory practices that can prevent many problems. These include:

- *Cross-training staff*: A pool of several staff members who are cross-trained, documented in terms of proficiency, and retrained as needed can prevent the loss of critical testing when staffing is reduced for any of a number of reasons. This is particularly helpful when staff can be rotated regularly so that emergency retraining is not necessary when they are called on.
- *Inventory management*: While the concept of "just in time" inventory is appealing on an accounting level, shortages of some key supplies do occur, and it is wise to weigh the benefit of investing in a several week supply of some materials, particularly those that have been difficult to obtain in the past, or those for which required contracts are difficult to renew.
- *Exercise*: Any plan that is developed should be tested. The more real world the exercise, the more useful it is. An after action report is critical to obtaining useful improvements. PHLs become involved in many exercises because each public health program that it serves is likely to develop an exercise. Participation pays off in several ways: It shows the direction a program is going and the needs it may have in an emergency situation, which can be valuable for planning in the laboratory). It also means that the participants get to know each other. That kind of familiarity, even if superficial or role based, can be useful after hours during a real emergency.
- *Back-up staff*: It is important that roles and responsibilities are independent of personalities. In any plan that considers the possibility of excessive overtime or a loss of staff, back-up staff becomes desirable. A laboratory needs to consider not only the testing, of course, which can require time-consuming training and updates in training, but also how staff can quickly be used in the preanalytical stage (accessioning, extracting, preparation steps, etc.) and the postanalytical stage (reporting, autoclaving, cleaning, etc.). These pre- and post-analytical phases can free up trained technologists who can then concentrate on the much more technical aspect of analytical testing.

Impact of Influenza A H1N1 on Laboratory Operations and Staff

In an unprecedented approach, the CDC and FDA developed a rapid, high-throughput method for detecting an expected avian influenza pandemic. The method was vetted through state PHLs that sent representatives for training at CDC on specific instrument models using specific methods and specific primers/probes and reagents. The result was a method released in February 2008 and found on the FDA's Web site under *Establishing Performance Characteristics of In Vitro Diagnostic Devices for Detection or Detection and Differentiation of Influenza Viruses*.[11]

The method was a real-time reverse transcription polymerase chain reaction (rRT-PCR). It was developed for qualitative detection from viral RNA extracts of:

- Influenza virus type A or B from nasopharyngeal swabs or nasopharyngeal washes,
- Subtype seasonal human influenza A virus as seasonal A/H1 or A/H3, and
- Presumptive identification of influenza A sub-type A/H5 (Asian lineage).

The purpose was epidemiological, not diagnostic, as the performance characteristics were developed when seasonal influenza were the dominant circulating strains. Even so, the in vitro diagnostic (IVD) status from CDC was useful because it is expected that the results would be reported to clinicians as well as epidemiologists.

As it happened, however, it was swine origin influenza that developed in 2009 and quickly became pandemic. Suddenly, the IVD, the only test around, was not sufficient for the emergency need as it was not approved for diagnostic purposes. The FDA quickly issued an emergency use authorization (EUA) for the method that allowed it to be used for presumptive diagnosis.[12] To quickly put the method out with the correct primers and probes, CDC developed a rapid evaluation program.

Those PHLs that had sent a technologist to be trained at CDC on the IVD test were to analyze specimens in their labs until they had at least five presumptive positive novel A/H1N1 specimens (identified as "probable"), send them to CDC for confirmation, and then the laboratory could report "confirmed" novel H1N1 influenza. Many state PHLs had not sent a staff member for the training. That could be somewhat embarrassing given the public recognition of the importance of

preparedness, but certainly understandable. Even CDC was caught off guard, but much to their credit, they had a method if not the specific primers for the specific influenza. Those state laboratories could develop the method, obtain primers/probes and reagents from CDC, and then work with approved state laboratories to test five positive specimens to ensure performance. At that point, the state laboratory was approved to report "confirmed" novel H1N1 influenza.

Although PHLs often emphasize surveillance over clinical diagnostic testing (individual patient care), the expectation of the public (including clinicians) is that all tests will be performed on all patients when there is a crisis or emergency, real or perceived. In April through July of 2009, the IDPH laboratories tested more than 7000 specimens, and almost all positive specimens were novel A H1N1 (unpublished data). While the normal influenza season tapers off through the summer and does not pick up until late fall, the novel H1N1 influenza remained, although in lower numbers in terms of laboratory submissions. The IDPH laboratories did not have sufficient instrumentation for the throughput that was needed to meet expectations, nor the clerical staff to hand-enter specimen information and then manually prepare reports, and fax them individually. The number 7000 takes on great significance when it includes preanalytical, analytical, and postanalytical processes. This was especially significant as the normal workload continued. Normally, influenza PCR work was not performed; only viral culture. The laboratory staff, as employees and their supervisors everywhere tend to do, rose to the occasion, putting in long days, 7-day weeks, and many weeks of overtime. As a result, many people were reassured, or received confirmation of their expectation.

Laboratory operations and management are time-consuming, varied in nature, and comprehensive. Individuals in management positions need some degree of skill in personnel management, budgeting and accounting, and knowledge of testing procedures. This last allows for better allocation of prioritization of resources and to better justify program costs and benefits. All these skills, knowledge, and abilities are important to maintain day-to-day operations and perform strategic planning for the unexpected. In times of economic hardship, such skills are especially critical to maintaining continuity of operation for required testing, and ensuring that analyses remain both timely and accurate. Given that the health of individuals, and national security via surveillance, are dependent on laboratory results, system interruptions are to be strenuously avoided through management and planning.

Discussion Questions

1. What role does reassurance of the public play in a PHL's mission?
2. What are some of the potential personnel issues concerning roles, titles, and responsibilities? How might they be resolved?
3. State PHLs often utilize a combination of BSL-2 and BSL-3 laboratories. What are some of the differences, and in what situations would each be preferred over the other?
4. AHRFs were devised to be able to safely handle almost any sample for almost any analyte. Under what circumstances would they be useful, and how many (if any) might be useful nationally and worth the expense?
5. Discuss why CLIA was enacted and why compliance is still important.
6. Discuss potential HIPAA concerns with disseminating laboratory results by telephone, fax, and email. How might these be resolved?
7. Laboratories use both FDA-approved and non–FDA-approved tests. What are the possible benefits of each type of test?
8. Laboratory management is frequently a matter of addressing scientific needs through a government process, and these are not always compatible. What options might a manger have when the analytical equipment their staff want for a specific test is not the lowest bidder for the state contract?

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Additional Resources

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11

Laboratory Data: Uses and Communication

Introduction

There are almost as many potential uses for laboratory data as there are data elements. Uses are essentially limited only by the extent of need, individuals' imagination, and privacy regulations. As in other parts of this text, this section is not meant to be an exhaustive listing of all data uses. It is more a summary of the most common and important uses and a description of some technologies and specialties that use them.

Public Health Information Network

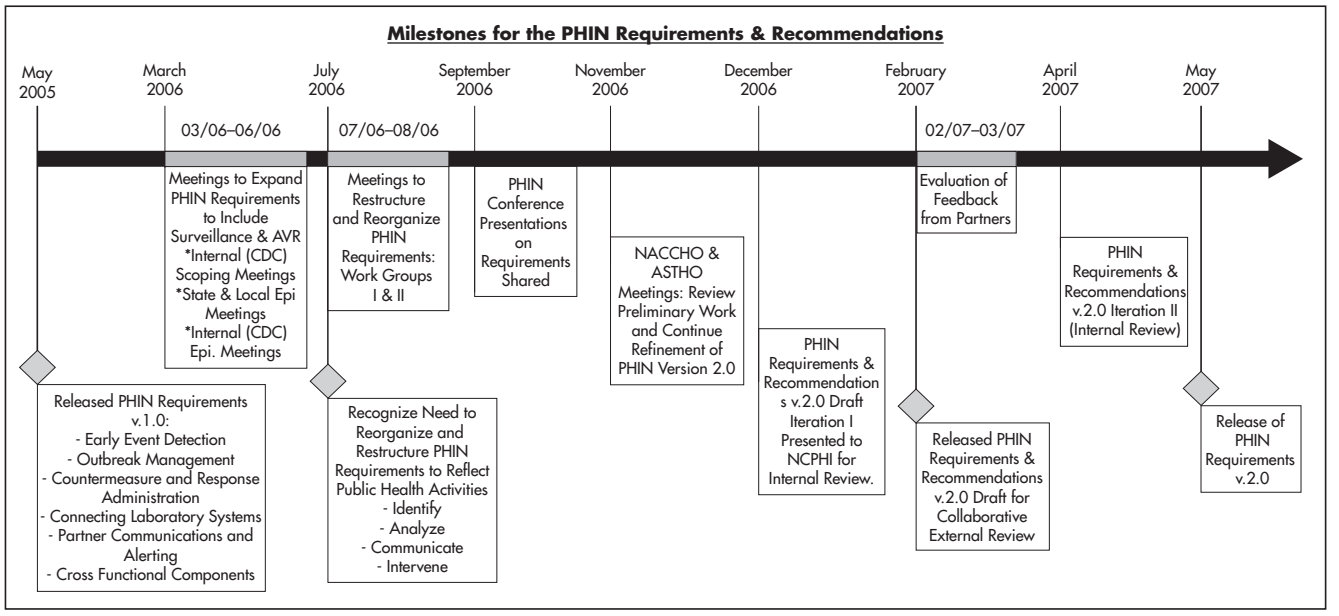
The Centers for Disease Control and Prevention (CDC) has been a driving force in the modernization and expansion of public health laboratories (PHLs) in many ways, and information handling and infrastructure is one important aspect easy to overlook. The explosive expansion of use of the Internet and electronic records has created unprecedented opportunities (and dangers) for public health in general and laboratories in particular to both manage their data internally and share with their outside partners. Internal uses of laboratory data include the generating of reports, quality control/quality assurance, and resource management. External partners benefit in many ways, from swifter results reporting to greater opportunities for disease surveillance at the national level. To coordinate and standardize many of these endeavors, the CDC has created the Public Health Information Network (PHIN). The PHIN is a "*national initiative to improve the capacity of public health to use and exchange information electronically by promoting the use of standards, defining functional and technical requirements*."[1]

The PHIN was originally launched in 2005 as part of national preparedness efforts. The intent was to help public health agencies more effectively respond to emergency events by becoming better able to communicate swiftly (re: electronically). Since then, events and technologies have evolved to the point where different and more comprehensive requirements are needed. Of great importance is the need for public health data system compatibility with the developing National Health Information Network (NHIN). The CDC undertook a collaborative requirements-building effort in 2006 to update and revamp the PHIN requirements to meet these multiple needs. The result is Requirements 2.0. The timeline for PHIN requirements development is shown in Figure 11-1.[2]

The PHIN is applicable to all aspects of public health, and much of it has direct impact on laboratories. Perhaps the most pervasive are the PHIN requirements. These are designed to promote interoperability between data systems such that different agencies and disciplines can "talk" to each other electronically. In order for a system to be PHIN compliant, it must be able to:

- Compose electronic messages using standard protocols, formats, and terminologies
- Send such messages to one or more recipients

Figure 11-1 Timeline of recent PHIN requirements and recommendations. (Courtesy of CDC.)



- Receive and process such messages from other entities
- Enter, edit, and retrieve data from a public health directory that adheres to standard protocols, formats, and terminologies
- Ensure that the system is secure at all times and allows access only to those authorized[2]

It is important to note that these requirements do not necessarily apply to an individual laboratory instrument (e.g., gas chromatography [GC]/mass spectrometry [MS]). However, they do apply to the laboratory information management system to which the instrument may be connected. The CDC has created several applications that rely, in part, on laboratory data.

The *National Electronic Disease Surveillance System* (NEDSS) is an Internet-based set of applications providing infrastructure for public health surveillance and data exchange. These applications are provided by the CDC and developed by state agencies and private vendors. The CDC has created a NEDSS Base System (NBS), which states may adopt and use as opposed to developing their own, perhaps unique, system. Both the NEDSS and NBS use standard terminology code sets (e.g., health level 7 [HL7]) and rely on data from laboratories, providers, state and local health departments, the CDC, and others. To date, there are in excess of 35 states that have a surveillance system based on NEDSS or NBS.[3]

Sidebar 11-1 Assessment of NEDSS Integration[4]

In August 2007, the Council of State and Territorial Epidemiologists (CSTE) conducted a survey assessment of all 50 states to determine the status and characteristics of state electronic disease surveillance systems. While states may adopt the NBS provided by the CDC, they may also purchase their own base system and other standalone disease modules (e.g., HIV/AIDS) and task modules (e.g., electronic laboratory reporting). The assessment found that only 16 states reported using the NBS, with the others using some combination of commercial, CDC, and state-designed systems. In spite of this variation in systems (and degree of implementation), 35 states reported they were able to send a communicable disease message to CDC in HL7 format. Automated electronic laboratory reporting was the most integrated (includes all separate disease modules in one system) component assessed, with 20 states reporting it as integrated, 4 as standalone, and 4 unspecified. This was followed by generally communicable disease surveillance at 23 integrated, 15 standalone, and 2 unspecified. The use of diverse modules and systems is much of a double-edged sword for state health departments. The ability to use and customize modules associated with specific diseases or functions allows a high degree of specialization for each state's specific needs. It also becomes much more expensive to maintain and become integrated and interoperable with other state and federal systems.

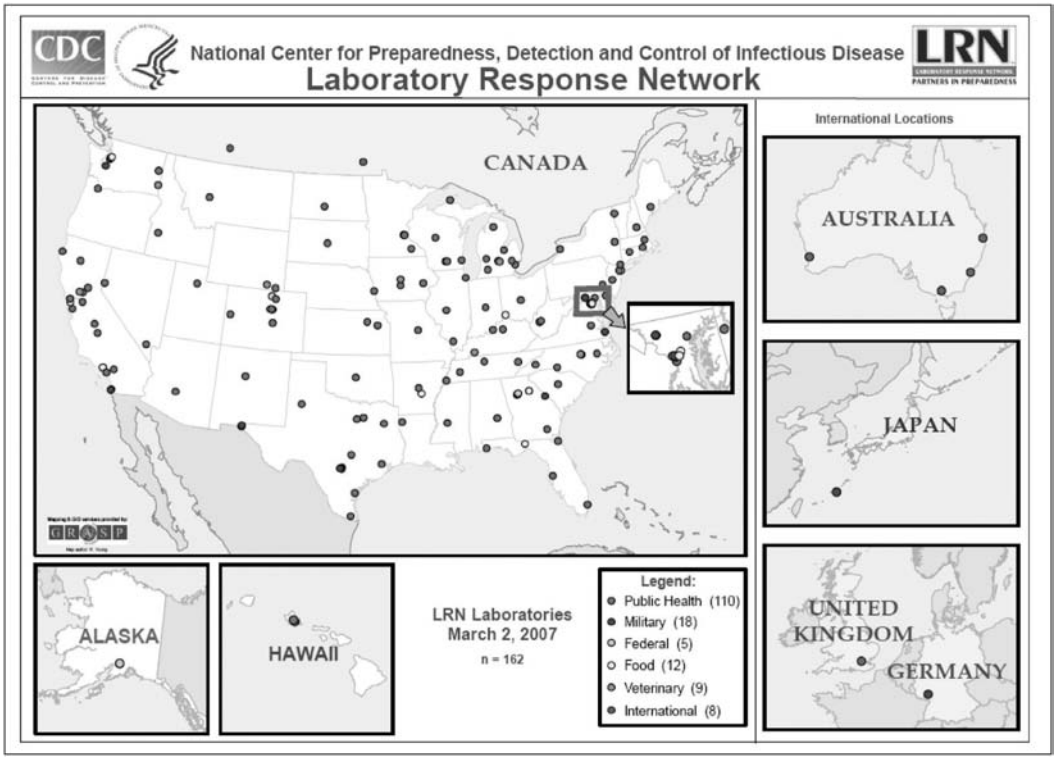
BioSense is real-time surveillance of human health data that is used to monitor emerging and existing disease trends. The BioSense application is available through a secure Web site and collects data from healthcare organizations, state syndromic surveillance systems, laboratories, and others and submits them to advanced statistical analyses. The purpose is to provide real-time analysis of the health of a population, whether at the local or national level. It can therefore help to confirm or refute the existence of a disease outbreak and monitor the size, location, and rate of an existing outbreak. Laboratory data is provided by national and BioWatch laboratories, many of which are located in state laboratory facilities.

The Laboratory Response Network's *Results Messenger* (LRN RM) provides a framework for the secure management and sharing of sample data and results between LRN partner laboratories. By using RM, laboratories can enter sample information and results, which are then analyzed real time by CDC. It uses the standard terminology code sets Logical Observation Identifiers Names and Codes (LOINC) and Systematized Nomenclature of Medicine (SNOMED). RM is a first step toward the goal of developing a comprehensive standards-based electronic data exchange system for the network and has been installed in over 150 LRN laboratories. Figure 11-2 shows where these laboratories are located.[5]

RM is designed to be a complement, not a replacement, for laboratory information management systems (LIMS). While the RM allows the sharing of data between LRN labs, an LIMS allows for the complete data management between all testing sections within a laboratory. It is through this LIMS (if present) that data is shared with outside partners. An effort parallel to RM, LIMS Integration (LIMSi) seeks to guide the adoption and development by laboratories of LIMS, which are PHIN compliant and allow direct data exchange between partners without the RM application. Figure 11-2 shows the distribution of LRN partner laboratories.

LIMS come in many designs and operate on many different operating and database systems. The LIMS is the core backbone of the laboratory data flow. A well-designed LIMS allows laboratory personnel to do their work while tracking data, data changes, and workload management in an effective manner. Many LIMS have been created by professionals over the years to work in a specific PHL environment. Today, many software vendors have modified

Figure 11-2 Location of Laboratory Response Network laboratories in the United States and worldwide. (Courtesy of CDC.)



or written specific laboratory systems geared toward public health. Whether creating a system in-house or purchasing one, many hours are needed to create or configure the system to work in a PHL. Considerations when creating a system today include the ability to be PHIN compliant, work with CDC's other data collection systems, analytical test equipment interfacing, and be standardized enough to share information with other states in the event of a national or regional public health emergency. The system also needs to be flexible enough to adapt to an ever changing disease environment. Creation of new tests for newly discovered strains of disease that was not considered a public health risk previously makes flexibility mandatory instead of desirable. The pace at which new outbreaks are discovered and tracked makes adaption of new testing as quickly as possible extremely critical. Add to this the rapid development of new and more effective testing instrumentation that feeds result information a new LIMS and the result is a need for a highly flexible, extremely efficient data collection and dissemination system for your customers.

Data Communication

The previous sections dealt with the subject of the different uses of laboratory data. This section will focus more on the methods of data communication. There are many factors involved in both the methods of data transmission and who is authorized to view them. In many cases, much of the data associated with laboratory testing is protected under multiple statutes, laws, and regulations. These can be defined by federal, state, and agency statutes. Some of the more common regulations that lab data can be protected by include Health Insurance Portability and Accountability Act (HIPPA), Clinical Laboratory Improvement Amendments of 1988 (CLIA), US Environmental Protection Agency (EPA), and State of Illinois law. Also, many specific tests are covered under the laws and regulations associated with that disease (e.g., HIV).

Those who have submitted a sample usually have first priority (or shared first priority) for receiving data associated with the sample's analysis. The important point to remember is that the data is returned to the *submitting entity*, not necessarily the person or location from where the sample was collected. This is often a source of confusion for private individuals from whom some samples originate. They are often under the assumption that since the sample is associated with them or their property, or that they themselves had brought the sample in for analysis, that the results should be given directly to them. Instead, the results are usually sent to the local health department, hospital laboratory from where the sample came, and/or to other official agencies as the situation warrants. There are various good reasons why this is usually the standard operating procedure.

One reason is simple expediency. Laboratory staff members may personally perform thousands of analyses, and many state laboratory systems generate more than 1 million test results each year. The three Illinois Department of Public Health (IDPH) laboratories performed over 2.1 million tests July 2007 through June 2008 (unpublished data). If lab staff were required find and report test results to the individuals to which the samples are associated on a piecemeal and random basis, they would have little time available for actual sample analysis. Likewise, this method of result delivery cannot be delegated to laboratory support staff, at least not at the current levels.

Secondly, test results may often not make sense to the layman and need to be explained. The explanation includes the result interpretation(s) and implications. These types of explanations are too time-consuming and complex for laboratory staff. Indeed, they often do not themselves know the implications and next steps associated with any particular test result. For example, a laboratory microbiologist does not have the time or training to inform an individual, they are positive for chlamydia, counsel them on the short- and long-term implications of the infection, direct them to treatment, and perform contact tracing and notification. These data are therefore sent to the sexually transmitted diseases section, which coordinates such activities with their staff and local health department personnel who are properly trained.

Water Analysis for Nitrate and Nitrite

Water is analyzed for the presence of these two ions on a regular basis, and there are EPA rules concerning allowable levels in drinking water (10 mg/L nitrate [NO_3] and 1 mg/L nitrite [NO_2]). Municipal supplies provide their own analyses, but homeowners utilizing private wells for drinking water must arrange their own. The analysis itself is fairly simple and swift with results usually available within 24 hours. This test is one of the few where laboratories may accept samples directly from homeowners, who often collect the sample themselves. However, the results are not sent back to the homeowner, but to the local health department.

The results produced by the laboratory need to be understood by the homeowner, and the actual numbers do not tell everything that must be known. First, there must be a determination of whether the results are within

allowable levels, which may change with time and which the laboratory analyst may not be aware. Secondly, if any of the results are beyond the allowable level, the homeowner must be made aware of the implications of the test and what remediation steps are needed and available locally. Again, this is information that the analyst does not possess. Finally, there is the matter of result security. By sending the results to a known and legitimate agency (local health department), the laboratory ensures that they have fulfilled their part in delivering the results to the authorized recipient. It is difficult to ensure proper result delivery when sending results by mail to private addresses and/or calling people on the phone.

Laboratory data is often used for multiple purposes. As discussed previously, the primary recipient is usually the submitting entity and their clients or partners. There are many other uses to which the data is usually put. For example, sample results are tracked as a measure of instrument performance to ensure that the results remain within a verifiable range for the instruments. They are also tallied as a performance measure for the laboratory for a given time frame (e.g., 1000 analyses annually), and used to measure disease prevalence in a given population. These measures in particular are critical to the identification of where best to target limited resources.

Use of Laboratory Data to Create Screening Guidelines

In 2006, the number of reported cases of chlamydia in the United States exceeded 1 million for the first time.[6] Since significant illness is much more likely in women than men, intervention efforts are most heavily targeted to that gender, and screening (testing) women for chlamydial infection is the best and most applicable tool. However, there are limitations when considering implementing screening at the national scale. There are in excess of 150 million women in the United States, and screening them all on an annual basis is simply impractical and prohibitively expensive. The question then becomes, "Which women would benefit the most from screening services?" Laboratory data answers this question in two different ways. First, the data show us that the greatest rate of infection is in those ages 16 to 25. By limiting regular screening to those who are sexually active and within that age range, we both increase the predictive power of the test (see Chapter 12 for a description of positive and negative predictive values) and focus our efforts toward those at greatest risk. Laboratory data also allows us to determine the actual prevalence of infection in specific populations. That is, of all the samples from a specified group submitted, how many are positive? This measurement may be used to compare different interventions for relative effectiveness in reaching those infected. If Intervention A reaches 100 individuals, 5 of who test positive, and Intervention B reaches the same number of individuals, but 10 are positive, then Intervention B is more effective at actually reaching a higher prevalence population (those at increased risk).

Use of Laboratory Data for Epidemiological Studies

One use of laboratory data is associated with epidemiological studies of food-borne outbreaks. In this section we will explore an increasingly utilized method termed biomonitoring. In essence, it is the analysis of human samples for the presence of chemical components. Of particular interest are those known or suspected to have toxic effects. Typically, the analyses involve determining the presence of particular compounds and/or their metabolites in blood and urine. Though this concept may be new to many, it has actually been used successfully in the past. Factory workers in the 1890s at risk for lead poisoning routinely had their blood and urine measured to avoid dangerous levels.[7]

The strength of biomonitoring lies in the actual analysis of human samples for chemical compounds. In previous studies and times, epidemiologists were quite hampered when it came to determining the effects of various compounds to human health. There were many significant factors that had to be assumed as there was no direct measurement. For example, there have been many studies examining the relationship between occupational exposure to farm chemicals (pesticides) and subsequent health. Researchers often had to estimate human exposure in terms of an individual's personal habits; measurements of the compound in the surrounding air, soil, water, or food; how the compound was applied; and the duration and timing of application. There was no real way of determining just how much of the particular pesticide actually made it into the human body and its subsequent fate (e.g., swift metabolism, storage in adipose tissue). With biomonitoring, a researcher may take blood and urine samples from the farmer and measure the actual absorbed dose. This measure allows for a more confident estimation of the compound's effects as we can actually determine which measured values correspond to which health effects. This is even more important when attempting to determine the level of indirect exposure, such as for secondhand smoke.

Nationally, CDC's Environmental Health Laboratory is responsible for the National Biomonitoring

Program. In 2003, the CDC funded three grantees to perform biomonitoring studies (New Hampshire, New York, and the Rocky Mountain Consortium [six states]). While individual grantee activities vary, they all include the expansion of capabilities to perform biomonitoring for different compounds (e.g., PAHs, As/Hg) and the instigation of pilot projects and programs. In addition, state laboratories that have received CDC Preparedness Grant funding for chemical terrorism are encouraged to use their equipment and resources to perform biomonitoring activities in their own states. Direct federal funding for these specific activities is quite limited.

Sidebar 11-2 Biomonitoring[8,9]

Biomonitoring has been utilized in various important ways. In 1997 it was discovered that the pesticide methyl parathion (MP) was illegally applied indoors in at least 968 homes in the Chicago area. Environmental samples were analyzed from 903 homes, with MP detected above levels of concern in 596 of them (average wipe concentration > 15 μg/100 cm^2). Individuals from these homes were offered urine tests to determine extent of exposure, and 1913 were tested. The results indicated that 550 residents had elevated levels of a MP metabolite (> 600 μg/L). They were relocated while 100 homes were remediated. This type of knowledge lessened the health concerns of many residents. Biomonitoring has also been used in recent studies to determine the levels of benzophenone-3, triclosan, bisphenol-A, arsenic, and polyfluoroakyl compounds in the US population.

Use of Laboratory Data to Measure Program Effectiveness

One of the great success stories of public health in the 20th century was the recognition of lead as direct cause of decreased mental development in children and the subsequent reduction in lead use and environmental exposure. It was this recognition that led to the abolishment of leaded gasoline and lead use in paint.[10] In fact, one of the Healthy People 2010 goals is the removal of lead poisoning as a health problem in children. To this day, children younger than the age of 6 years are still tested on a regular basis for the presence of lead in their blood as an indicator of environmental exposure. This program is supported by the CDC's Childhood Lead Poisoning Prevention Program, which was initiated by the Lead Contamination Control Act of 1988. While the program has many activities (including prevention and education programs), one of the largest and most expensive has been the support of state health department laboratories to analyze blood samples.[11]

The CDC estimates that in excess of 310,000 US children ages 1 to 5 have blood lead levels above 10 μg/dl. Levels above 10 trigger a response by the state's lead program. Through this widespread and consistent testing, the CDC has been able to show the dramatic reduction in child blood lead levels since these (and other) interventions took place. For example, the rates of elevated blood lead levels in Illinois children has dropped from 17.5% of all tested children in 1997 to 4.8% in 2003.[11] From these data, we can also show quite clearly that the intervention efforts are indeed having a positive, and in this case rather dramatic, effect.

Disease Diagnosis and Health Care

Laboratory data with possibly the most direct impact on the individual are those associated with disease test results. These analyses are performed to confirm or deny the presence of microorganisms, toxins, or chemical/elemental compounds. These results have direct and immediate effect on the direction of treatment and the health of the individual. Some example data types and their uses are listed in the following sections.

Sexually Transmitted Infections

The confirmed presence of a sexually transmitted infection (STI) organism in a sample activates a multitude of response activities. In Illinois, the results are relayed to the STI section and from there to the local health department to arrange for treatment. This in itself may be as simple as a single dose of azithromycin (for chlamydia infection [CT]) to a potentially lifelong treatment regimen (for HIV). The data is also used to assist contact tracing and partner notification services so that others who are potentially infected may be identified and tested. Swift analysis and reporting are thus especially important for diseases with short latency periods before becoming infectious. In 2007, there were 55,470 cases of chlamydia, 20,813 cases of gonorrhea (GC), and 1906 cases of HIV (non-AIDS) reported in Illinois.[12] Though the state laboratory analyzes less than half of the samples associated with these cases, it still tested 117,664 CT, 51,142 GC, and 60,905 HIV samples July 2007 through June 2008 (unpublished data). Managing and disseminating this volume of data is an involved process between the laboratory, commercial and private laboratories, the STI section, and local health departments.

Blood Lead

Lead poisoning is the number one environmental illness in children. In Illinois, approximately 25,000 children are found to have elevated blood lead levels (exceeding 10 µg/dl) and the greatest source of exposure is from homes painted before 1978.[13] The IDPH laboratory analyzed 85,203 samples for blood lead July 2007 through June 2008 (unpublished data). It has been determined that even trace levels of lead in the blood may have a significant, and lifelong, adverse impact on an individual's mental development. Children are therefore screened for lead on a regular basis, and those found to have lead levels in excess of 10 µg/dl are referred to the Lead Program. Evaluations are done on their living environments to determine appropriate interventions (e.g., removal of lead-based paint).

Tuberculosis

While there are several clinical symptoms that may lead to an accurate presumptive identification of active tuberculosis (TB), laboratory confirmation is important for confirmed identification. There were 521 reported cases of TB in Illinois in 2007 and 13,299 in the United States. Of special importance is the testing of the organism for antimicrobial resistance, as ~1.1% of cases are multidrug resistant.[14] While the majority of active TB cases in the United States are not drug resistant, the increased popularity and availability of international travel has contributed to an increase in the number of multiple drug resistant and extremely drug resistant TB cases (MDR- and XDR-TB). Identification of these strains has significant impact on the treatment regimen and the public health response.

Program Evaluation

In order to show the progress or success of any program, there must be some form of evaluation. These can range from the purely qualitative and descriptive to the solely quantitative. For state health departments, and indeed almost any organization relying in whole or part on state, federal, and grant funding, it is often the case that the more hard and fast quantitative results the better. Laboratory data is often ideal for such purposes as test results are usually unambiguous and straightforward. Further statistical analyses, where data are grouped together and added to other data sets, may be more open to questioning.

Water Supply Quality

Some health departments have varying oversight of drinking water quality. In Illinois, for example, municipal water quality assurance is overseen by the Illinois EPA. However, other nonmunicipal public water supplies, such as water pulled from a well and provided to guests at a campground, are regulated by the IDPH and are required to have their water tested on a regular basis. The laboratory performs these analyses and reports back to the program office and the sample submitter. In this way, the program can show that it is performing its required activities (requiring testing and performing inspections) and that these activities are effective (through remediation done when water quality parameters are beyond acceptable levels).

Epidemiology

Laboratory data is quite often aggregated and used for various statistical analyses. This is done for a multitude of reasons, some of which have a direct impact on public health functions, and others that impact policy and drive legislative efforts. Again, one of the strengths of laboratory data is that they are often unambiguous, though meaning attributed to those results may be. In this section we will explore some of the uses to which large amounts of data may be put. Such uses include determining which factors do/do not contribute to disease, developing effective and cost effective public health policy, and locating the source or cause of adverse health events.

Disease Epidemiology

Food-borne outbreak investigations are typically complex and may vary in extent from a few individuals in a single town to multiple areas across the United States. The result of a global food supply chain has caused challenges in ensuring the safety of food imported from abroad. The widespread adoption of just-in-time food delivery and a shortened "farm-to-fork" time frame have also made increased demands on workers to quickly inspect products. The result is that the effective screening of fresh products for known pathogens is inconsistent. Indeed, testing requirements vary by both food type and contaminant, but no food type must be 100% tested all the time, just on a determined basis. Holes in this surveillance can be compounded by inappropriate food storage, handling, and preparation. When outbreaks do occur, it is therefore somewhat difficult to determine who is affected, the identification of the pathogen, and the source of contamination.

Investigations are typically initiated at the laboratory end by the submission of clinical samples collected from individual(s) suffering from an apparent food-borne illness. These are analyzed for various pathogenic organisms.

The analysis may be substantially narrowed based on the preliminary rule-out of some organisms based on presented symptoms. Based in part on the laboratory results, a timeline will be created and an attempt made to identify the food associated with the infection based on the time between exposure (ingestion) and the presentation of symptoms. Food samples corresponding to the appropriate time may then be collected, if available, and analyzed for the presence of the identified pathogen.

Oftentimes, however, the suspected food is no longer available. In this scenario, data from the individual is matched with that from other individuals who have either presented with the same symptoms (and same laboratory diagnosis) or eaten the same food items. Though somewhat complex, the investigation attempts to match those who are ill with food sources that are common to all. An example may be a summer office cookout. If 95% of those ill consumed the bratwurst, and 88% of those without symptoms did not, then the bratwurst may be the culprit in the absence of other strong associations. At this point, the investigation may look at how the bratwurst was handled, and where it was purchased and produced. Laboratory identification, particularly subtyping by pulsed-field gel electrophoresis, is particularly valuable in linking together illnesses caused by food distributed in a wide network. A large fast food chain may buy hamburger in bulk and distribute it across multiple states. This specific laboratory subtyping analysis allows investigators to determine that an individual in Ohio is infected with the same strain of *Salmonella* as others in Pennsylvania. This data is often a critical component of food-borne outbreak investigations.

Policy Development

We will detail in Chapter 12 how the statistical usefulness of a screening test for CT can be increased by limiting testing to certain groups of people more likely to be infected. Because CT is largely asymptomatic, confirmatory diagnosis is based on laboratory analyses. Data from the state health departments are shared with the CDC on a continuous basis so that trends and prevalence in CT infection may be monitored. In this fashion, it has been shown that CT rates of infection have steadily increased for the past 10 years. Screening is still the most widely used and evidence-based intervention, and laboratory data is used to form the current screening guidelines.

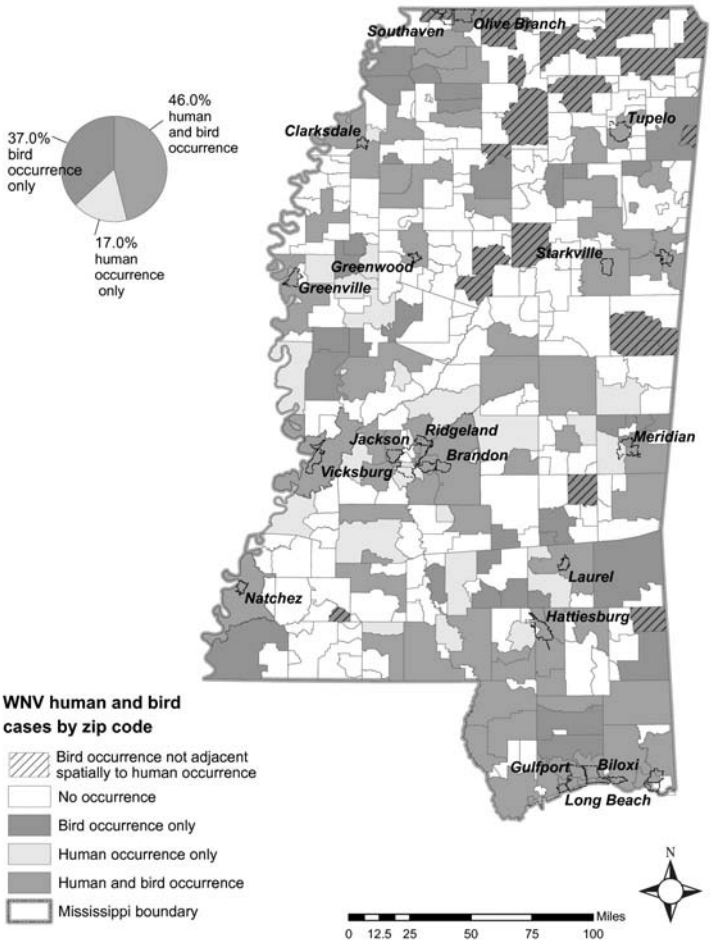
Geographic Information Systems

Geographic information systems (GISs) are becoming more popular and useful every year. On the one hand, the maps they are capable of producing may be quite visually compelling, in much the same manner as "a picture is worth a thousand words." The ability to graphically illustrate the extent or levels of disease in communities may contribute much to policy decisions. In addition, the software and statistical analyses now available make these maps quite useful when looking for clusters of disease that are more significant than would appear by chance alone. They are also useful for making policy decisions such as locating services, where spatial analysis may indicate the best location for a new facility that would be available to the greatest number of people. Laboratory data lends itself to use in GIS because of its often high levels of both quality and quantity.

An excellent example of how laboratory data can interact with a GIS is a study performed by Cooke, Grala, and Wallis in Mississippi in 2006.[15] They were attempting to estimate the likelihood of West Nile virus (WNV) infection in the state by mapping avian and environmental data. In this way, they hoped to locate suitable habitats for WNV-carrying mosquito breeding where they could then potentially intervene (e.g., by insecticide spraying) before humans became infected. They mapped avian infection and environmental data and developed models to predict risk. The test of the system came when they mapped actual human cases of WNV and the results of dead bird tests. This is shown in Figure 11-3. They found that there was a correlation between the occurrence of dead birds and increased risk of human infection. This study could not have been completed without laboratory analyses, which were used to conduct the research and assist in policy development.

In this chapter we have discussed some of the ways in which laboratory data is distributed and used. There are, of course, a number of other uses to which different data sets are routinely put. They are used for investigative research into the causes of disease, the testing of different policies and interventions for effectiveness, and for the surveillance for outbreaks of infectious disease. The increasingly global nature of our world puts an increasingly heavy burden on laboratories. Not only must their analytical capability be top-of-the-line, all the time, but their data reporting systems must be increasingly linked with other agencies such as the CDC. System compatibility allows for a much greater area of surveillance, at much greater detail, than would be possible with individualized reports examined by hand. PHL data have an importance for both national security and various research areas than most people might imagine.

Figure 11-3 Map of West Nile virus infections categorized by the type of occurrence. (Source: Cooke, W. H., Grala, K., & Wallis, R. C. [2006]. Avian GIS models signal human risk for West Nile virus in Mississippi. *International Journal of Health Geographics, 5*, 36.)



Discussion Questions

1. How does laboratory data assist public health professionals in isolating contributing factors to disease?
2. The CDC has provided requirements for systems to be compliant. Is this push for standardization in data handling good or bad for laboratories? Why or why not?
3. Does laboratory health data have to be protected like other healthcare data?
4. Can laboratory data trending analysis be used to better pinpoint where financial resources can be used to make treatment and prevention more effective? Describe how or why not.
5. Because diseases are not confined by borders, is making laboratories more effective in transferring data to one another a good idea?
6. The speed of transferring result information has become a priority and assists in treatment of patients more quickly than in the past. How might technology further assist in this area?
7. Laboratory data contributes to the mapping and development of risk factors for West Nile virus in Mississippi. What other laboratory data would also be useful for GIS purposes and why?
8. Go on the Internet and access PubMed (http://www.ncbi.nlm.nih.gov/PubMed). Enter "public health laboratory information [disease]" as a search. (The

disease should be one discussed in this book, such as TB.) Choose one article and describe how the authors used PHL information in their research. Do this for two different diseases/conditions.

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Additional Resources

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12

Links Between Laboratory Work and Other Public Health Disciplines

Introduction

There persists to this day within the field of public health that laboratories are the proverbial "black box" of which few know the workings or importance. That is, laboratories are often in locations isolated from other public health units, their activities are largely speculative, and assumed use of results are solely to make a diagnosis or populate a table with data. The truth is that laboratory work, and the data resulting from it, are of vital importance to most aspects of public health practice. Without laboratory work, we would not have the germ theory of disease, and from that, water treatment systems that are effective in preventing disease. Without laboratory work, many newborn infants would die from nondiagnosed metabolic syndromes that are now detected during newborn screening and effectively treated. Without laboratory data to support their causes, legislators would have great difficulty passing some legislation, such as limiting the use of lead in processes where children might be exposed. Finally, laboratory results are often the signal events for preparedness responses and community action. In this chapter we will explore how laboratory work and its results interact with the five traditional concentrations in public health: biostatistics, community health and education, environmental and occupational health, epidemiology, and health policy and administration.

The importance of laboratory work may be surmised by its contribution to many of the 10 great public health achievements listed by the Centers for Disease Control and Prevention (CDC) in 1999.[1] Table 12-1 lists six achievements where laboratory work contributed significantly. While many of the laboratory activities may be considered investigative research versus the more (seemingly) mundane routine sample analysis and result reporting that is the bread and butter of most public health laboratories (PHLs), the personnel, the required skills, and facilities are remarkably similar. Modern PHL staff are highly trained and often actively contribute to the investigation of new techniques and analysis methods to meet emerging threats to health.

There is often a division seen between the practices of medicine and public health. After all, physicians typically deal with individual patients while public health practitioners deal with populations. This division was not always so, and public health activities were an extension of medicine at the beginnings of the 20th century. The Johns Hopkins School of Hygiene and Public Health was started in 1916, followed by other schools of public health at Columbia, Yale, and Harvard by 1922. Degrees were originally designed to be a complement to an existing medical degree, but as time passed, a greater proportion of students were seeking public health as their primary degree. This is seen as the proportion of public health students who were also physicians declined from 35% in 1944 to 1945 to only 11% in 1978.[2] The proportion is likely much lower today, due at least in part to the rapidly increasing interest in public health as a field of study and work. We are also seeing increasing interest in the reconvergence of public health and medicine as there at least 29 accredited programs offering the MD and MPH as a dual degree program (with more under development).[3] Finally, there are an increasing

Table 12-1 Great Public Health Achievements in the United States, 1990–1999 and How Laboratory Work May Have Been a Contributing Factor to Each

Achievement	Laboratory Contribution
Vaccination	Laboratory work is an inherent component of vaccine development and production.
Control of infectious diseases	Laboratories identify infections and infectious organisms, develop medications, and assist outbreak investigations.
Safer and healthier foods	Laboratories identify pathogenic organisms, assist in outbreak investigations, and provide monitoring services.
Healthier mothers and babies	Laboratories provide newborn screening services and also analyses for potential congenital infections such as syphilis.
Fluoridation of drinking water	Laboratories monitor water supplies for appropriate fluoride levels.
Recognition of tobacco use as a health hazard	Laboratory analyses of tobacco and cigarette smoke were important contributors.

number of opportunities for education and training in PHL practices, as well. Examples include the Emerging Infectious Disease fellowship and the National Center for Public Health Laboratory Leadership through the Association of Public Health Laboratories, the Public Health Laboratory Sciences Masters degree program at Southern Illinois University School of Medicine, and technical training through the National Laboratory Training Network.

Biostatistics and Laboratory Work

Biostatistics may be defined as the application of statistical methods to the analysis of medical and/or biological data. Statistics are familiar to us in everyday life, even if we do not understand how they are calculated. We see examples of statistics in such things as earned run average, economic predictors, and weather forecasts. In the field of public health, statistics are used to calculate such things as the prevalence of disease in a population, rates of progression, cost-effectiveness, and study significance. Of the five traditional areas of public health study, biostatistics and epidemiology are the most closely aligned. Epidemiologists make much use of statistics in their studies to show significance, power, and associations. Biostatisticians often use epidemiological data in their research as well. In some significant ways, laboratory work would not be nearly as meaningful or important without the use of statistics. They are used to assist in method design and validation, devise testing criteria and guidelines, and assess significance and likelihood to various results. In this section we will show how laboratory work and results are intertwined with the field of biostatistics.

Sensitivity, Specificity, and Positive and Negative Predictive Value

Our common language incorporates uncertainty. When we say "usually," we are implying that there is an assumed chance of things not turning out as expected. Indeed, the study of physics tells us that in an infinite universe, anything is possible. Some things are just more or less likely. In spite of this possibility of anything being possible, and as a matter of practicality, some things are essentially "100%" or "always." However, it is important to know when this is not the case, and that is not always obvious. For example, if you jump out of an airplane at 3000 feet you will fall to the ground 100% of the time, but your odds of perishing are not 100% (people occasionally survive their parachute not opening). Do laboratory results belong to the category of falling to the ground, or surviving the fall? There are four criteria that assist us in determining how much stock to place in a test result.

- Sensitivity: This is the ability of a test to correctly identify if a sample contains the target of interest.
- Specificity: This is the ability of the test to correctly determine if a sample does not contain the target of interest.
- Positive predictive value (PPV): This is the proportion (percentage) of all positive test results that really do contain the target of interest.
- Negative predictive value: This is the proportion (percentage) of all negative test results that really do not contain the target of interest.

Figure 12-1 Basic 2 × 2 table as used for laboratory work showing its basic elements.

Test Result	Target Analyte: Present	Target Analyte: Absent	
Positive	true positive (A)	false positive (B)	total positive tests (G)
Negative	false negative (C)	true negative (D)	total negative tests (H)
	total samples with analyte (E)	total samples without analyte (F)	total samples (I)

- Prevalence: This is the proportion of samples which contain the analyte. (In epidemiological terms, this refers to the number of individuals within a defined population with a given condition at a given time [e.g., 2000/100,000].)

At this point we will introduce the 2 × 2 table. This is used much in statistics and epidemiology. While it is usually used to estimate odds rations for disease occurrence and risk, we can amend it to assess laboratory tests. Figure 12-1 shows a basic 2 × 2 table with associated elements.

Let us work through an example of how this may be used to assess the utility of a test, and how the test's values may, in turn, be used to develop sampling guidelines. For our example, we will examine a nucleic acid amplification test (NAAT) for chlamydia (CT), and also assume the prevalence in our sampling pool is 5%. As seen previously in the section on sexually transmitted infections (STIs), the expected sensitivity and specificity of a modern NAAT can be ~95% and ~98%, respectively. From this data, we know that out of a pool of 10,000 people:

- Five hundred are infected and 475 will be correctly identified (95%; true positives), and;
- Nine thousand five hundred are not infected and 9310 will be correctly identified (98%; true negatives).

Figure 12-2 shows how the known data fits into the table.

With simple arithmetic we can complete the table (Figure 12-3).

We now see the likelihood of errors in using this test. Of the 500 people in our sample who are infected, we find that 25 of them (box C) will be missed and return a negative result (false negative). We also find that of the 9500 people who are not infected, 190 of them (box B) will return a positive test (false positive). The implication for these false results varies by the test and condition/infection. For CT, a false positive might result in treatment, which is currently a single dose of antibiotic. For other conditions, a positive result may lead to invasive procedures (e.g., biopsy) to emotional trauma (being told they have HIV). This is why it is important to understand the limitations of a test and adjust conditions to maximize its utility.

Now comes the fun part, determining just how useful this test is. The PPV will tell us just how many of the positive results are truly positive (remember that a number of the positive results are false). The PPV is the proportion of all positive tests that are *truly* positive and is represented by PPV = (A/G)100 = (475/665)100 = 71.4%. This means that for our example, almost one third of the tests reported as positive are in fact negative. In a

Figure 12-2 Known data entered into the table.

Test Result	Target Analyte: Present	Target Analyte: Absent	
Positive	475	false positive (B)	total positive tests (G)
Negative	false negative (C)	9310	total negative tests (H)
	500	9500	10,000

Figure 12-3 The table and elements now completed.

		Target Analyte: Present	Target Analyte: Absent	
Test Result	Positive	475	190	665
	Negative	25	9310	9335
		500	9500	10,000

similar fashion, the NPV is determined by (D/H)100 = (9310/9335)100 = 99.7%. Bottom line, if the test result is negative, we can be pretty certain the individual is indeed infection free, but if the test is positive, it is only correct ~two thirds of the time.

At this point, there are several options available. First, we can simply treat all positives (single dose of azithromycin). Because of cost and concerns about increasing microbial resistance, this may not be best. Two other options use the effect that prevalence has on the PPV. In the previous example, the prevalence of infection in the sample pool was 5% (500 out of 10,000). What if we were to increase it fourfold? A prevalence of 20% might seem quite high, but it can sometimes be found in juvenile detention centers. Utilizing the same sensitivity and specificity, with a 20% prevalence, we know that out of a pool of 10,000 people:

- Two thousand are infected and 1900 will be correctly identified (95%; true positives), and;
- Eight thousand are not infected and 7840 will be correctly identified (98%; true negatives).

Figure 12-4 shows the table element values have changed.

A quick calculation shows that the PPV (1900/2060) now equals 92%! Our confidence in the positive test result has greatly increased. In a similar fashion, we can perform confirmatory testing on the positives from the original test. That is, we take all the samples testing positive (where 28.6% are actually false positives) and perform another analysis utilizing a different method. This has the effect of taking the sample pool prevalence of 5% (of all samples collected) and increasing it to 71.4% (number of true positives from the pool of test positives). Using a test with the same sensitivity and specificity, we find that the PPV for the second analysis is 99.2%.

It is through this process that guidelines for chlamydia screening are developed. In fact, the current United States Preventive Services Task Force (USPSTF) guidelines specify annual screening for all women ages 16 to 24 because they are at the highest risk for infection.[4] This sample pool is therefore likely to have the highest prevalence and therefore increase the PPV in the analyses done in PHLs. The guidelines also identify other risk factors for infection to assist if younger/older women and men should be tested (e.g., change in sex partners, risky sexual behavior). These guidelines are designed to maximize the usefulness of testing procedures and also be the most cost-effective. Similar processes are utilized in determining the screening guidelines for other conditions, such as breast and colon cancer.

Community Health and Laboratory Work

Community health is the study of, and intervention in, the health of identified communities; however, a

Figure 12-4 Element value differences when a disease's prevalence changes.

		Target Analyte: Present	Target Analyte: Absent	
Test Result	Positive	1900	160	2060
	Negative	100	7840	7940
		2000	8000	10000

community may be defined. Thus, they look at the influences and determinants of health for a town, school, nursing home, or other collection of individuals. In fact, this field of study is what often gives public health its most visible character and greatest strength. Community health practitioners look at data concerning the community, or specific portions of it, and devise methods to reinforce positive health behaviors and intervene in unhealthy ones. They employ behavior models such as the Healthy Belief and Transtheoretical (Stages of Change) models in individual and group settings to affect local health outcomes. Laboratory data is important to direct these efforts, support their activities, and provide a means to measure progress. For example, laboratory analysis of local well water, showing inadequate levels of fluorine, may support a community fluoridation campaign to promote dental health.

Syphilis

The goal of the CDC's Syphilis Elimination Effort (SEE) can be directly surmised from its name. Recent data of infection trends show that the incidence of syphilis in the United States is on the rise. Factors contributing to this rise may be more frequent and better testing, the increase in risky sexual behaviors, and the basic etiology of syphilis. The symptoms of primary and secondary syphilis are relatively mild, resemble other infections, and resolve on their own without treatment. Infection is passed through sexual contact with a sore, but such a sore may not be recognized for what it is (small and painless) or even observed (if in the rectum). Syphilis, as an infection and laboratory analyses, is discussed in more detail in Chapter 5; testing for syphilis is therefore important for two distinct reasons. First is to identify those who are in the latent stage and unaware of their infection. Left untreated, infection may progress to the late stage, which may result in severe illness and death. Being able to identify these individuals in time for adequate treatment is quite important. Another aspect is to identify and treat those who are still infectious and identify their partners. Knowing their disease status allows infected individuals to alter their behavior as needed to prevent further spread. Partner tracing and notification is an important component of community health services addressing STIs and is a major portion of syphilis activities. Figure 12-5 shows an advertisement designed to involve and educate the community.

Laboratory identification of infected individuals is an important aspect of syphilis elimination efforts and complements community outreach programs. One group in Baltimore used laboratory data to determine which specific area of town had the highest concentration of cases. They then formed community partnerships in those areas and placed teams of workers on the streets to meet and speak with local residents. By performing this kind of intensive, on-the-scene activity, they were able to find additional infected individuals (who may have gone unrecognized otherwise) and educate the local high-risk population of the local risks and sexual health services.[5]

Figure 12-5 Example of community engagement in the SEE through print advertising. (Courtesy of CDC.)

Pandemic Influenza

One of the greatest infectious disease threats in the 21st century has been the potential for a new influenza pandemic. There have been three in the 20th century, with

the 1918 outbreak (H1N1), the most deadly with up to 50 million deaths worldwide. While many people have limited immunity for many influenza strains, and receive short-term vaccines for different seasonal strains, concern still remains. This is because of the potential for avian strains to undergo genetic drift/shift. At that point, a human would be capable of harboring the virus and have little natural immunity to a very virulent disease strain.

PHLs serve as the sentinels for disease watch in the United States and worldwide. Selected laboratories worldwide partner with the World Health Organization (WHO) in the WHO Global Influenza Surveillance Network.[6] In the United States, funding for influenza surveillance comes through the CDC's Public Health Emergency Preparedness grant to the states and territories.[7] Surveillance is key to identifying new outbreaks of virus with the potential for human-to-human transmission. Community health workers from the local to global level have significant roles in managing potential outbreaks. Responses to outbreaks to date include the mass culling of infected domestic poultry, vaccination promotion efforts, and large-scale education about influenza. These are often quite complex and involve large numbers of stakeholders including federal response agencies, school districts, emergency management, police and fire agencies, healthcare service providers, and major employers.

Of particular interest for community health (and policy) practitioners in preparing for pandemic response are the large number of ethical and logistical issues involved. Three in particular have come to the fore:

- *Legal basis for forcible quarantine*: What is to be done with someone who is likely infectious, but does not comply with voluntary isolation and quarantine? It has been found that many states have inadequate legislation regarding the legal basis for forcible quarantine (essentially the involuntary detaining of someone without due process) and these laws vary widely by state. The federal government has some statutory authority, but most local efforts are left to the local and state authorities.[8] Many states rely upon laws that were passed in the 19th century. While all states have examined their current legal status in this matter as part of their pandemic preparedness planning, revising these laws in modern terms is quite often slow and difficult.

- *Rationing of vaccines*: Producing a vaccine for a new influenza strain remains (as of this writing) a long process. The time from disease identification to vaccine production is at least 6 months, and can be much longer for large-scale production. This is too long to deal with a new pandemic and other medical measures will need to be implemented. States and nations are therefore currently maintaining stores of antiviral medication. The problem comes about when there is not enough of this medication or other healthcare resources for those in need. Especially during a pandemic, demand may significantly outpace supply. Even in nonpandemic years, the supply of vaccine is often insufficient. Health response planners need to decide before the event how they will distribute a potentially insufficient supply. Do they make a priority to those at greater risk of serious morbidity and/or death (e.g., the very young and elderly)? Or do they first treat those responsible for keeping public order and infrastructure in place (e.g., police/fire, healthcare workers)? The denial of potentially lifesaving medication and treatment to anyone is a serious action that demands rigorous examination.[9]

- *Closing of schools*: One potentially important strategy for reducing the risk of infection spreading is the closing of schools during an epidemic. Schools by design place a large number of people from a relatively wide geographic area into a situation of extended and close contact. The chance of infection spreading in such circumstances is obviously substantial. Closing schools would therefore rule out this particular venue for infection. However, there are several serious issues that must be resolved before this can occur. For example, who actually has authority to close schools? District superintendents are not usually under the authority of the state health department. Also, what would be the impact of this closing on the local workforce? Would parents stay home from work to care for children (in a situation where many workers are already missing because of illness)? Would this keep police, fire, and healthcare workers at home when they are needed most? Would this cause smaller businesses to close and eventually go out of business? A recent study shows that, in at least the short-term, school closings were generally approved by parents and did not cause widespread loss of work, but its effects during a true pandemic are yet to be seen.[10]

It is for these reasons, and others, that community health practitioners work closely with multiple partners. It is also why they rely on timely and accurate laboratory

results, in this case results from sentinel surveillance, to provide as much information as early as possible to prepare and implement the best plans for the community. Laboratory typing of influenza identifies new cases as either being of a relatively mild, known strain, or a new and potentially pandemic new strain. This is critical in terms of the levels of response and resources used.

Environmental Health and Laboratory Work

Environmental health is the study of how the built and natural environments impact human health. The built environment includes those items that have been constructed as part of human living, such as home and building design and workplace safety. The natural environment includes nature itself and the organisms and compounds contained therein. The field therefore examines how an individual's surroundings impact health, and how these factors may be augmented or mitigated.

Laboratory analyses are often guiding, if not driving, factors in environmental health programs aimed at infectious diseases. Laboratory results provide the objective determination that a specific course of action has succeeded (e.g., successful decontamination of a commercial kitchen from *Salmonella*). They also provide supporting data for future courses of action. This section will discuss the emergence of Legionnaires disease as a specific public health concern and how environmental health practitioners use laboratory analyses to prevent and mitigate outbreaks.

Legionnaires Disease

Legionnaires disease acquired its name from an outbreak that occurred at the American Legion's convention in Philadelphia in 1976.[11] Within days of the convention start, there were a number of attendees experiencing symptoms of pneumonia. It is thought that there were as many as 221 people treated with 34 subsequent deaths. The symptoms are quite similar to other lung infections and it is difficult to determine exactly how many were infected. It was determined in 1977 that the bacterium *Legionella pneumophila* was responsible. This is one of the more than 46 *Legionella* species identified, 20 of which have been linked to disease in humans. Those causing disease are collectively referred to as Legionnaires disease bacteria (LDB). The combination of the large number of afflicted individuals with identification of the environmental conditions supporting bacterial growth caused the disease to gain widespread attention in the field of public health.

The initial investigation focused on more traditional sources of infection. These included examining the food consumed and the medical histories of attendees and staff. When this failed to reveal a likely source of infection, the CDC began more closely examining environmental conditions. The *Legionella* bacterium is widely found in the environment, and it was discovered that the cooling towers used in the hotel's air conditioners provided an excellent growing environment. As the bacterium must be inhaled to be infectious, the combination of positive growing conditions and easy introduction into the air handling system provided an excellent scenario for the spread of this disease. It was because of this investigation that new rules concerning air handling systems were introduced worldwide.

Legionnaires disease is fairly common in the United States, now that we are looking for it. There are an estimated 25,000+ cases every year resulting in 4000+ deaths.[11] More precise figures are difficult to obtain as the disease is often misdiagnosed because of its similarity to other respiratory features. In addition, it may present in a less severe form call Pontiac disease. Those stricken with this form may not even seek medical attention, and wait for the symptoms to resolve on their own.

Laboratory work continues to assist in maintaining safe environments by providing different types of analyses for the presence of LDB. The analysis of clinical samples from individuals is often the first clue that environmental conditions warrant examination. Follow-up investigations are supported and guided by the analysis of water and swab samples for the presence of LDB. These results indicate where or if contamination above allowable limits exists. This is important to determine as upward of 20% of diagnosed cases are actually associated with travel and efforts need to be focused toward the correct infection source.

Epidemiology and Laboratory Work

Epidemiology is the study of the causes, control, and distribution of disease in populations. It borrows heavily from biostatistics to help determine if disease clusters and outbreaks, or things thought to be associated with disease, are in fact significant. The alternative is that a cluster of cases or an association between an exposure and event is just a random occurrence. The knowledge of what is significant (real) versus insignificant (random) is quite important as it has direct implications to how

resources are allocated and how people alter their behavior. Laboratory analyses often contribute directly to these studies with direct evidence of either exposure (e.g., lead in blood) or disease (e.g., specific strain of *Salmonella*).

Food-borne Outbreaks

The traditional view of food-borne outbreaks has the source of infection associated with a local source, with local individuals infected. However, as occurrences in the past few years have shown, church potluck dinners are unlikely to be the culprit when considering an event whereby thousands may become ill in multiple states. Many people in a relatively small area becoming ill with the same symptoms within the same time period is often a highly visible red flag to authorities that there is a source of infection. But what if the same numbers of people, or many more, become ill in different towns, counties, or states? How would a physician or public health official in Springfield, IL, link the illness he sees with illnesses in Columbia, MO, and Evanston, IN? Or, if they do identify similarly affected individuals in these disparate locations, how do they know they were infected by a common bug from a common source?

Two tools in particular merge laboratory data with epidemiological analyses. One is the Salmonella *outbreak detection algorithm* which monitors *Salmonella* serotypes reported by state laboratories to the CDC. By collecting this information weekly along with historical data for the specific serotypes by state and region, epidemiologists are able to locate large and diffuse multistate outbreaks that are linked by a common type. *PulseNet* is a somewhat similar program involving state laboratories (and other partners such as the US Department of Agriculture) that perform molecular subtyping of selected food pathogen isolates using pulsed-field gel electrophoresis (PFGE). This procedure produces a "fingerprint" of the isolate's genetic material and is very specific to an organism. These patterns are collected and analyzed via PulseNet and allow epidemiologists to match isolates from different areas, providing links and patterns of exposure that may not be found otherwise. Both these tools have allowed the detection and investigation of outbreaks that would have been otherwise missed.[12]

Chlamydia Cluster Analysis

The endemic and intractable nature of chlamydial infection causes practitioners to search for novel methods of intervention. Successful infectious disease interventions must halt the transmission of disease (through education, mechanical barriers such as condoms, etc.) and/or eliminate sources of the disease (identifying and treating those infected). Many traditional STI intervention methodologies have been quite broad in scope in terms of the target population and geographic area. Many of these have failed to cause a reduction in the incidence of new infection. New methods which more narrowly focus resources, for a hoped-for greater impact, are being investigated.

One such method relies in great part on laboratory analysis of specimens. There is a growing body of research investigating the geographical distribution and "clustering" of CT cases, and how that knowledge may be combined with other disciplines (e.g., social networking theories) to develop geographically targeted interventions. The idea is that an intervention might be designed for a specific population, in a specific area, and therefore have a greater result than one applied more widely. While usual CT analysis only identifies the genus (*Chlamydia*) and species (*trachomatis*), Wylie et al. had samples from Manitoba, Canada, analyzed at the genotypic level. They discovered that there were numerous, geographically distinct clusters of cases in this province. Additionally, they found that many of them were genotypically distinct, even if they were geographically close.[13] The implication of this is that there are distinct social networks at play here, otherwise, the mix of genotypes would be more heterogeneous. From this they may be able to design an intervention to target a specific area and social/demographic population. These distinct social sexual networks would not likely have been discovered without the aid of laboratory analysis.

Health Policy and Administration and Laboratory Work

The field of health policy and administration studies and implements legislation, rules, and policies that work to mitigate adverse health and promote good health. The field is broad, and includes such activities as proposing legislation to limit pollutant emissions, using laboratory analyses to evaluate the effect of a program, and altering health programs based on evaluations of their effectiveness. Of the five public health fields, laboratory data may have the least direct impact here as the data are most often used by programs in other fields, which then support policy and administration. For example, environmental health specialists may use the analysis of air samples (described in Chapter 8) to show that federal legislation limiting the emission of pollutants has caused a significant decrease in the concentration of these pollutants. Lead

concentrations in particular have decreased substantially since it was banned for use as an additive to gasoline in 1986. One may therefore state that the legislation is indeed working and producing the intended effect.

Regulation of DDT

Rachel Carson's seminal work *Silent Spring* brought the environmental dangers of DDT to the public's attention by showing how its use was associated with a dramatic decline in the number of predatory birds (eagles, hawks). She described how this pesticide entered the environment and was ingested and stored in the bodies of small animals, particularly fish. As these fish were consumed by larger fish, their stored DDT was passed to the predator and concentrated, repeatedly, up the food chain. Eagles, hawks, and other top predators subsequently consumed sufficient DDT to cause a resultant decrease the thickness of their egg shells, leading to increased egg loss and decreased population. Legislation was enacted that eventually banned the use of DDT in the United States. Laboratory analyses of water, soil, and animal tissue showed where the DDT exposure was occurring and how it became more concentrated higher in the food chain.

We hope to have shown to the reader the importance of laboratory work in the wider practice of public health. The results generated by these laboratories are not solely used for diagnoses. They support investigations, ensure continuing good practices and safety, and inform policy. No field of public health can be effective in a vacuum, but must collaborate with other disciplines. Laboratories are often the source of hard or concrete data in an often fuzzy world. We do well to recognize the importance of their contributions to many fields of study and policy. Laboratories, in turn, are indebted to the programs that they serve. Data are no good if not put to use, and laboratories are not equipped to use data to educate, train, or effect change. Collaborative efforts between analyses, practice, and policy are what will continue improvements in public health for the coming future.

Discussion Questions

1. Besides prevalence, what other factors might contribute to the development of targeted screening guidelines?
2. What data would you need to determine if screening children for hypertension is worthwhile?
3. State health department laboratories are the sentinels for identifying new strains of influenza in their community (be it local or state). They are often extremely busy with sample analyses when a new virus begins to spread, but usually have much fewer samples for analysis as time passes. Why might fewer samples be submitted during an epidemic than nearer the beginning?
4. Environmental sample analysis for LDB is a part of environmental health inspections. Name three other areas where the practice and research of environmental health are dependent on laboratory analysis.
5. Describe how laboratory analysis and "fingerprinting" allow epidemiologists to determine the extent of a food-borne outbreak.
6. The chlamydia example showed how serotyping was useful in determining social–sexual networks to target for intervention. What might be some of the shortcomings with performing this type of analysis in a larger, more heavily populated area?
7. Many laboratories routinely perform analyses to show the absence/level of a regulated substance in a sample (e.g., benzene in water). Besides the obvious use of this testing to ensure safety from exposure, what is another benefit to this type of monitoring?
8. How might the reporting of a sample(s) positive for influenza A H1N1 or West Nile virus in a particular county potentially change policies or strategies at the local and state level?

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Public Health Laboratories

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40 Tall Pine Drive
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978-443-5000
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