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Tonya M. Colpitts
Editor

West Nile Virus

Methods and Protocols

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West Nile Virus

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Preface

Protocols for West Nile Virus Research is a collection of research methods, techniques, and approaches to investigate the molecular biology of West Nile virus (WNV). The target audience is comprised of researchers such as virologists, molecular biologists, microbiologists, and other scientists working with WNV. The methods provided here will serve as a comprehensive guide for those conducting research in the field of WNV biology, including life cycle, pathogenesis, impact of infection, and possible treatment/prevention development. A brief introduction along with a discussion of WNV laboratory safety is also included here.

Columbia, SC, USA ***Tonya M. Colpitts***

Contents

Preface v
Contributors ix

1 A Brief Review of West Nile Virus Biology 1
Berlin Londono-Renteria and Tonya M. Colpitts

2 Overview of West Nile Virus Transmission and Epidemiology 15
Andrea Troupin and Tonya M. Colpitts

3 Propagation and Titration of West Nile Virus on Vero Cells 19
Alexander J. McAuley and David W.C. Beasley

4 Generating West Nile Virus from an Infectious Clone 29
Rianna Vandergaast and Brenda L. Fredericksen

5 Functional Analysis of West Nile Virus Proteins in Human Cells 45
Pakieli H. Kaufusi, Alanna Tseng, and Vivek R. Nerurkar

6 Lessons from the Murine Models of West Nile Virus Infection 61
Brenna McGruder, Vandana Saxena, and Tian Wang

7 Exploration of West Nile Virus Infection in Mouse Models 71
Penghua Wang

8 Examination of West Nile Virus Neuroinvasion and Neuropathogenesis in the Central Nervous System of a Murine Model 83
Hameeda Sultana

9 In Vitro and In Vivo Blood–Brain Barrier Models to Study West Nile Virus Pathogenesis 103
Mukesh Kumar and Vivek R. Nerurkar

10 Methodology for Identifying Host Factors Involved in West Nile Virus Infection 115
Manoj N. Krishnan

11 ELISA and Neutralization Methods to Measure Anti-West Nile Virus Antibody Responses 129
Brian M. Friedrich and David W.C. Beasley

12 Safe Handling of West Nile Virus in the Insectary 143
Dexter R. McKellar and Michael J. Conway

13 Techniques for Experimental Infection of Mosquitoes with West Nile Virus 151
Yang Liu and Gong Cheng

14 Field Surveillance Methods for West Nile Virus 165
Samuel B. Jameson and Dawn M. Wesson

15 Detection Protocols for West Nile Virus in Mosquitoes, Birds, and Nonhuman Mammals 175
Elizabeth B. Kauffman, Mary A. Franke, and Laura D. Kramer

16 Field Methods and Sample Collection Techniques for the Surveillance of West Nile Virus in Avian Hosts 207
Sarah S. Wheeler, Walter M. Boyce, and William K. Reisen

17 Statistical Tools for the Interpretation of Enzootic West Nile virus Transmission Dynamics 221
Kevin A. Caillouët and Suzanne Robertson

18 Role of Immune Aging in Susceptibility to West Nile Virus 235
Yi Yao and Ruth R. Montgomery

19 An Overview of Current Approaches Toward the Treatment and Prevention of West Nile Virus Infection 249
Dhiraj Acharya and Fengwei Bai

Index *293*

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Chapter 1

A Brief Review of West Nile Virus Biology

Berlin Londono-Renteria and Tonya M. Colpitts

Abstract

West Nile virus (WNV) is an arbovirus with increased global incidence in the last decade. It is also a major cause of human encephalitis in the USA. WNV is an arthropod-transmitted virus that mainly affects birds but humans become infected as incidental dead-end hosts which can cause outbreaks in naïve populations. The main vectors of WNV are mosquitoes of the genus *Culex*, which preferentially feed on birds. As in many other arboviruses, the characteristics that allow *Flaviviruses* like WNV to replicate and transmit to different hosts are encrypted in their genome, which also contains information for the production of structural and nonstructural proteins needed for host cell infection. WNV and other *Flaviviruses* have developed different strategies to establish infection, replication, and successful transmission. Most of these strategies include the diversion of the host's immune responses away from the virus. In this review, we describe the molecular structure and protein function of WNV with emphasis on protein involvement in the modulation of antiviral immune responses.

Key words West Nile virus, Flavivirus, WNV protein, WNV structure, Virus immune evasion

1 Virion Structure

WNV is an important member of the family *Flaviviridae* closely related to yellow fever virus and dengue virus. All members of this family have single-stranded, positive-sense RNA genomes with similar gene order and conserved nonstructural protein motifs [5]. In general, the virion is comprised of a host-derived membrane envelope and a nucleocapsid containing the virus genome. Later, we describe each of the proteins encoded by the genome of the WNV and their role in the virus life cycle. Here, we will start with the proteins and nucleic acid found in intact virions.

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2 Viral Envelope

The outer shell of the virion is formed by the specific interaction of 180 copies of the membrane protein (MP) and 180 copies of the envelope glycoprotein (EP) creating three distinct symmetry environments resulting in icosahedral symmetry. The external domains of the EP dimers recline close to the outer surface of the virion membrane. The MP contains two membrane-spanning domains and a short ectodomain. The EP encoded by most strains of WNV is glycosylated at a single N-linked glycosylation site. Interestingly, the capsid protein (CP), located inside virions, has no discernible nucleocapsid symmetry and no established contacts with either EP or MP [6, 7].

2.1 Viral Nucleocapsid

2.1.1 Genomic Structure

The WNV virion is approximately 50 nm in diameter with a positive-stranded RNA genome. A single copy of the viral RNA is enclosed within a nucleocapsid formed by the multiple copies of the capsid protein, later enclosed by a lipid bilayer derived from the host cell [8]. CP dimers are displayed with half of the basic residues located on the internal side of the virion interacting with the genomic RNA and a conserved hydrophobic region on the opposite side that faces the inner side of the virion envelope [9]. However, there have been no reports of an actual contact between the CP and the other structural proteins located in the envelop membrane [10].

The WNV genome has a length of 11,000 nucleotides [11], a single open reading frame (ORF), and two noncoding regions (NCR), one at the 5′ end (96 nucleotides) and a larger NCR region at the 3′ end (631 nucleotides). The three viral structural proteins, capsid (C), membrane (prM/M), and envelope (E), are encoded within the 5′ end of the genomic ORF, while the seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are encoded within the 3′ end. Their RNA genome contains a 5' cap structure that is methylated at the guanine N-7 and the ribose 2′-OH positions of the first transcribed nucleotide (type 1 cap structure) [12]. Like the other viruses of the *Flaviviridae* family WNV genome lacks a 3′ poly-A region or tail. Instead, the 3′ end of the genome terminates with CUOH [13, 14].

2.2 Noncoding Genomic Regions

The noncoding regions (NCRs) located at the 5′ and 3′ ends of the WNV genome contain conserved secondary RNA structures that play an important role not only in genome replication but also in protein translation enhancement [15]. They are also important for mediating RNA interactions with respect to genome cyclization and replication [16]. The 5′ NCR may act as a template for recognition by the enzyme responsible for methylation reactions required for cap assembly [17]. The 3′ NCR may function as an

interaction site for the replication complex assembly and for initiation of negative-sense RNA synthesis [18, 19]. Interestingly, vertebrate host protein elongation factor 1α, the T cell-restricted intracellular antigen-related protein (TIAR), and the TIA-1 host proteins have been shown to be involved in WNV replication through demonstration of direct interactions with the genome NCRs [20–22].

2.3 Genome Cyclization and Replication

An essential role of genome cyclization for replication of *Flaviviruses* has been demonstrated [23]. Typically, RNA-dependent RNA polymerase binds to a stem loop at the 5′ NCR. Consequently, cyclization allows the interaction of the polymerase with the 3′ end facilitating the synthesis of the negative-RNA strand. However, the balance between circular and lineal forms of the intracellular viral genome is essential for efficient RNA replication and modulation of translation initiation [24, 25]. The current model for WNV negative-RNA synthesis includes the NS5 protein binding at the 5′ end of the viral positive-RNA genome and the relocation of the polymerase to the 3′ initiation site after the RNA-RNA interactions mediate long-distance genome cyclization [25].

3 Encoded Proteins

The WNV genome encodes for ten viral proteins that are processed to single proteins by the viral serine protease, NS2B-NS3, and various cellular proteases after the translation of a single polyprotein [26–28]. These proteins are divided into two groups according to their role in the virus life cycle: (1) structural proteins (proteins that are part of the virion particle) and (2) nonstructural (proteins responsible for replication and virus survival that are not incorporated in the virion particle). A summary of these proteins is described below:

3.1 Structural Proteins

Capsid protein: The capsid (CP), also called the core protein, is implicated in viral assembly and replication and is mainly composed of charged amino acids. The CP dimerizes and tetramerizes to build an electron-dense nucleocapsid (RNA + CP) that is later enclosed by the lipid bilayer envelope [29]. The N- and C-terminal parts of the CP may play a role in RNA folding during viral replication by conferring RNA chaperoning activity to the capsid [30], while the central part of the protein structure reveals the presence of four α-helices likely involved in direct interaction with the viral RNA [31].

Envelope: The envelope protein (EP) is the most immunogenic protein of the virus and the target for most neutralizing antibodies [32, 33]. The EP is a transmembrane protein, is glycosylated on

position 154 on most WNV strains, and is anchored to the lipid membrane by a C-terminal α-helical hairpin [27, 34]. Glycosylation of the EP is important for efficient transmission to both vertebrate and invertebrate hosts. EP glycosylation modifications have also been associated with human neurological disease [35]. The atomic structure of the E glycoprotein shows the typical folding of the *Flavivirus* E glycoproteins, organized in three domains known as DI, DII, and DIII [36, 37]. DI contains approximately 120 residues in three segments (residues 1–51, 137–189, and 285–302). The dimerization domain of EP is achieved through the two loops formed between these three segments [38]. Additionally, DII contains a virus fusion loop that mediates the homodimerization of EP on the cellular membrane through the insertion of the distal β-barrels into the cellular membrane [39]. DIII, an immunoglobulin-like domain involved in receptor binding, contains multiple epitopes that are recognized by neutralizing antibodies, which makes it an attractive candidate for vaccine development [40]. Conformational rearrangements, such as switching from dimers to trimers, expose the fusion loop and enable viral envelope fusion with endosomal target membranes [41].

Membrane protein prM/M: The prM/M is a short transmembrane glycosylated protein associated with the lipid bilayer of the virion. In the immature virion, the prM proteins cover the fusion loop of the EP. The prM is linked to the EP (prM-EP heterodimer) in the immature virus at a neutral pH [42]. Virion maturation occurs when furin-like proteases cleave off the prM/M, turning inert virus into infectious particles within the trans-Golgi network [43]. At low pH (i.e., 6.0), the immature viral particles undergo a reversible conformational change that allows this cleavage by furin [44]. Mature virions have no exposed M on the surface. In short, the function of the prM protein is to protect the EP proteins from an early fusion with host membranes inside acidic vesicles of the Golgi complex [11].

3.2 Nonstructural Proteins

NS1: Nonstructural protein 1 is a glycoprotein that contains three extremely conserved N-linked glycosylation sites and multiple cysteines, which form disulfide bonds essential for virus viability [45]. NS1 may have differing oligomeric states depending on the cellular location or whether secretion occurs. The state of NS1 can vary between dimers, its primary form, to monomers and/or hexamers [46, 47]. For instance, viral NS1 monomers are soluble and hydrophilic while the secreted NS1 by mammalian cells exists in primarily hexamers formed by three dimeric subunits [48]. Previous studies suggest that maturation and secretion of hexameric NS1 from *Flavivirus*-infected cells depend on the glycosylation status of the host cell [48]. NS1 is mainly located on the luminal side of the ER membrane. However, it can also localize with viral replication

complexes located on the other side of the ER membrane. The location of NS1 on either side of the ER membrane is determined by the C-terminal E protein signal sequence [11, 49]. Intracellular NS1 functions as an essential cofactor for viral replication, where it localizes to WNV replication sites, while cell surface and secreted NS1 may act as an immunomodulator antagonizing complement activation and Toll-like receptor signaling [50].

NS2A: The NS2A protein is a hydrophobic transmembrane protein of approximately 25 kDa involved in viral RNA replication, induction of membranous structure formation, and virion assembly [51–53]. NS2A is also involved with regulation of host immune responses and viral dissemination [54, 55]. NS2A is primarily located within vesicle packages (VP) associated with the viral dsRNA. NS2A binds with high specificity to the 3′ untranslated region (UTR) of viral RNA and to other components of the replication complex [56].

NS2B: This small hydrophobic protein acts as a cofactor of the NS3 protease and may function as a membrane anchor for viral protease. In the case of dengue virus (DENV), a central hydrophilic domain of 40 residues is required for protease activity and can directly interact with NS3 [57]. Three membrane domains at both N- and C-terminus are important for the association of the protease complex into the virus-induced membranes [58]. The specific interaction between NS2B and NS3 possibly allows NS3 to unwind the WNV RNA [59, 60].

NS3: The NS3 is a highly conserved protein in which the N-terminal portion encodes the viral trypsin-like serine protease. The NS3 protease is able to cleave the viral polyprotein to release both structural and nonstructural proteins [58]. NS3 is only active in the presence of its cofactor, NS2B [61]. Membrane association of the NS3-NS2B complex is required for efficient polyprotein processing. NS3 is associated with the ER membrane through its interaction with the NS2B protein or by directly interacting through amino acids 31–33 forming an anchor that stabilizes the N-terminal NS3 protease domain. The NS3 protein also presents other enzymatic activities within its sequence (helicase, nucleoside triphosphatase, RNA triphosphatase) that are important for WNV replication. The NS3/NS2B protease complex has been localized within paracrystalline arrays (PC) or convoluted membranes (CM), suggesting that these are the membranes involved in proteolytic cleavage [62, 63].

NS4A and NS4B: They are both hydrophobic proteins with several transmembrane domains bound to the viral replication complex in virus-induced membranes. NS4A may act as a cofactor regulating ATPase activity of the NS3 helicase [64]. Cleavage of its C-terminal region (2 K fragment) may be responsible for membrane

rearrangements in infected cells [65]. NS4A is also associated with immune evasion. NS4B protein plays a major role in the inhibition of WNV interferon signaling [66]. Mutations in NS4B have been shown to result in attenuation of WNV replication in vivo [66]. Previous studies have proposed that cleavage at the 4A/4B site is mediated by a cellular signal peptidase since the preceding NS4B amino acid sequence is very similar to the sequence of other signal peptides used for translocation of proteins into the endoplasmic reticulum lumen [68].

NS5: NS5 is the largest protein encoded by the WNV genome. NS5 co-localizes with genomic RNA at viral replication complex sites [69]. It has two different enzymatic activities: the N-terminal encodes the methyltransferase (MTase), while the C-terminal encodes the viral RNA-dependent RNA polymerase responsible for the replication of the virus genome [69]. Since NS5 lacks proofreading activity, WNV populations can express high sequence diversity, which favors advantageous variants in response to environmental selective pressures. The methyltransferase and polymerase activities make NS5 a promising antiviral target because its activity is necessary for capping the 5′ end of the viral RNA involved in viral replication. The NS5 mediates N-7 and 2′-O methylations and is therefore essential for *Flavivirus* 5′-cap formation. The NS5 methyltransferase domains use S-adenosylmethionine as a methyl donor. Mutagenesis studies with viral MTases suggest that NS5 requires distinct amino acids for its MTase activities. Correct methylation of the cap structure at the guanine N-7 and the ribose 2′-OH positions of the first transcribed adenine is necessary for optimal WNV infectivity. WNV deficient in the N7 methylation mechanism are non-replicative, while virions lacking the 2′-OH methylation are replicative but attenuated [70].

4 Molecular Aspects of WNV Life Cycle

4.1 Cell Tropism

In vitro studies reveal that WNV is able to replicate in various types of both primary and immortalized cells from a wide variety of hosts, including birds, mammals, amphibian, and insect species. These findings suggest that WNV is using highly conserved receptors, or entry molecules, to invade cells and move from host to host [71, 72]. In mice and humans, the virus undergoes a first replication cycle at the mosquito bite site and then travels to the draining lymph nodes. In these two hosts, viral cell tropism attracts the virion to monocytes, macrophages, dendritic cells, and endothelial cells [73, 4]. Replication in mononuclear cells in lymph nodes results in a primary viremia that is then followed by the infection of peripheral tissues. Once the virus enters circulation, it is able to cross the blood–brain barrier and infect neurons [74].

WNV infection is initiated by the binding of virions to the cellular receptors, which have been proposed to include glycosaminoglycans (GAGs), c-type lectins, and integrins [75, 76]. Viral particles are internalized into host cells via clathrin-dependent pathways and are then transported via endosomal compartments [75]. WNV first attaches to the cell surface using the EP, which allows the virus to enter the cells by a receptor-mediated endocytosis process [39]. Low pH in the endosomal vesicle triggers fusion between the viral and the host cell membranes through structural reorganization of the E protein [77]. Optimal fusion of WNV with liposomes occurs very fast at pH levels between 6.3 and 6.9. Membrane fusion leads to the release of the nucleocapsid and viral RNA into the cell cytoplasm [77].

4.2 Membrane Rearrangements

Most *Flavivirus* life cycles are associated with intense host cell membrane alterations that result in the formation of membranous microenvironments facilitating virus replication. In most cases, the 70- to 100-nm membrane "vesicles" house the replicating viral RNA. The cellular origin of such membranes in the case of WNV is the endoplasmic reticulum (ER) [78, 67]. Three well-defined structures can be seen in WNV-infected cells: (1) CM, (2) PC, and vesicle packets (VP) or membrane sacs containing small vesicles [79]. Localization studies suggest that components of the virus protease complex localize specifically in the CM/PC, while viral double-stranded RNA (dsRNA) and the viral RNA-dependent RNA polymerase (RdRp) NS5 localize primarily to VP [80, 81]. The CM and PC structures are modified membranes of the intermediate compartment and rough ER, whereas the VP appears to be derived from *trans*-Golgi network membranes [79]. In some WNV strains, the NS4A protein is able to induce the formation of CM and PC structures [82]. The induction of these structures during WNV replication offers partial protection from viral clearance by interferon (IFN)-induced antiviral factors [50].

5 Mechanisms of Immune Evasion

In order to establish an infection, viruses need to either suppress or escape from host immune defense systems. Evasion mechanisms can be summarized into two categories: (1) those directed to the *innate immune response* and (2) those able to inhibit *adaptive immune responses*. Recent immunological research has focused on innate immunity as the first line of host defense against invading pathogens, especially pattern recognition molecules identifying microbial components called pathogen-associated molecular patterns (PAMPs). These PAMPs frequently induce inflammatory responses mediated by interferon, the hallmark of antiviral immune responses [83, 84]. *Flavivirus* infection may result in the modification

or suppression of immune responses and several viral coded proteins have been associated with diverse "escape" mechanisms. One of the mechanisms for WNV is the production of membrane rearrangements as discussed earlier in this review. The membrane rearrangements hide the viral genome delaying recognition by immune surveillance and conferring protection against the IFN-induced antiviral responses [78].

In most cell types, WNV-induced cell death occurs following several rounds of replication and recent research has pointed to a CP-dependent survival of the infected cell. WNV CP can be detected in the cytoplasm, nuclei, and nucleolus of infected cells. Its presence has been associated with protection against apoptosis by a process that involves activation of the kinase Akt, a pro-survival kinase that blocks apoptosis [85]. The capsid protein has also been implicated in potentiating WNV dissemination by modulating claudin protein degradation, a major component of the tight junction [86]. Literature suggests that the CP alone may induce the disruption of epithelia barrier in vitro and this process is mediated by lysosomal proteases [87].

Other WNV proteins also have immunomodulatory properties. For instance, the protein NS2A has the ability to inhibit α/β interferon production, which increases viral virulence. Previous work has demonstrated that an alanine-to-proline substitution at position 30 of the NS2A gene confers attenuated virulence of WNV. It also prevents the virus from downregulating the production of IFN-α/β [88]. Inhibition of interferon signaling has also been observed by the presence of NS2A, NS4A, NS4B, and NS5 [54, 55]. The type I IFNs appear to inhibit infection by preventing the transcription of minus-strand RNA [89] and by stimulating the generation of proinflammatory molecules and nitric oxide [90].

Complement inhibition by *Flaviviruses* has also been described. For instance, the NS1 from WNV is able to bind the complement factor H, a major regulator of complement activation, disrupting the formation of the alternative pathway C3 convertase (C3bBB). NS1 can also interact with C4 and reduce C4b deposition and formation of the C4b2a, the C3 convertase of the classical pathway [91, 92].

Antibodies are the core of the adaptive immune response against any pathogen. Neutralizing antibodies against *Flaviviruses* are mainly directed against the EP, although antibodies directed against NS1 are also important in conferring protection [93, 94]. Viral mutation and antigenic variation are the main viral weapons against neutralizing antibodies. However, previous studies have shown that mutations on the EP may significantly alter the replication efficiency and virulence of WNV in infected animals such that these "improved" viral versions are able to replicate to higher levels while reducing TNFα induction by immune cells [95].

6 Conclusion

WNV is a neuroinvasive arbovirus with a very complex structure that facilitates its replication and transmission while enabling it to avoid detection and destruction by the host. Mortality by WNV is escalating, though the majority of infections are still asymptomatic. Further investigation is necessary to unravel the mechanism by which some strains of the virus selectively induce severe pathogenesis while others cause mild disease.

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Chapter 2

Overview of West Nile Virus Transmission and Epidemiology

Andrea Troupin and Tonya M. Colpitts

Abstract

West Nile virus (WNV) is a mosquito-borne flavivirus that can cause mild-to-severe disease in humans and horses. WNV was first documented in Uganda in 1937 and passed through the majority of Africa, West Asia, and Europe before arriving in the USA (with infections in New York City in 1999). After the spread of the virus on the US east coast, it traveled westward, northward, and southward through the USA and into Central and South America. WNV can cause fever, rashes, nausea, vomiting, and potentially neuroinvasive disease or death. The virus is sustained through a mosquito-bird-mosquito cycle and there are many species that are competent vectors. Unfortunately, there are no vaccines and the only treatment is supportive care. This chapter highlights the epidemiology and transmission of WNV and provides insight into some of the challenges of controlling WNV disease.

Key words West Nile virus, Transmission, Epidemiology, Mosquitoes, *Culex* sp.

1 Introduction

West Nile virus (WNV) is a mosquito-borne flavivirus that was first identified in Uganda in 1937. It now can be found in Africa, Europe, West Asia, North America, and Australia [1–3]. In 1999, WNV made its first appearance in North America by the way of New York City causing an epidemic of encephalitis. By 2002, the disease was reported in 39 states with ~4000 cases and almost 300 deaths (Fig. 1; [5]). Most human WNV infections are asymptomatic but a small percentage of people present with symptoms such as fever, rash, nausea, and vomiting [1, 5]. Less than 1 % of infected people may develop severe disease, which can include headaches, disorientation, coma, paralysis, and potentially death. Individuals over the age of 50 have a higher incidence of severe neuroinvasive disease [3, 5]. The only available vaccines are for horses and there is no specific treatment other than supportive care.

Birds are the natural reservoir and *Culex* sp. are the natural vectors of transmission, and WNV is maintained in nature in this

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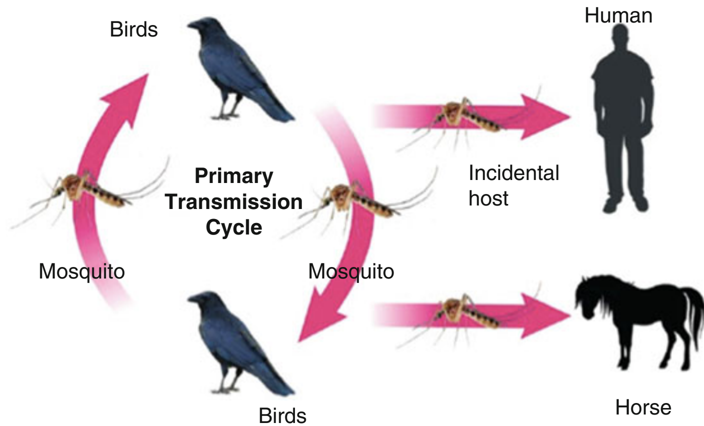

Fig. 1 Incidence of West Nile virus in the USA from 1999 to 2004 [4]

mosquito-bird-mosquito transmission cycle (Fig. 2). WNV is primarily transmitted by *Culex* sp. mosquitoes but other mosquito genera can be involved if they favor people and their dwellings. In North America alone, 59 mosquito species have been identified that can become infected with WNV; however, only 10 out of the 59 are competent transmission vectors [1, 4]. The dominant vector species for WNV varies across the USA. For example, the northern house mosquito, *Culex pipiens*, an efficient transmission vector, made up at least half of the WNV-positive mosquitoes in the northern region of North America during initial viral spread [1, 4]. In 2002, *Culex pipiens* still made up a large portion of confirmed WNV-positive mosquitoes though *Culex quinquefasciatus*, also known as the southern house mosquito, emerged as the leading WNV transmission vector in the southern USA. *Culex tarsalis* is an efficient laboratory vector but it has also been found to be the principal vector west of the Mississippi River [4].

Birds are the natural reservoir, or amplifying hosts, for WNV, and in North America over 100 bird species are capable of becoming infected. Most bird hosts are able to survive the disease and develop permanent immunity [1, 4]. Infected birds have transient high viremic titers consequently allowing transmission of WNV to ornithophilic mosquito species. In North America, crows and jays from the family Corvidae do not usually survive WNV infection and are useful in tracking virus spread through dead-bird surveillance programs [1, 3, 4]. WNV can be transported through different regions by viremic migratory birds along established flyways [1].

Unfortunately, humans and horses can become incidentally infected and develop a mild-to-severe disease. As incidental or "dead-end" hosts, both humans and horses are unable to transmit the virus to other mammals or mosquitoes during infection.

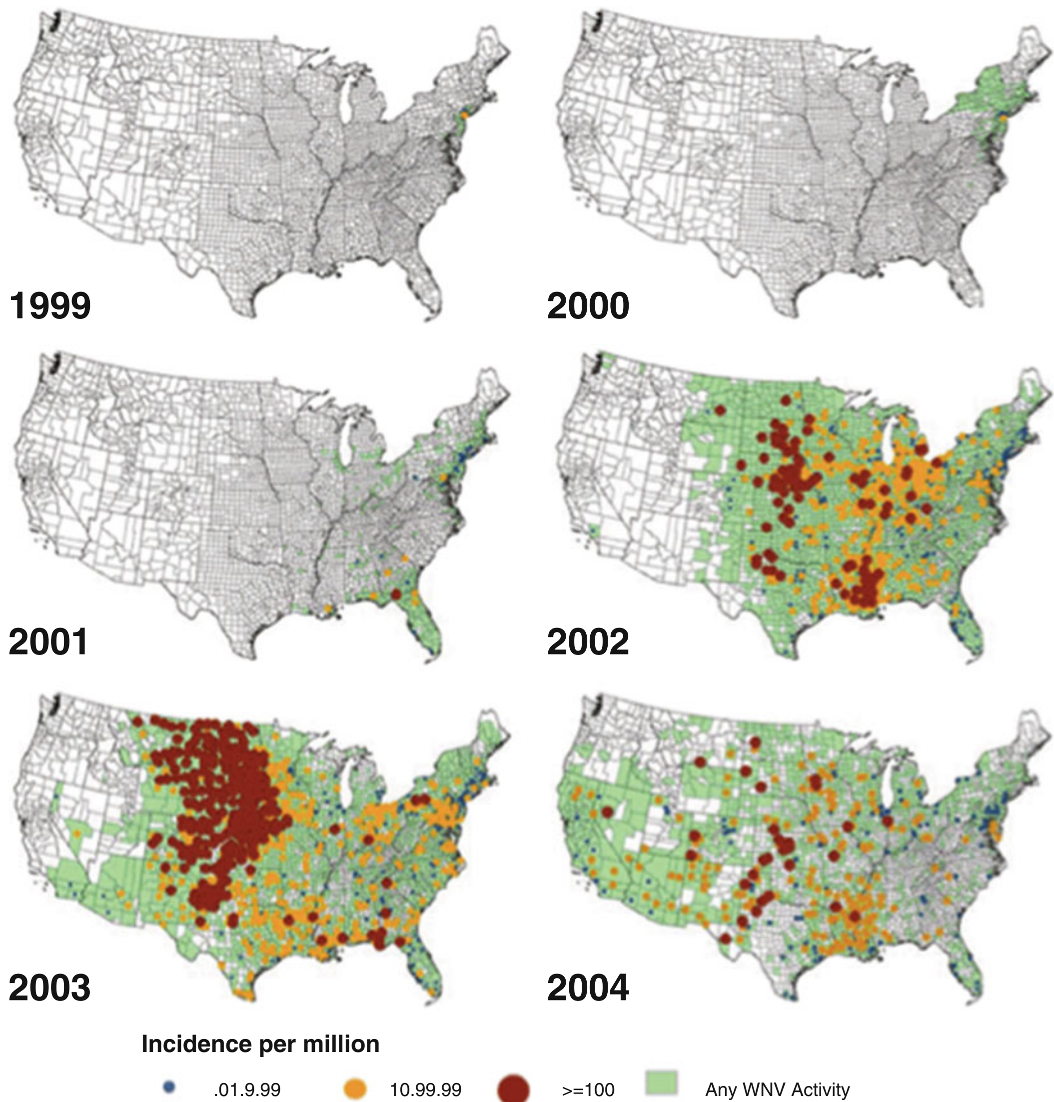

Fig. 2 West Nile transmission cycle [6]

Interestingly, eight other animal species including cats, rabbits, skunks, squirrels, chipmunks, alligators, lake frogs of Russia, and some bats may become infected with WNV but these are usually asymptomatic [1, 3, 4]. The role that these animals play in the transmission cycle is unknown, but it is highly unlikely that they are natural reservoirs as viremia titers are quite low [4].

Little was known about WNV upon its detection in 1937 but the virus has become the primary cause of arboviral neuroinvasive disease on every continent excluding Antarctica [2]. In 2002, reports of transmission pathways through blood transfusions, organ donations, and from mother to neonate during childbirth

or breastfeeding were discovered [2, 3]. The virus is able to overwinter year after year, indicating that it has an efficient "hibernation" method. Reducing the risk of WNV transmission will require intense emphasis on sustained mosquito control and public education [1, 3]. Providing bed nets, applying insect repellant, and encouraging people to remain inside during peak mosquito biting times are crucial community programs [3]. In addition, environmental modifications to destroy mosquito-breeding sites will lower transmission rates. Sadly, the risk of outbreaks remains high in cities with unsanitary conditions and poor economic environments [1]. Many of these cities lack the infrastructure for proper surveillance or vector control programs creating a higher risk of disease and thus continuing the transmission cycle and incidental human infections.

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Chapter 3

Propagation and Titration of West Nile Virus on Vero Cells

Alexander J. McAuley and David W.C. Beasley

Abstract

The propagation and titration of viruses are key virological techniques. Unlike other flaviviruses, such as the dengue viruses, West Nile virus (WNV) grows and plaques very efficiently on Vero cells, usually inducing strong cytopathic effect (CPE) and forming clear plaques. Here, we outline the steps for propagating WNV from culture supernatant stocks and homogenized organ/mosquito samples, as well as for determining virus titers in samples by serial-dilution plaque assay using neutral red or crystal violet stains.

Key words West Nile virus, Virus propagation, Vero cells, Virus titration, Plaque assay

1 Introduction

Unlike many other mosquito-borne flaviviruses, most strains of West Nile virus (WNV) grow rapidly and to high titers in mammalian and mosquito cell cultures. In addition, naturally occurring WNV strains generally have consistent plaquing characteristics which facilitates quantification of infectious virus particles. In the USA, WNV is classified as a biosafety level 3 (BSL3) virus and all quantities of potentially infectious WNV cultures must be handled in a laboratory approved for that level of containment. Strains of the closely related Kunjin virus, now classified as a subtype of WNV, may be handled in BSL2/PC2 laboratories [1].

WNV grows in a wide range of mammalian, avian, and mosquito cell types and it should be noted that, as for most flaviviruses, serial passaging in a single cell type is often associated with generation of adaptive mutations (e.g., mosquito cell adaptations [2]; mouse cell-adapted NS4B mutation [3]) that may result in phenotypic shifts affecting growth in other cell types or virulence in animal models. This chapter provides the standard methods used in our laboratory for preparation and plaque titration of WNV stocks using Vero cells. Starting materials for the virus propagation may be culture supernatants, serum/plasma, or clarified and/or filtered animal tissue or mosquito homogenates.

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2 Materials

2.1 Virus Culture in Vero Cells

1. Vero cells (ATCC #CCL-81; ATCC, Manassas, VA).
2. Growth medium: MEM + 8 % FBS + 1% L-glut + 1 % NEAA + 1 % pen/strep. To one bottle of MEM (Corning), add 40 ml FBS (Hyclone), 5 ml 100× L-glutamine (Gibco), 5 ml MEM non-essential amino acid solution 100× (Sigma), and 5 ml 100× penicillin/streptomycin (Corning). Store at 4 °C until use. FBS should be heat inactivated at 56 °C for 30 min prior to use. After inactivation, FBS can be aliquoted in suitable volumes for intended use and stored at −20 °C.
3. Maintenance medium: MEM + 2% FBS + 1% L-glut + 1% NEAA + 1% pen/strep. To one bottle of MEM (Corning), add 10 ml FBS (Hyclone), 5 ml 100× L-glutamine (Gibco), 5 ml MEM non-essential amino acid solution 100× (Sigma), and 5 ml 100× penicillin/streptomycin (Corning). Store at 4 °C until use.
4. 1× Sterile phosphate-buffered saline (PBS; Gibco).
5. 5, 10, and 25 ml Serological pipettes.
6. T-75 Tissue culture flask (Corning).
7. 1.5 ml Eppendorf tubes.
8. 1.5 ml Cryovial tubes.
9. Pipettes and aerosol-resistant pipette tips.
10. Microcentrifuge.

2.2 Plaque Titration on Vero Cells

1. Growth medium (described above).
2. Maintenance medium (described above).
3. Overlay medium: 2× E-MEM + 4% FBS + 2% L-glut + 2% NEAA + 2% pen/strep. To one bottle of 2× E-MEM (e.g., Quality Biological, catalogue# 155-073-101), add 20 ml FBS (e.g., Hyclone, catalog# SH30071), 10 ml 100× L-glutamine (e.g., Gibco, catalog# 25030), 10 ml MEM non-essential amino acid solution 100× (e.g., Sigma, catalog# M7145), and 10 ml 100× penicillin/streptomycin (e.g., Corning Cellgro, catalog# 30-001-CI). Store at 4 °C.
4. Neutral red solution: 0.33% neutral red in PBS (Sigma). Neutral red is light sensitive, store in the dark at 4 °C.
5. 0.25% Crystal violet solution: Add 250 mg crystal violet (Sigma) to 100 ml 10% formalin solution. Store at room temperature until needed.
6. 2% Agar: Dissolve 2 g agar per 100 ml deionized water. Autoclave to sterilize prior to use. Autoclaved agar may be stored at room temperature until needed.

7. 1× PBS (Gibco).
8. 10% Formalin solution (Sigma).
9. 6-Well tissue culture plates (Corning).
10. 96-Well tissue culture plates for sample dilutions (Corning).
11. 1.5 ml Eppendorf tubes.
12. 5, 10, and 25 ml serological pipettes.
13. 8- or 12-channel multichannel pipette.
14. Pipettes and aerosol-resistant pipette tips.
15. Aluminium foil.
16. 52 °C Water bath.
17. 37 °C Water bath.
18. White-light transilluminator.

3 Methods

Propagation of Vero cells and seeding of those cells into flasks or plates that are to be used in the following procedures should be performed under BSL2 conditions. Flasks/plates of cells should be transferred to BSL3 for work with live WNV. All work involving cell cultures and/or virus materials should be performed in a biosafety cabinet using standard aseptic techniques and universal precautions to ensure integrity of the cultures and safety of the person performing the work.

3.1 Virus Culture in Vero Cells

1. Prepare an appropriately sized flask of Vero cells. Seed cells at sufficient density so that they will be 80–90% confluent the next day. Plating at 1.2×10^5 cells/ml is usually appropriate for use the following day, while 6×10^4 cells/ml is suitable for a 2-day incubation. Cells are usually plated in growth medium, as described in Subheading 2.1.
2. Prepare virus inoculum. If virus is from a previously grown stock/culture supernatant, the sample may be used neat or, if the titer is known, diluted in maintenance medium to give an MOI of 0.01–0.001. If virus is from an organ/mosquito homogenate, 1:10 or 1:100 starting dilutions are better, as concentrated tissue material can affect plaque formation. The inoculum volume should be sufficient to cover the monolayer, but no more than about 10% of that of the usual culture medium volume for the flask being used. For the majority of WNV culture work, a T-75 flask is appropriate, providing sufficient supernatant for 10–30 virus aliquots (0.2–1.0 ml volumes), depending upon intended use. For inoculation of this sized flask, a 0.5–2 ml volume is usually appropriate.

3. Remove culture medium from cells.
4. Using a sterile serological pipette, add 5–10 ml of sterile PBS to the flask, and rock gently to wash the cells. Remove the PBS by pipetting or pouring off.
5. Add the previously prepared inoculum.
6. Replace the cap on the flask and incubate for 30–35 min, gently rocking the flask every 10–15 min. Incubation for this infection step can be performed at 37 °C or at room temperature.
7. After incubation, remove flask from incubator.
8. (Optional; *see* **Note 1**) Remove inoculum and wash cells again with 1× PBS. Remove PBS.
9. Add an appropriate volume of maintenance medium. For a T-75 flask, 13–18 ml of medium in addition to the 2 ml of inoculum is appropriate. If inoculum removed, add normal culture volume (15–20 ml).
10. Incubate flask at 37 °C with 5% CO_2 and observe daily for onset of cytopathic effect (CPE).
11. Peak WNV titers generally occur when CPE is first developing (typically around 2–3 days post-infection), so harvesting when cells start to detach is preferred (*see* **Note 2** and Fig. 1). Delayed harvesting is possible, but titers usually decline approximately 0.5-log per 24 h following the onset of CPE and stocks harvested at later time points are likely to have a higher proportion of inactivated or noninfectious WNV antigen present and increasing quantities of cellular debris.

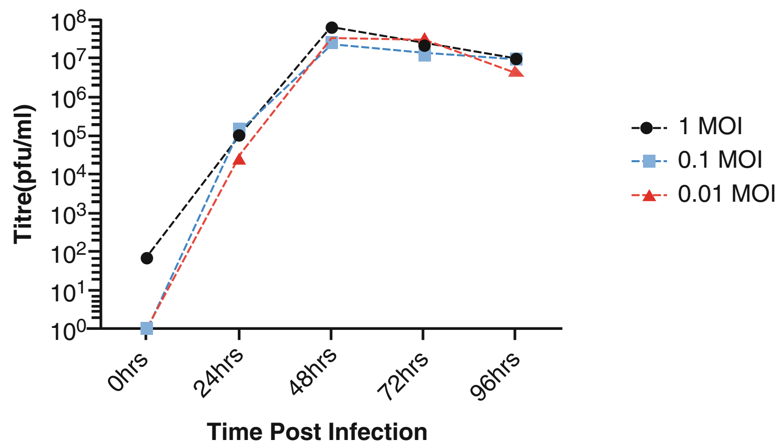

Fig. 1 Comparison of WNV NY99 growth kinetics in Vero cells following infection with varying MOIs

12. Harvest virus by removing supernatant to an appropriately sized sterile container, e.g., 15 ml conical tube.
13. Centrifuge supernatant for 5 min at $>3000 \times g$ to pellet cells and cell debris.
14. Aliquot cleared supernatant into appropriately labeled 1.5 ml cryovials (*see* **Note 3**).
15. Store aliquots at −80 °C until needed.

3.2 Plaque Assays

1. Seed wells of 6-well plates with Vero cells at 1.2×10^5 cells/ml to get 80–90% confluence the next day (*see* **Note 4**). Cells may be plated at BSL-2 (12-well plates may also be used, *see* **Note 5**).
2. Prepare 2% agar for overlays by melting pre-sterilized agar in the microwave with the lid loosened to relieve pressure within the bottle. Place molten agar-containing bottle in a 52–56 °C water bath until needed. Warm overlay medium in a 37 °C water bath until needed.
3. Depending upon the number of samples to be titrated, serial tenfold virus dilutions may be prepared in a column of a 96-well plate or in individual sterile plastic 1.5 ml Eppendorf-type tubes. Add 225 μl of maintenance medium to each well to be used. In the top well, add 25 μl of virus stock to the 225 μl of medium to make a 10^{-1} dilution. Mix by pipetting 8–10 times.
4. With a new pipette tip, transfer 25 μl of the 10^{-1} dilution to the 10^{-2} dilution well. Mix by pipetting 8–10 times.
5. Continue serial transfer until an appropriate maximum dilution (*see* **Note 6**), ensuring that a new tip is used each time. If multiple samples are being run, a multichannel pipette is useful, but care must be taken to ensure that correct volumes are transferred in all tips.
6. Remove cell culture plates from incubator, and remove medium from wells either by pipetting or inverting the plate over a waste container.
7. Wash cells with 1× PBS with a volume equivalent to 50–100% of the original culture medium volume.
8. Remove PBS by pipetting or inverting the plate over a waste container.
9. For a 6-well plate, transfer 200 μl of sample from the appropriate dilution well of the 96-well plate. The same pipette tip may be used if transfer is carried out from the highest dilution to the lowest, e.g., 10^{-7}–10^{-2}.
10. Rock the plate thoroughly to ensure even spread of the inoculum.
11. Place the lid on the plate, and incubate for 30–35 min, rocking flask every 10–15 min. Incubation may be performed at room temperature.

12. After approximately 25 min of incubation, prepare a 1:1 mixture of the previously melted 2% agar with overlay medium in a suitable disposable container (e.g., conical tube or tissue culture flask). Prepare sufficient volume to ensure 24 ml of overlay for each plate being prepared. Transfer the container with overlay media to the BSL3 and, at the end of the 30–35-min incubation, add 4 ml/well of the overlay to each plate.
13. Allow the overlay to set, and return the plate(s) to the 37 °C/5% CO_2 incubator (*see* **Note 7**).
14. Depending upon intended use, plaque plates may be stained with neutral red overlay, allowing observation of plaques in viable cells and potential recovery of live virus via plaque picking, or fixed and stained with crystal violet in buffered formalin. If staining with neutral red, after approximately 48-h incubation, prepare neutral red overlay: melt pre-sterilized 2% agar, combine 1:1 with overlay medium, and add neutral red at a 1:50 dilution. Ensure that the solution is protected from light, as neutral red is light sensitive. If crystal violet stain alone is to be used, incubate plates for a further 24 h, and then proceed to **step 18** (*see* **Note 8**).
15. Add neutral red overlay to the plate. For a 6-well plate, add 2 ml per well.
16. Wrap plate(s) in foil, and return to the incubator.
17. After a further 24 h, remove plate from the incubator and, using a while-light transilluminator, observe and count plaques in the monolayers of the wells. It is often easiest to flip the plate over to see them better. If plaques are not clear, plates may be returned to the incubator and read on day 4, or fixed and stained using crystal violet. Depending on the strain of WNV, plaque size may vary (Fig. 2). WNV NY99 usually has plaques approximately 3–5 mm in diameter after 3-day incubation. Some other WNV strains/variants display small (pinpoint) plaque phenotypes. If plaques are clear and quantifiable, proceed to **step 23**.
18. If the plates are to be stained using crystal violet, fill the agar-containing wells with 10% buffered formalin solution, and incubate at room temperature for 1 h to inactivate the virus and fix the cells.
19. Discard the formalin solution from the wells into an appropriate chemical waste container, and carefully remove the agar plugs using a spatula. It is very important to avoid damaging the cell monolayer.
20. Add 2 ml 10% formalin solution to each well, and incubate at room temperature for a further 10–15 min.
21. Discard the formalin, and wash the cells twice with 1× PBS. Remove the PBS, and add 200 μl of 0.25% crystal violet solution to each well. Incubate for 5 min at room temperature.

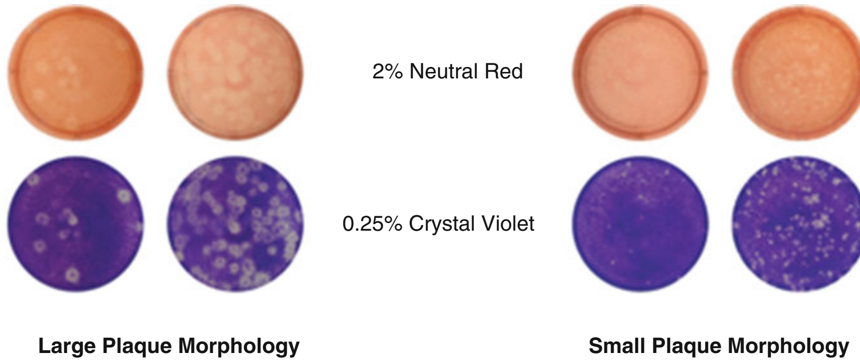

Fig. 2 Comparison of small and large plaque morphology with 2 % neutral red and 0.25 % crystal violet stains

22. Remove the crystal violet, and wash the wells twice with 1× PBS. Allow the plate to dry overnight, and count plaques in the monolayer using a white-light transilluminator.
23. Record the number of plaques and the dilution for each well. As an internal control for the dilution series, the plaque counts for the final two wells in the series with visible plaques (others will usually be too numerous to count) should be approximately tenfold different (Fig. 3).
24. Using the wells with countable plaques, calculate the titer per ml of the stock. This is done by multiplying the number of plaques by 5 if a 6-well plate was used (as 200 μl of sample was added to the well), and then by the inverse of the dilution factor. For example, if there are five plaques in the 10^{-7} well, the titer per ml would be $5 \times 5 \times 10^7$, or 2.5×10^8 pfu/ml. Titers should be calculated based upon wells with a plaque count between 5 and 50 for best accuracy.

4 Notes

1. Inocula may be removed and cells washed with PBS after incubation, prior to addition of maintenance medium or MEM/agar overlay. This step is optional, as the majority of infectious virus particles should have attached to the cells during the infection step. Washing the cells at this stage should have little bearing on the outcome of the assay.
2. The optimal virus harvesting time may vary slightly depending upon the virus strain and the input concentration. With MOIs ranging from 0.01 to 1, growth kinetics and peak virus titer with WNV NY99 vary little (Fig. 1). In Vero cells infected with WNV, CPE presents with the cells appearing slightly vacuolar with membrane projections. Cells readily detach, so that after

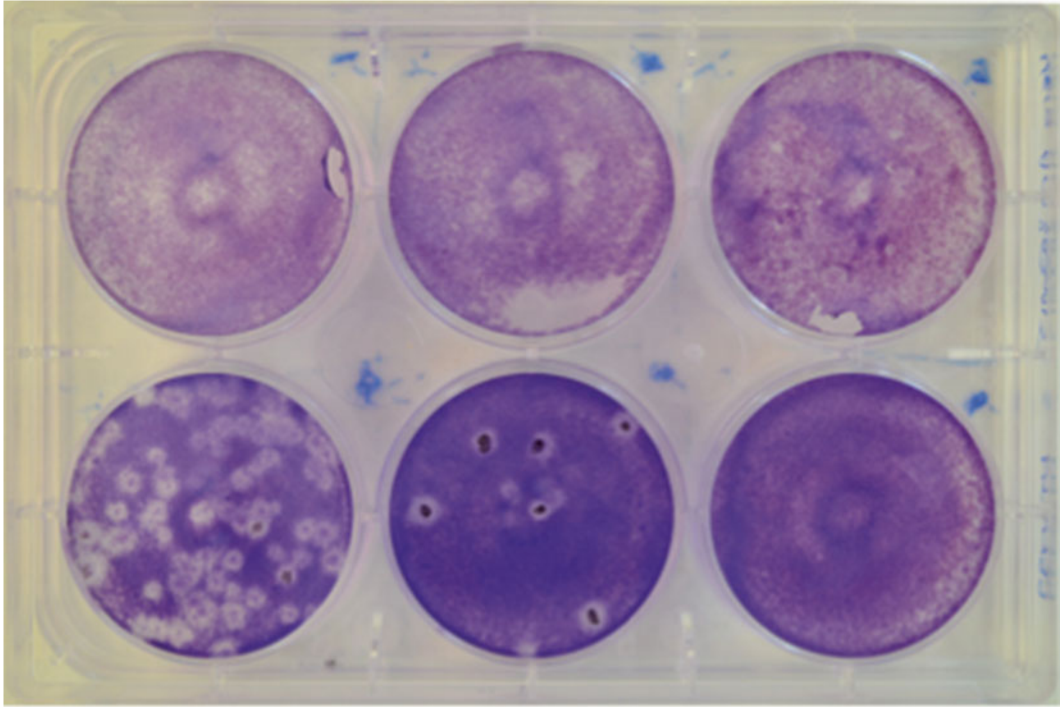

Fig. 3 Crystal violet-stained plate showing tenfold dilution series

2–3 days, the medium will begin to look cloudy. Characterizing the growth kinetics of the particular virus strain in Vero cells may be appropriate, allowing for harvesting at a time equivalent to the peak virus titer.

3. For long-term storage of virus stocks, some laboratories add 20–30 % FBS as a cryopreservative. In our experience this is not necessary for WNV, as virus in supernatant has been shown to be stable at −80 °C for 4 years without appreciable changes in titer. Indeed, in our experience, even a single freeze-thaw of WNV stocks has only a minimal effect on titer, with any reduction less than the inherent variability seen between titration runs.
4. In our experience, the ATCC CCL-81 Vero cells can be passaged approximately 30 times and still remain useful for plaque assays. Cells at higher passages tend to behave poorly, with indistinct and less consistent plaquing. Therefore preparation of cryo-preserved master and/or working cell banks is recommended.
5. Plaque assays run in 6-well plates are usually easiest to read, and allow for greater confidence in the data. Titration may be carried out in 12-well plates, but plaques are often less distinct, with a tendency to overlap. If 12-well plates are to be used, halve the volumes stated for 6-well plates. When it comes to calculating titers, remember that 100 μl of diluted sample was

added to the well, so the count must be multiplied by ten, rather than five, to give the titer per ml.

6. The range of dilutions appropriate for titrations varies depending on the sample. Peak virus titers for WNV NY99 grown in Vero cells rarely exceed 10^9 pfu/ml. Accordingly, a dilution range between 10^{-2} and 10^{-7} should be sufficient for the titration of most samples. If the particular sample to be titrated is expected to have a very low titer, a dilution range of 10^0–10^{-5}, or 10^{-1}–10^{-6}, may be more appropriate.
7. Some WNV isolates and mutants may have temperature-sensitive phenotype, resulting in lower titers or smaller plaque sizes when grown at higher temperatures. These may be identified by comparing plaque counts and morphology following incubation at 37 and 41 °C. Temperature-sensitive phenotypes have been associated with mutations in structural proteins affecting virion stability and in nonstructural proteins involved in viral replication. Such assays have been described previously [4, 5].
8. While neutral red staining is usually sufficient for WNV, plaques may occasionally be indistinct. Some laboratories choose to formalin-fix the plates and stain with crystal violet solution. For viruses with small plaque morphologies this may aid visualization of plaques. If it is desired, plates may be fixed and stained with crystal violet after neutral red staining. This allows for the decision about crystal violet staining to be made after the plates have been inspected with neutral red staining.

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Chapter 4

Generating West Nile Virus from an Infectious Clone

Rianna Vandergaast and Brenda L. Fredericksen

Abstract

WNV infectious clones are valuable tools for elucidating WNV biology. Nevertheless, relatively few infectious WNV clones have been generated because their construction is hampered by the instability of flaviviral genomes. More recently, advances in cloning techniques as well as the development of several two-plasmid WNV infectious clone systems have facilitated the generation of WNV infectious clones. Here we described a protocol for recovering WNV from a two-plasmid system. In this approach, large quantities of these constructs are digested with restriction enzymes to produce complementary restriction sites at the 3′ end of the upstream fragment and the 5′ end of the downstream fragment. These fragments are then annealed to produce linear template for in vitro transcription to synthesize infectious RNA. The resulting RNA is transfected into cells and after several days WNV is recovered in the culture supernatant. This method can be used to generate virus from infectious clones encoding high- and low-pathogenicity strains of WNV, as well as chimeric virues.

Key words West Nile virus, Infectious clone, Infectious RNA, Electroporation, In vitro transcription, Two-plasmid system

1 Introduction

Infectious clones are useful tools for studying multiple aspects of virology. Not only does this approach allow specific mutations to be introduced into individual viral products in order to probe their function, but also chimeras containing regions from multiple strains can be used to identify virulence determinants. Traditionally, making infectious clones for flaviviruses has been difficult. This difficulty is primarily due to the fact that flaviviral genomes are highly unstable in bacteria, often resulting in an inability to recover clones containing the full genome using traditional cloning methods. Furthermore, generated plasmid constructs were difficult to maintain due to the fact that the viral sequences often

This chapter was prepared based on the work conducted while Brenda Fredericksen was employed at the University of Maryland. The opinions expressed in this chapter are the author's own and do not reflect the view of the National Institutes of Health, the Department of Health and Human Services, or the US Government.

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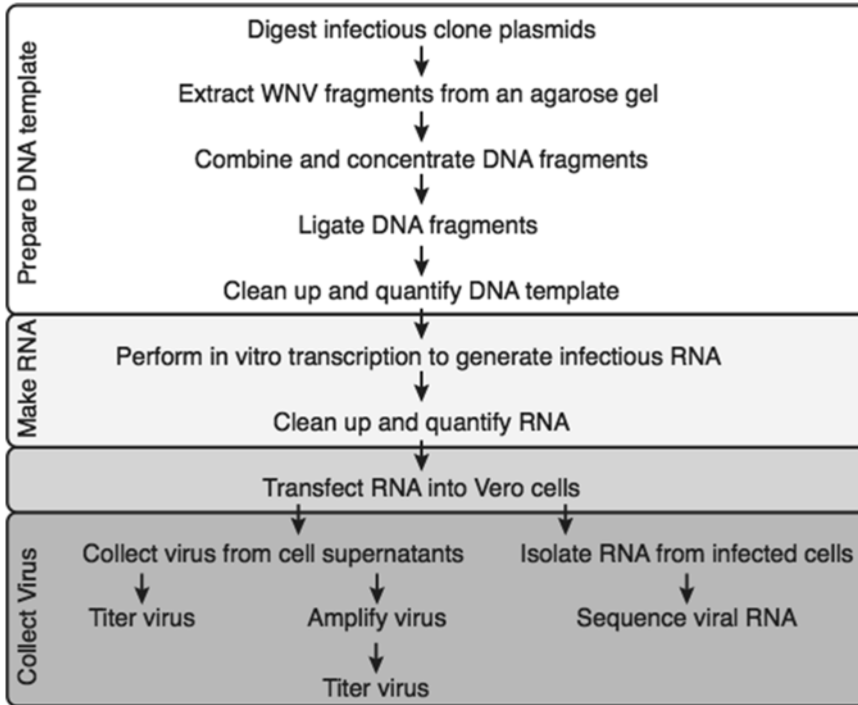

Fig. 1 Schematic of workflow. Overview of the steps required to generate recombinant virus from a two-plasmid infectious clone system

acquired mutations during amplification of the plasmid in bacteria. Several approaches (*see* **Notes 1–7**) have helped to overcome these difficulties [1–19], including the use of a two-plasmid system, which limits the required cloning steps, the use of Gibson Assembly cloning methods or chemical synthesis of portions of the genome, the use of low or very low copy plasmids, and the maintenance of infectious clone plasmids in bacterial strains with low mutation rates [20]. Additionally, modern electroporation technologies have increased the efficiency of recovering virus from infectious clones. Here we describe the use of a two-plasmid system to generate infectious WNV RNA and recover virus particles (*see* Fig. 1).

2 Materials

All reagents used for the preparation of the template RNA production or the manipulation of infectious RNA should be RNase free.

2.1 Nucleic Acid Manipulation/Generation

1. RNase decontamination reagent.
2. 6× DNA loading dye: 30% Glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF.

3. 1 kb DNA Ladder.
4. 1× TAE (Tris-acetate-EDTA): 40 mM Tris, 20 mM acetic acid, 1 mM EDTA.
5. 0.8% Agarose gel: 0.8% Agarose, 1× TAE.
6. Ethidium bromide staining solution: 10 μg/mL Ethidium bromide, 1× TAE.
7. Gel and PCR cleanup kit (*see* **Note 8**).
8. 3 M Sodium acetate pH 5.2 (adjust pH with glacial acetic acid).
9. 100% Ethanol.
10. 70% Ethanol.
11. Ultralow-volume spectrophotometer (*see* **Note 9**).
12. 10× Ligase buffer.
13. T4 DNA Ligase.
14. 20% Sodium dodecyl sulfate (SDS).
15. Proteinase K: 200 μg/mL Proteinase K.
16. TE-saturated phenol:chloroform: 50% TE-saturated phenol, 50% chloroform.
17. Chloroform.
18. T7 in vitro transcription kit or reagents (*see* **Note 10**) including:

    RNase-free water.

    10 mM ATP mix.

    10 mM CTP mix.

    10 mM GTP mix.

    10 mM UTPmix.

    100 mM DTT.

    RNase inhibitor.

    T7 RNA polymerase.
19. 5 M Ammonium acetate.
20. 2× RNA Loading Dye.
21. ssRNA Ladder.
22. 100% Isopropanol.
23. 75% Ethanol.

2.2 Tissue Culture Manipulations

1. Vero cells [21].
2. Dulbecco's phosphate-buffered saline (DPBS): 2.7 mM KCl, 1.5 mM KH_2PO_4, 136.9 mM NaCl, 8.9 mM $Na_2HPO_4 \cdot 7H_2O$.
3. Trypsin.
4. Unsupplemented Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate.

5. Fetal bovine serum (FBS).
6. 100× Antibiotic/antimycotic: 10,000 IU Penicillin, 10,000 μg/mL streptomycin, and 25 μg/mL Amphotericin B1.
7. 100× Nonessential amino acids.
8. Complete DMEM (cDMEM): Unsupplemented DMEM, 10% FBS, 1× nonessential amino acids, 1× antibiotic/antimycotic solution.
9. Transfection system (*see* **Note 11**).
10. RNA extraction kit/reagent (*see* **Note 12**).

3 Methods

To protect the integrity of the infectious WNV RNA, all steps should be carried out using RNase-free reagents and common practices to prevent RNase contamination. These practices include wearing gloves, wiping surfaces and pipets with an RNase decontamination reagent, such as RNase AWAY™ (Life Technologies), and using RNase-free plasticware and barrier pipet tips. RNA manipulation steps should be performed on ice, unless otherwise noted. RNA should be divided into single-use aliquots and stored at −80 °C.

In the following protocol, the linear DNA template used for RNA transcription consists of ligated fragments from two separated plasmids, NY-pA and NY-pB (*see* Fig. 2) [22, 23]. This two-plasmid system separates the 5′ and 3′ regions of WNV onto two distinct plasmids, thereby simplifying the cloning process. Generation or acquisition of the appropriate plasmids is required to carry out the procedures described below. *See* **Notes 1**–**7** for general guidelines relating to plasmid design and preparation.

3.1 Preparation of the DNA Template Using a Two-Plasmid System

1. Digest each infectious clone plasmid with the appropriate restriction enzymes (*see* **Notes 13**–**16**).
2. Add 6× DNA Loading Dye to completed digests, to a final concentration of 1×.
3. Load the digested samples on a 0.8% agarose gel in 1× TAE. Use gels with two small wells and one large well. In the small lanes load 1 kb DNA ladder and a small volume of the digested plasmid. Use the minimal amount necessary to be visualized by UV (*see* **Note 17**). Load the remaining digested plasmid into the large well.
4. Run the gel for 1–1.5 h at 100 V.
5. Carefully separate the lanes containing the 1 kb ladder and the small-volume sample from the remainder of the gel.

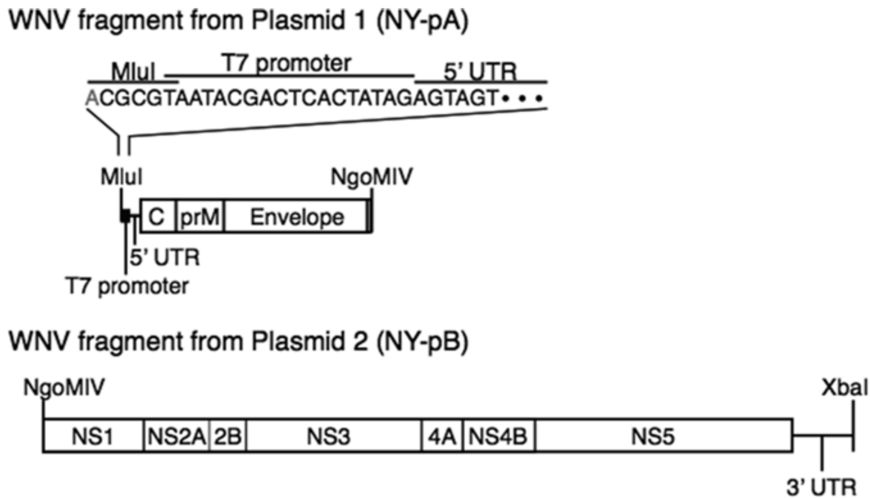

Fig. 2 Organization of WNV infectious clone fragments. Scale representations of the two WNV fragments generated from a two-plasmid infectious clone system using the plasmids NY-pA and NY-pB [22, 23]. The location of the ten WNV genes and the 5′ and 3′ untranslated regions (UTRs) are shown, as are the restriction sites used for generating the linear fragments. The placement of the T7 promoter sequence relative to the start of the WNV 5′UTR is shown at the *top*. Ligation of the complementary NgoMIV sticky ends on the two fragments produces the full-length DNA template

6. Stain the portion of the gels containing the ladder and the small sample by submersing them in ethidium bromide staining solution for 5 min. Care should be taken to follow institutional safety and disposal protocols for ethidium bromide.
7. Using a UV light, visualize the DNA bands and mark the locations of the appropriate DNA fragments on the gel using a razor blade.
8. Align the marked gel sections with the remainder of the gels. Using a clean razor blade, excise the corresponding sections of the unstained gels.
9. Place the gel slices in pre-weighed 1.5 mL microfuge tubes and determine their weights (*see* **Note 18**).
10. Extract the DNA using a gel cleanup kit (*see* **Notes 8** and **18**).
11. Determine the concentrations of the DNA fragments using an ultralow-volume spectrophotometer (*see* **Note 9**). At this point, the digested DNA fragments can be stored at −20 °C (*see* **Note 19**).
12. Verify the purity of the digested DNA by running 2 μL on a 0.8 % agarose gel, as described above. Only a single band of the appropriate length should be visible for each plasmid.
13. Combine the digested plasmid 1 (2–6 μg) and plasmid 2 fragments at a 1:1 M ratio in a 1.5 mL microfuge tube.

14. Ethanol precipitate the DNA by adding one-tenth volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of 100% ethanol and incubate at −20 °C for 2 h. Centrifuge at 16,000 × *g* for 20 min at 4 °C and carefully remove liquid. Wash the pellet with 1 mL of 70% ethanol. Centrifuge for 5 min at 16,000 × *g* at room temperature and carefully remove all traces of liquid. Air-dry the pellet for 5–10 min and resuspend in 17 μL of water.
15. Add 2 μL of 10× DNA ligase buffer and 1 μL (400 U) of DNA Ligase and incubate at room temperature for 12–18 h.
16. Add 73.5 μL of water, 2.5 μL 20% SDS, and 4 μL Proteinase K, and incubate at 37 °C for 1 h.
17. Perform a phenol:chloroform extraction using freshly prepared TE-saturated phenol:chloroform. Add 150 μL water and 250 μL phenol:chloroform. Vortex for 15 s and spin at 16,000 × *g* for 2 min. Carefully remove the top (aqueous) layer and transfer to a fresh Eppendorf tube. Repeat extraction with chloroform, noting the amount of aqueous phase recovered.
18. Ethanol precipitate the DNA as described in **step 14**, except resuspend the final pellet in 6 μL of water (*see* **Note 20**). This is the linear DNA template for in vitro transcription. Remove 1.5 μL for the analyses described in **steps 19** and **20** and store the remaining template at −20 or −80 °C until needed (*see* **Note 21**).
19. Determine the concentration of the DNA template by spectrum analysis of 1 μL on an ultralow-volume spectrophotometer.
20. Perform agarose gel analysis on 0.5 μL of the DNA template to verify efficient ligation of the two WNV fragments (*see* **Notes 22–24**).

3.2 Synthesis of Infectious RNA

While it is not necessary to use a commercially available kit to perform the in vitro transcription step of this procedure, these kits contain all the required reagents and are optimized for maximum yield (*see* **Note 10**). Listed below is a standard in vitro transcription reaction. The volumes may vary depending on the reagents provided by the manufacturer.

1. Assemble the transcription reaction in a total volume of 20 μL. The components should be added in the following order:
   - X μL RNase-free water.
   - 1 μg DNA template (*see* **Note 25**).
   - 2 μL Transcription buffer (10×) (provided with the T7 polymerase).
   - 1 μL ATP mix (10 mM).

- 1 μL CTP mix (10 mM).
- 1 μL GTP mix (10 mM).
- 1 μL UTP mix (10 mM).
- 2 μL DTT (100 mM).
- 0.5 μL RNase inhibitor.
- 2 μL T7 RNA polymerase.

2. Incubate at 42 °C for 2 h (*see* **Note 26**).
3. Add 1 μL of DNase I (1 U/μL) and incubate at 37 °C for 15 min.
4. Perform a phenol:chloroform extraction using freshly prepared TE-saturated phenol:chloroform. Add 179 μL water and 200 μL phenol:chloroform. Vortex for 15 s and spin at 16,000 × *g* for 2 min. Carefully remove the top (aqueous) layer and transfer to a fresh microfuge tube. Repeat extraction using chloroform, noting the amount of aqueous phase recovered.
5. Add an equal volume of 5 M ammonium acetate and incubate on ice for 15 min.
6. Centrifuge at 12,000 × *g* at 4 °C for 15 min and carefully remove liquid.
7. Wash pellet with fresh 70% ethanol.
8. Centrifuge at 12,000 × *g* at room temperature for 2 min and carefully remove liquid.
9. Air-dry pellet for approximately 5 min (do not overdry) and resuspend in 20 μL water. This is the infectious RNA. Remove 2 μL for the analyses described in **steps 10** and **11** and separate the remaining RNA into single-use aliquots (*see* **Note 27**). Store the RNA at −80 °C.
10. Determine the RNA concentration by analyzing 1 μL of RNA on an ultralow-volume spectrophotometer (*see* **Note 28**).
11. Dilute 1 μL of RNA with 2 μL of water and 3 μL of 2× RNA Loading Dye. Load sample along with an ssRNA ladder into wells of a 0.8% agarose gel in 1× TAE buffer. Run the gel for approximately 2 h at 100 V, and then stain the gel by submersing it in ethidium bromide staining solution for 5 min. Visualize the RNA bands under UV light (*see* **Note 29**).

3.3 Transfection of Infectious RNA into Vero Cells

WNV is a Biosafety Level 3 pathogen. Therefore, transfection of infectious WNV RNA and subsequent steps should be carried out in BSL3 facilities using appropriate institutional safety procedures.

There are several methods for transfecting RNA into cells. Electroporation has been the method of choice because it is typically more efficient at transfecting large RNAs into cells. However, traditional electroporation systems utilize high-voltage pulses that

result in substantial cell death. More modern electroporation systems are capable of efficiently transfecting large RNAs with minimal cell death. The protocol for transfecting cells will vary depending on the transfection system that is used. *See* **Notes 30–37** for general considerations for transfecting cells. The following protocol is for transfecting Vero cells with the Neon® transfection system (Life Technologies); however other systems can be used to recover virus.

1. Prior to beginning the transfection protocol, ensure that Vero cells are at an appropriate density in T75 flasks (*see* **Notes 31**).
2. Aspirate media off of Vero cells and wash once with DPBS.
3. Add 3 mL trypsin to each T75 flask. Incubate at room temperature with occasional rocking until the cells detach.
4. Add 3 mL cDMEM. Mix cells thoroughly by pipeting gently up and down and transfer to a 15 mL conical tube. Remove a small sample of cells for counting (*see* **Note 32**).
5. Place 1.5×10^6 cells into fresh conical tubes (*see* **Note 33**). One tube is needed for each infectious RNA to be transfected (*see* **Notes 34** and **35**). Centrifuge tubes at $300 \times g$ at room temperature for 5 min.
6. Meanwhile, place 10 mL of cDMEM without antibiotic/antimycotic (*see* **Note 36**) into 100 mm tissue cultures plates (one plate for each RNA), and place in a 37 °C/5 % CO_2 incubator.
7. Carefully remove supernatant from cells. Wash cells gently with 1 mL DPBS and repeat centrifugation.
8. Place 18 μg infectious RNAs into 1.5 mL Eppendorf tubes (do not use smaller tubes) (*see* **Notes 34**, **35**, and **37**).
9. Carefully aspirate supernatant off of cells. Resuspend cells in Neon® Transfection System Buffer R and transfer to tubes containing RNA. The total volume of Buffer R and infectious RNAs should equal 150 μL.
10. Use the Neon® Pipet to pull up 100 μL of the cell/RNA mix (*see* **Note 38**) and electroporate cells using the following settings: 1150 V, 20 ms, and 2 pulses.
11. Eject cells onto the plates from **step 6**. Gently swirl plates and return to 37 °C/5 % CO_2 incubator.

3.4 Collection and Analysis of Infectious Clone Virus

1. Four days after transfection (*see* **Note 39**), pipet culture supernatant off of cells and transfer to 15 mL conical tubes. Do not discard the cells; they are used in **step 4**.
2. Centrifuge culture supernatant at $500 \times g$ at room temperature for 5 min.
3. Carefully transfer the virus-containing supernatant to a fresh conical tube. This is the pass 0 virus; it should be aliquoted and stored at −80 °C (*see* **Note 40**). The titer of the recovered virus

should be determined by plaque assay on the cell line to be used for experiments.

4. Wash cells once with 3 mL DPBS and extract RNA using an RNA extraction kit/reagent (*see* **Note 12**). The recovered RNA can be used as the template for RT-PCR, and the resulting DNA sequenced to confirm the identity of the recovered virus (*see* **Notes 41–44**).
5. Amplify the pass 0 virus to generate working stocks (*see* **Note 45**). Add 0.1–1 mL of pass 0 virus to a T75 flask of Vero cells approximately 70% confluent. Add unsupplemented DMEM to bring the total volume to 1 mL. Incubate cells at 37 °C/5% CO_2, rocking thoroughly to ensure that cells do not dry out. After 1 h, add 9 mL cDMEM to the cells and return to a 37 °C/5% CO_2 incubator. Collect viral supernatants 24–48 h after infection as described in **steps 1–3** (*see* **Notes 39** and **45**).

4 Notes

Plasmid considerations

1. This protocol describes the recovery of WNV from a two-plasmid infectious clone system. Both single- and two-plasmid systems exist for generating infectious WNV RNA. While it is easier to prepare the template for in vitro transcription using single, full-length RNA plasmids, they are typically more difficult to generate and unstable in *E. coli*. Because the two-plasmid system significantly reduces cloning time and complexity and is more stable in *E. coli*, it is often the preferred method for generating infectious clones.
2. WNV sequences are highly susceptible to mutation during cloning. Traditional cloning methods can be used to generate infectious clone plasmids; however, significant time can be saved by using newer cloning strategies, such as Gibson Assembly® (New England Biolabs).
3. Because WNV sequences are highly susceptible to mutation, the relevant coding sequences should be sequenced every time the plasmid is isolated from *E. coli*.
4. To help maintain the integrity of cloned WNV plasmids, the use of low-mutation-rate strains of *E. coli*, such as the MDS™ 42 strain (Scarab Genomics LLC), are recommended for use in cloning and maintaining the plasmids.
5. Proper placement of the T7 promoter at the 5′ end of the WNV genome is essential. The recommended T7 promoter sequence *taatacgactcactatag* should be immediately followed by the WNV 5′UTR. No additional nucleotides

should be inserted between the promoter and the WNV 5′UTR (*see* Fig. 2) [14].

6. The restriction site at the end of the WNV genome should be precisely placed so a 3′ UTR of appropriate length is generated during in vitro transcription. Alternatively, a ribozyme coding sequence can be incorporated such that it will generate the precise 3′ end in the resulting RNA.
7. Infectious clones can be used to generate both chimeric viruses as well as reporter viruses. However, attempts to add reporter genes into the WNV genome have been only moderately successful, as the recovered viruses exhibit either reduced replication or loss of the added reporter sequences [5, 11, 22].

Reagent considerations

8. The Wizard® SV Gel and PCR Clean-Up System (Promega) works well for this protocol. However, similar kits sold by other manufacturers, such as Qiagen and Life Technologies, should yield similar results.
9. Accurate quantification of DNA and RNA intermediates is important for success. The use of a Nanodrop Spectrophotometer (Thermo Scientific) or an equivalent device is recommended as it provides accurate quantification and spectral analysis while requiring only 1 μL of sample.
10. A commercially available kit is recommended for the generation of infectious viral RNA. Typically, all the components of the in vitro transcription reaction described in Subheading 3.2 (**step 1**) are provided as part of the kit. The AmpliCap-Max™ T7 High Yield Message Maker Kit from Cell Script is capable of producing high yields of large, capped RNAs. While capping the viral RNA is not an absolute requirement, it may increase the stability and translation efficiency of the transfected RNA.
11. The use of newer, high-efficiency electroporation transfection systems will improve virus recovery and is highly recommended.
12. TriZol® Reagent (Life Technologies) works well for isolating total RNA from cells (Subheading 3.4, **step 4**). However, other commercially available options should yield similar results.

Generating linear DNA template

13. Different infectious clone plasmid systems require different restriction enzymes to generate linear DNA templates. Sequential digest of plasmids with different enzymes may be required depending on the enzyme pairs being used and their buffer activities.
14. When digesting the plasmids it is important to consider the size of the WNV fragment relative to the unwanted vector

backbone fragment. If the sizes of the two pieces are similar, digestion with an additional restriction enzyme that cuts the vector backbone and not the WNV fragment is recommended in order to make excision of the WNV fragment from the agarose gel more precise.

15. Efficient generation of infectious RNA from the linear DNA template requires relatively large amounts of template. Since much of the template DNA is lost during the digestion and ligation processes, it is important to start the digests with sufficient quantities of plasmid DNA. We recommend using approximately 10 μg of plasmid for each 1000 nucleotides of WNV sequence present in the digested fragments.
16. Although large quantities of plasmid DNA are needed for digestion, the restriction digests proceed more efficiently when performed in smaller volumes. Using 60 μL or less per tube is recommended. If the template concentration requires using larger volumes, multiple tubes of identical digests are recommended.
17. In order to decrease the potential for DNA damage, care should be taken to minimize exposure of the DNA template to UV light. This protocol uses a duplicate lane to visualize the position of DNA fragments on a gel and serve as a marker for locating the appropriate DNA fragments on the unexposed portion of the gel. Newer methods for visualizing DNA that do not require the use of UV light may also be used.
18. To accommodate the large amount of DNA required for some plasmids, such as plasmid 2 depicted in Fig. 2, it is recommended that the dissolved gel slices be applied to two different gel cleanup columns and then pooled following elution.
19. The digested WNV fragments can be stably stored for long term at −20 °C.
20. Most *in vitro* transcription kits recommend using at least 1 μg of DNA template. To generate adequately concentrated template it is important that the ligated DNA be resuspended in a small volume following ethanol precipitation.
21. The long-term stability of ligated template stored at −20 or -80 °C has not been assessed.
22. Contamination with uncut plasmid can significantly reduce or even inhibit production of infectious RNA. Partially cut or uncut plasmids will not anneal efficiently to create a full-length linear template and will substantially decrease the yield of full-length genomic RNA.
23. It is important to accurately determine the concentration of the template and the relative purity of the DNA. High-quality DNA can be recovered using this protocol; A_{260}/A_{280} ratios for the DNA template should be at least 1.8.

24. Efficient ligation of the WNV fragments is imperative for successful generation of full-length WNV infectious RNA. In particular, the presence of unligated WNV fragments containing the T7 promoter (the plasmid 1 fragment) may lead to the preferential production of truncated WNV RNAs instead of full-length WNV RNA. Properly ligated template should appear as a single, approximately 11 kb band.

Generating infectious RNA

25. Efficient transcription requires the use of a full 1 μg of template. Using less template may result in tenfold or greater reductions in RNA yields. If less than 1 μg of template is available, repeat the steps in Subheading 3.1 to generate additional template. If the template is too dilute, it should be concentrated through ethanol precipitation prior to use.
26. A standard in vitro transcription reaction is carried out at 37 °C for 30 min. The conditions recommended here will increase the yield of full-length genomic RNA.
27. The integrity of the RNA decreases with each freeze-thaw cycle it undergoes. For best results, it is recommended that RNA undergoes no more than one freeze-thaw cycle prior to transfection into cells. However, successful transfections have been achieved with samples that have undergone multiple freeze-thaw cycles.
28. Relatively high concentrations of RNA (greater than 1 μg/μL) can be obtained using this method. Spectrum analysis should also confirm the purity of the RNA. A_{260}/A_{280} values of over 2 are expected. Lower A_{260}/A_{280} values indicate that the RNA quality is poor and may not be usable for recovering virus.
29. Agarose-gel analysis of the infectious RNA product can provide useful information. A single RNA product of 11 kb is usually produced when the template for in vitro transcription is derived from a single full-length infectious clone template. However, when using a two-plasmid system, a wide variety of RNA products are often generated, which are visible as a smear on the denaturing gel. Virus can often be recovered from RNA preparations containing large quantities of truncated RNA; therefore, it is generally recommended that any RNAs of sufficient quality and quantity, as determined by spectrum analysis, be transfected into cells to attempt virus recovery.

Transfection considerations

30. RNase-free reagents should be used for this procedure.
31. Vero cell are highly permissive for WNV and commonly used to isolate virus; however, other cell lines have been successfully used. Vero cells should be approximately 95% confluent for optimal transfection efficiency.

32. Addition of trypan blue when counting cells is recommended in order to exclude dead cells.
33. The number of cells per transfection will depend on the transfection system that is being used. See the manufacturer's protocol for recommendations.
34. Whenever possible, it is recommended that a positive control RNA be used to ensure efficient transfection. Any RNA that has previously been successfully transfected can be used. To prevent any possible contamination, it is recommended that control RNAs are always transfected last.
35. Different strains of WNVs replicate at different rates and therefore must be harvested on different days after transfection. If the optimal day of virus collection is in doubt, as is generally the case when generating chimeric viruses, transfections should be done in duplicate so that two separate plates of each clone are available for harvesting.
36. Transfections should be performed with serum-free medium to prevent degradation of the RNA.
37. 10–12 μg of in vitro-transcribed RNA should be used for each transfection, though additional RNA is needed to accommodate the requirement for larger working volumes (*see* **Note 38**).
38. Bubbles in the pipet tip will inhibit efficient electroporation. To avoid introducing bubbles into the tip it is important to prepare more than the required 100 μL of RNA/cell mix. As described in this protocol, 150 μL of RNA/cell mix is recommended. If infectious RNA is a limiting reagent, a volume of 125 μL of RNA/cell can be used, though extreme care must be taken to avoid bubbles when drawing up the sample into the pipet tip.

Clonal virus isolation

39. Because different WNV strains replicate at different rates, the optimal time for recovering virus depends on the viral strain that was used to generate the infectious clone. In general, the optimal day of harvest is dependent only on the virus strain and not on the amount of transfected material. For highly pathogenic strains, cytopathic effects (CPE) are often visible several days after transfection and indicate that it is time to collect virus. Transfection of infectious RNA of less pathogenic strains may not result in CPE despite the presence of virus. For best results, supernatants should be collected no later than 7 days post-transfection.
40. WNV stocks should be stored at −80 °C. Many strains can be thawed and refrozen three times without noticeable reductions in the initial titers. However, it is recommended that the number of freeze-thaw cycles be limited. Therefore, the recovered virus should be divided into aliquots immediately after collection

41. Viral RNA can also be isolated from culture supernatants; however, the low level of virus present in the pass 0 virus stock may make it difficult to detect using standard techniques.
42. If TriZol Reagent® (Life Technologies) is used for RNA isolation, the recovered lysate from **step 4** can be stored at −80 °C until RNA extraction can be completed.
43. The identity of the recovered virus should be confirmed by sequence analysis. This is especially important when generating multiple infectious clones at the same time. It is not always necessary to sequence the entire viral genome; rather, sequencing specific regions may be sufficient to confirm identity.
44. The isolated total RNA can be used as a template for reverse transcription. Reverse transcription using WNV-specific primers as well as random hexamers has been used successfully to generate cDNAs. The resulting cDNA is then used as a template for PCR to generate fragments for sequencing. Depending on the viral genome region to be sequenced, subsequent PCR fragments can be generated using a wide variety of primer pairs and conditions. In general, PCR products under 1 kb are generated more efficiently.
45. Recovered infectious clone viruses typically need to be passaged once to generate high-titer working stocks. However, if titers of the pass 0 virus are high, amplification may not be required. For most efficient amplification, a T75 flask of cells should be infected at an MOI of 0.1. The time after infection at which viral titers will be highest varies depending on the WNV strain. For pathogenic strains, highest titers are generally recovered 24–48 h after infection. In contrast, low-pathogenicity strains may not reach highest titers until 5–6 days after infection.

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Chapter 5

Functional Analysis of West Nile Virus Proteins in Human Cells

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Abstract

West Nile Virus (WNV) lineage 2 strains have been responsible for large outbreaks of neuroinvasive disease in the United States and Europe between 1999 and 2012. Different strains in this lineage have previously been shown to produce either severe or mild neuroinvasive disease in mice. Phylogenetic and amino acid comparisons between highly or less virulent lineage 2 strains have demonstrated that the nonstructural (NS) gene(s) were most variable. However, the roles of some of the NS proteins in virus life cycle are unknown. The aim of this chapter is to describe simple computational and experimental approaches that can be used to: (1) explore the possible roles of the NS proteins in virus life cycle and (2) test whether the subtle amino acid changes in WNV NS gene products contributed to the evolution of more virulent strains. The computational approaches include methods based on: (1) sequence similarity, (2) sequence motifs, and (3) protein membrane topology predictions. Highlighted experimental procedures include: (1) isolation of viral RNA from WNV-infected cells, (2) cDNA synthesis and PCR amplification of WNV genes, (3) cloning into GFP expression vector, (4) bacterial transformation, (5) plasmid isolation and purification, (6) transfection using activated dendrimers (Polyfect), and (7) immunofluorescence staining of transfected mammalian cells.

Key words WNV, Computation, Protein topology, Sequence similarity, Cloning, Transfection, Immunofluorescence

1 Introduction

The West Nile virus (WNV) genome consists of a single-stranded, positive-sense RNA of approximately 11-kb that encodes a single polyprotein precursor, which is processed by cellular and viral-encoded proteases into three structural proteins and seven non-structural (NS) proteins [1]. The roles of the NS proteins in the WNV life cycle are not clearly characterized, except for NS2B-3, which is the viral protease (Pro) [1, 2], and NS5, which serves as the methyltransferase and the soluble RNA-dependent RNA polymerase (RdRp) [1, 3]. Although the NS proteins have been shown to be components of the virus replication complex [2], defining

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the individual roles of the NS proteins in viral pathogenesis has been one of the major challenges in the flavivirus field.

Phylogenetic analysis between highly or less virulent WNV strains has demonstrated that certain amino acid changes in the NS gene(s) were associated with large outbreaks of neuroinvasive disease in the United States and Europe [4–6]. However, the exact mechanistic roles of these changes and their contributions to WNV pathogenesis are still unknown. Here, we describe computational and experimental approaches that can be utilized to investigate the functional role of WNV NS proteins in virulence and pathogenesis.

Computational methods are techniques that bioinformatics researchers use to assign biological or biochemical roles to proteins. Proteins with comparable sequences are usually homologous and may thus have similar functions. The proteins will be annotated using the sequences of similar proteins of other genomes in the databases. If a newly sequenced gene is found to be similar to a previously sequenced gene, then an evolutionary relationship can be inferred and the function of the new gene is likely to be the same, or at least similar, to the function of the known gene.

Once a protein sequence has been obtained, whether it is the sequence of a single cloned fragment or of an entire WNV genome, a computer-based method can be used to search for specific sequence features associated with the genes. In this analysis, the protein databases are scanned to determine if the test sequence is identical or similar to any genes that have already been sequenced. NCBI BLAST (an acronym for Basic Local Alignment Search Tool) has programs used to generate alignments between a protein sequence, referred to as a “query” and protein sequences within a database, referred to as “subject” sequences. This protocol will include the programs and databases that are currently available online.

Experimental approaches involve cloning the NS genes and expressing the resulting plasmids in human cells. With the methods outlined in this section, any WNV genes can be amplified, fused to GFP, and be easily expressed in human cells, such as HEK293T, or other highly transfectable cell lines. Fusing WNV genes to GFP also allows for the visualization of the individual protein when antibodies directly against the WNV protein are not readily available. This information should allow any laboratory skilled in computer analysis and molecular virology methods to establish gene fusion expression system for studies of differences in protein localization, intracellular membrane association, protein secretion, and cytokine and chemokine induction profiling. This system can also be useful for studying other viruses that require high-level biocontainment or are inherently difficult to grow.

2 Materials

2.1 Computational Methods

No special materials or equipment, other than a computer with high quality Internet connection, are required to analyze nucleotide and/or protein sequence databases.

2.2 Isolation and Purification of WNV RNA

1. Vero cells (ATCC, Manassas, VA, Catalog Number CCL-81).
2. 1× M199 media supplemented with heat-inactivated fetal bovine serum (FBS), sodium bicarbonate, L-glutamine, and penicillin–streptomycin (*see* **Note 1**).
3. 1× Dulbecco's phosphate-buffered saline (DPBS).
4. 6-Well plates.
5. RNA isolation kit (Qiagen, Valencia, CA, Catalog Number 52904).
6. Humidified 5% CO_2, 37 °C incubator.

2.3 cDNA Synthesis and PCR Amplification of WNV Genes

1. Nuclease-free sterile water.
2. 10× Buffer.
3. 25 mM $MgCl_2$ (*see* **Note 2**).
4. 10 mM dNTP mix.
5. 10 μM Forward primer in sterile water.
6. 10 μM Reverse primer in sterile water.
7. 5 U/μL *Taq* DNA polymerase.
8. Automated thermal cycler.
9. cDNA Synthesis Kit.
10. Isolated WNV RNA
11. 0.2 mL PCR tubes.
12. *Optional*: Gel extraction kit (Qiagen, Valencia, CA, Catalog Number 28704).

2.4 Cloning into GFP Expression Vector, Bacterial Transformation, and Plasmid Isolation

1. GFP Fusion TOPO® TA Expression Kit (Life Technologies Grand Island, NY, Catalog Number K4820-01) (*see* **Note 3**).
2. LB agar plates containing 50–100 μg/mL of ampicillin.
3. LB medium containing 50–100 μg/mL of ampicillin.
4. 42 °C water bath or electroporator.
5. 37 °C nonshaking incubator.
6. 37 °C shaking incubator.
7. Plasmid DNA Maxi Kit (Qiagen, Valencia, CA, Catalog Number 12162).
8. Chemically competent TOP10 *E. coli* (provided in kit) or electrocompetent *E. coli*.

9. Inoculation loops.
10. *Optional*: Plasmid Mini Prep Kit (Qiagen, Valencia, CA, Catalog Number 27104).

2.5 Transfection Using Activated Dendrimers (Polyfect)

1. Human Embryonic Kidney 293T cells (HEK293T) (ATCC, Manassas, VA, Catalog Number CRL-11268).
2. Autoclaved round glass cover slips, 12 mm in diameter.
3. Poly-L-Lysine (PLL; molecular weight 70,000–150,000) solution: Dissolve PLL in sterile water to attain final concentration up to 100 μg/mL.
4. Plasmid DNA (from Miniprep or Maxiprep).
5. Complete Dulbecco's Modified Eagle's Medium, serum free (DMEM).
6. Complete DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin–streptomycin (5000 Units/mL).
7. Polyfect Transfection Reagent (Qiagen, Valencia, CA, Catalog Number 301105).
8. 24-Well plates.
9. Sterile Eppendorf 1.6 mL microcentrifuge tubes.
10. Humidified 5% CO_2, 37 °C incubator.

2.6 Immunostaining of Transfected Proteins

1. Dulbecco's Phosphate-Buffered Saline (DPBS), 1×.
2. Phosphate-Buffered Saline (PBS), 1×.
3. Paraformaldehyde (PFA) fixative: 3.7% PFA solution in 1× PBS.
4. Triton X-100 solution: 0.4% Triton in 1× PBS.
5. 5% bovine serum albumin (BSA) solution: Dissolve 0.5 g BSA in 10 mL 1× PBS.
6. Sharp-pointed forceps.
7. Primary antibody against host or viral protein of interest.
8. Fluorochrome-conjugated secondary antibody specific to host species of primary antibody.
9. Mounting medium with DAPI.
10. *Optional*: Anti-GFP antibody.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

Basic BLAST

Choose a BLAST program to run.

nucleotide blast	Search a **nucleotide** database using a **nucleotide** query *Algorithms:* blastn, megablast, discontiguous megablast
protein blast	Search **protein** database using a **protein** query *Algorithms:* blastp, psi-blast, phi-blast, delta-blast
blastx	Search **protein** database using a **translated nucleotide** query
tblastn	Search **translated nucleotide** database using a **protein** query
tblastx	Search **translated nucleotide** database using a **translated nucleotide** query

Fig. 1 The protein and nucleotide BLAST programs available using the NCBI database

3.1 Sequence Similarity-Based Methods

3.1.1 Protein BLAST

1. Visit the National Center for Biotechnology Information (NCBI) Blast website (www.ncbi.nlm.nih.gov) homepage and examine the following options (Fig. 1).
2. Click on the protein blast program "blastp" to run.
3. Enter the query FASTA protein sequence of interest to the query box and click on BLAST button at the bottom (*see* **Note 4**).

3.1.2 Translated BLAST

If only the nucleotide sequence of the amplified PCR product or cloned fragment is obtained, "Translated BLAST" can be utilized. The translated blast search employs a genetic code to translate either the "query," database "subjects," or both, into protein sequences, which are then aligned as in "blastp." The translations are conducted in the three forward as well as the three reverse reading frames so that no possible translation is missed.

There are three varieties of translated BLAST search available: "tblastn," "blastx," and "tblastx." In the first variant, "tblastn," a protein sequence query is compared to the six-frame translations of the sequences in a nucleotide database. In the second variant, "blastx," a nucleotide sequence query is translated into six reading frames, and the resulting six-protein sequences are compared, in turn, to those in a protein sequence database (*see* **Note 5**). In the third variant, "tblastx," both the "query" and database "subject" nucleotide sequences are translated in six reading frames, after which 36 (6×6) protein "blastp" comparisons are made (*see* **Note 6**). For more tutorials, visit the Wheeler D, Bhagwat M (2007) Example-Driven Web-Based BLAST Tutorial. Humana Press Inc. http://www.ncbi.nlm.nih.gov/books/NBK1734/.

3.2 Sequence Motif-Based Methods

The development of protein domain databases such as Pfam *by Sanger Institute* (http://pfam.xfam.org/search), MOTIF *by GenomeNet, Japan* (http://www.genome.jp/tools/motif/), Motif

Scan *by MyHits, SIB, Switzerland* (http://myhits.isb-sib.ch/cgi-bin/motif_scan), and others allow one to find known domains within a query sequence, providing evidence for likely functions. Within protein domains, shorter signatures known as motifs are associated with particular functions, and motif databases such as PROSITE (database of protein domains, families, and functional sites) can be searched using a query sequence. Motifs can, for example, be used to predict subcellular localization of a protein. Short signal peptides direct certain proteins to a particular location, such as the mitochondria, and nucleus and various tools exist for the prediction of these signals in a protein sequence.

Searching motif databases

1. Visit this website http://molbiol-tools.ca/Motifs.htm.
2. Pick one site from the list of protein domain databases. As an example, click the link for the "Pfam" site to reach the Pfam search engine.
3. Paste the protein sequence of interest into the query box to have it searched for matching Pfam families and press submit (*see* **Note** 7).

3.3 Protein Membrane Topology Prediction-Based Method

The Protein Membrane Topology Prediction Method integrates features for secondary structures, transmembrane helices, globular regions, coiled-coil regions, structural switch regions, protein–protein and protein–DNA binding sites, subcellular localization, domain boundaries, cysteine bonds, metal binding sites, and disulphide bridges.

1. Visit this website http://www.bioinformatics.utep.edu/BIMER/tools/transmembrane.html.
2. Pick one site from the list under "Predictions of Transmembrane Domains in Protein Sequences."
3. As an example, click on the highlighted "SOSUI" on the search sites to visit the "SOSUI" search engine.
4. Enter a title or comment for the sequence on the first rectangular box. Enter the sequence of interest with one-letter symbol to the query box or by copy and paste (*see* **Note 8**).
5. To execute the query press the button that says "Exec" (*see* **Note 9**).

3.4 Protein Expression Analysis

3.4.1 Isolation and Purification of WNV RNA

1. Seed the Vero cells at 10^6 cells per well of a 6-well plate in 1× M199 medium containing 10% FBS and 1% penicillin–streptomycin at 37 °C in a humidified 5% CO_2 atmosphere for 24 h.
2. Discard the old culture medium from each well.

3. Infect the cells with 1 mL of the WNV strain of interest at a multiplicity of infection of 1 (MOI-1) and incubate the cells at 37 °C in a humidified 5% CO_2 atmosphere for 1 h.
4. Discard the medium. Rinse cells with 1× DPBS and remove remaining DPBS with a pipet. Add 3 mL fresh growth medium per well and incubate the cells in the incubator for 48 h.
5. Collect the supernatant and follow the commercial viral RNA isolation kit protocol (QIAamp Viral RNA Mini Kit) or any similar commercial kit to isolate and purify viral RNA from the infected cells.

3.4.2 Designing Primers for PCR-Based Cloning into GFP Expression Vectors

1. Prior to designing primers to amplify the gene, review the sequence of the GFP expression vector (Life Technologies Grand Island, NY, Catalog Number K4820-01).
2. Using the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore), find a full-length genome of the WNV strain of interest to design the forward and reverse primers for WNV genes.
3. The forward primer will simply be the first 18–30 nucleotides of the gene. Incorporate the Kozak sequence containing the ATG start codon at the beginning of the 5′ end of the forward primer (*see* **Note 10**).
4. To design the reverse primer, use the reverse complementary sequence of the last 18–30 nucleotides of the gene. Insert an extra guanine nucleotide at the beginning of the 5′ region of the reverse primer to successfully clone the resulting PCR product in frame into the GFP expression vector.
5. Although these forward and reverse primers can be designed manually, use online tools such as Primer-BLAST to ensure that the characteristics of the primer (primer length, annealing temperature, melting temperature, GC content, etc.) are all optimized (*see* **Note 11**).
6. Once the primer design is complete, submit and order the primer sequences online (*see* **Note 12**).

3.4.3 cDNA Synthesis and PCR Amplification of WNV Genes

1. Use any commercial cDNA Synthesis Kit to synthesize cDNA using the RNA extracted from WNV-infected cells (*see* **Note 13**).
2. Prepare a standard 25 μL reaction in a PCR tube on ice. *See* Table 1.
3. Gently mix the reaction and briefly spin down in microcentrifuge.
4. Run the PCR sample according to the cycling conditions in Table 2 (*see* **Note 14**).

Table 1
Volumes and concentrations of PCR reaction components

Components	Volume (μL)	Final concentration
Water	to 25	
10× Buffer	2.5	1×
25 mM $MgCl_2$	2.0	1.5–2 mM
10 mM dNTPs	0.5	200 μM
10 μM forward primer	1.0	0.1–1 μM
10 μM reverse primer	1.0	0.1–1 μM
Taq DNA Poymerase[a]	0.25	0.05 Units/μL
cDNA template	Variable	Less than 10 μg
Total volume	25	

[a]Use a commercial high-fidelity *Taq* DNA Polymerase that is capable of amplifying PCR products up to 5-kb in size and has the ability to add single 3′ adenine overhangs at the end of the PCR product

Table 2
PCR cycling conditions

Cycle step	Temperature (°C)	Duration	Cycle
Initial denaturation	95	1–5 min	1
Denaturation Annealing Extension	95 55 72	30 s to 1 min 30–45 s 1 min per 1 kb	25–35
Final extension[a] Hold	72 4	15–30 min ∞	1

[a]The long final extension time is critical to ensure that the PCR products are full length and completely adenylated for subsequent TA cloning

5. The size of the amplified PCR product can be verified using agarose gel electrophoresis (*see* **Note 15**).
6. If there appears to be multiple bands on the gel, scale up to a 50 μL PCR reaction and use a commercial gel extraction kit to excise the DNA corresponding to the expected size of the fragment.
7. Adenylate the gel extracted PCR product. Using the 10 μL PCR reaction in Table 3, perform an extra extension cycle at 72 °C with *Taq* Polymerase and the purified DNA fragment (*see* **Note 16**).

Table 3
Volumes and concentrations for 10 μL PCR reaction

Components	Volume (μL)	Final concentration
10× Buffer	1.0	1×
25 mM $MgCl_2$	1.0	1.5–2 mM
10 mM dNTPs	0.2	200 μM
Taq DNA Poymerase	0.2	0.05 Units/μL
DNA	7.6	Up to 10 μL
Total volume	10	

3.4.4 Cloning into GFP Expression Vector, Transformation into E. coli, and Plasmid Isolation

The following protocol takes advantage of TOPO® cloning technology to fuse PCR products to a GFP vector. After cloning and verification of the correct orientation, the resulting plasmid can be purified and used directly for transfection into mammalian cells [7–9].

1. Follow the overall protocol included in the GFP TOPO® TA Expression Kit (https://tools.thermofisher.com/content/sfs/manuals/gfptopo_man.pdf).
2. Perform "TOPO® cloning reaction and Transformation" on pg. 9–10 of the TOPO® manual (*see* **Note 17**).
3. After transformation, incubate the plate at 37 °C overnight (*see* **Note 18**).
4. Verify the correct orientation of the inserted gene by using the methods under "Analysis of Positive Clones" on pg. 12 of the TOPO® manual. Streak a patch plate to preserve colonies (*see* **Note 19**).
5. Once a positive clone has been identified, streak the positive transformant on LB agar plates with ampicillin and incubate at 37 °C overnight.
6. Prepare a starter culture by using an inoculating loop to transfer bacteria from the colony into 5 mL of LB containing ampicillin. Shake at approximately 300 rpm at 37 °C overnight for 12–16 h (*see* **Notes 20** and **21**).
7. Dilute the starter culture 1/1000 into a larger volume of 100–500 mL of LB broth containing ampicillin (*see* **Note 22**). Shake at approximately 300 rpm at 37 °C overnight for 12–16 h.
8. Harvest the bacteria cells by centrifugation at 6000 rpm for 20 min at 4 °C.

9. Follow the commercial Qiagen Maxi Kit protocol or any similar commercial Maxi Prep Kit to isolate and purify the plasmid from bacterial cells.
10. Send the plasmid DNA for sequencing and store purified plasmid in 1× TE Buffer at −20 °C or below.

3.4.5 Transient Expression Using Activated Dendrimers (Polyfect)

The following method of transfection has been optimized for a variety of adherent cell lines such as COS-7, Hela, HEK293, HEK293T, and CHO cells in 24-well plates. The quick incubation times, high cell viability, and low toxicity of the transfection reagent makes it ideal for transfecting GFP fusion proteins into mammalian cells [7].

1. Place 1 autoclaved glass cover slip/well in a sterile 24-well plate.
2. Add approximately 300 μL of PLL solution to cover the surface of the well (*see* **Note 23**).
3. Incubate cover slips with PLL solution for 1 h at 37 °C.
4. Remove PLL solution from each well and rinse three times with sterile water.
5. Use PLL-coated coverslips for seeding immediately or store plate at 4 °C (*see* **Note 24**).
6. Seed 7×10^5 cells per well in 24-well plates containing PLL-coated cover slips. Grow overnight in a CO_2 incubator at 37 °C to 80 % confluency.
7. On the day of transfection immediately prior to preparing transfection complexes, aspirate old media and replace each well with 320 μL of fresh, prewarmed DMEM containing serum and antibiotics (*see* **Note 25**).
8. In a sterile Eppendorf tube, dilute 2 μg of DNA with DMEM without any serum, proteins, or antibiotics to a total volume of 100 μL. Mix and spin down solution to remove any drops of DNA or media from the top of the tube (*see* **Notes 26** and **27**).
9. Add 20 μL of Polyfect Transfection Reagent to the diluted DNA. Immediately mix by pipetting up and down approximately five times to thoroughly mix the DNA and the transfection reagent.
10. Incubate samples for 10 min at room temperature (20–25 °C) to allow for complex formation.
11. Add 600 μL of DMEM containing serum and antibiotics to tube containing the transfection complexes. Mix by pipetting up and down at least twice to terminate complex formation.
12. Dropwise add 180 μL of the transfection complexes + DMEM to each well to transfect a total of four wells in the 24-well

plate. Gently swirl the dish to promote even distribution of the complexes in the well.

13. Incubate cells with the transfection complexes at 37 °C and 5% CO_2 for 24 h to allow for gene expression (*see* **Note 28**).

3.4.6 Detection of Proteins in Mammalian Cells Using Immunofluorescence

Using the following protocol for immunofluorescence involves minimizing primary and secondary antibody solutions to conserve valuable antibodies. The separate permeabilization and blocking steps combined with thorough washing steps minimizes background to attain clear fluorescent microscopy images [7].

1. At 24 h after transfection, remove culture medium and wash cells once with 1× DPBS. Remove DPBS with vacuum.
2. Fix cells with 3.7% PFA solution for 5–10 min.
3. Vacuum off fixative and wash twice with 1× PBS (5 min/wash).
4. Permeabilize with 0.4% Triton X-100 for up to 10 min (*see* **Note 29**).
5. Vacuum off detergent and wash three times with 1× PBS (5 min/wash).
6. Block cells with 5% BSA at room temperature for 45 min to 1 h.
7. Vacuum off BSA and wash once with 1× PBS (5 min/wash).
8. Prepare a 50 μL primary antibody solution as follows: Dilute primary antibody in 2% BSA and 1× PBS. Vortex well and spin down before use (*see* **Note 30**).
9. Cut a piece of parafilm and pipet the 50 μL drop of primary antibody onto the parafilm. Using sharp-pointed forceps, carefully pick up the coverslip from the well and place the cover slip, face down, on top of the droplet (*see* **Note 31**).
10. Incubate cells with the primary antibody for 1 h at room temperature.
11. Using the forceps, carefully move the coverslip from the parafilm into the 24-well plate. Ensure that the cells on the coverslip (face down on the parafilm) are now faced up in the well.
12. Wash three times with 1× PBS (5 min/wash) on a horizontal shaker.
13. Prepare a 50 μL secondary antibody solution as follows: Dilute secondary antibody in 2% BSA and 1× PBS. Vortex well and spin down before use.
14. Repeat above **steps 9** and **10** to incubate cells with secondary antibody (*see* **Note 32**). Incubate at room temperature in the dark for 45 min.
15. Repeat **step 11** to carefully move the coverslip from the parafilm to the 24-well plate.

16. Wash three times with 1× PBS (5 min/wash) on a horizontal shaker.
17. Mount coverslips (face down) onto glass microscope slides using a drop of mounting medium containing DAPI. Incubate coverslips on slides for 5 min at room temperature. Use a kimwipe to wipe off and absorb any excess mounting medium.
18. Seal the edge of the coverslips with small drops of clear nail polish.
19. Examine slides under a confocal microscope to visualize antibody-tagged intracellular proteins (*see* **Note 33**).

4 Notes

1. To make 1000 mL of 1× M199 media, combine the following and then filter: 100 mL 10× M199, 50 mL inactivated FBS, 29.2 mL 7.5 % sodium bicarbonate, 10 mL L-glutamine, 10 mL penicillin–streptomycin (5000 Units/mL), and dH_2O up to 1000 mL. Store at 4 °C.
2. Some commercial PCR buffers may already conveniently include $MgCl_2$. If this is the case, do not add more $MgCl_2$. Excess of $MgCl_2$ will decrease the fidelity of the polymerase, which may produce unwanted gene products or promote misincorporation of nucleotides.
3. Using these kits, GFP will be fused to the C-terminal end of the amplified NS gene product.
4. BLAST “query” sequences are given as character strings of single letter amino acid codes, preceded by a definition line, beginning with a “>” symbol and this format is known as FASTA. The algorithm reveals the database sequence that most closely matches the sequence of interest. How similar the query sequence is to other WNV isolates will also help determine possible evolutionary changes.
5. A common use of the “tblastn” and “blastx” programs is to help annotate coding regions on a nucleotide sequence; they are also useful in detecting frame-shifts in these coding regions.
6. The “tblastx” program provides a sensitive way to compare transcripts to genomic sequences without the knowledge of any protein translation; however, it is very computationally intensive.
7. Sequence lengths must be less than 10,000 residues. Only residue symbols are allowed in the sequence and sequences containing other characters will not be accepted. Sequence search results will show the detailed description of the results.

8. Sequence length should not be less than 20 amino acids or more than 5000 amino acids.
9. The SOSUI result will provide the following: total protein sequence length, average of hydrophobicity, and a table detailing the total number and length of helices, the positions of the transmembrane helices in the sequence, and the type (primary or secondary). In addition, a display option will show the hydropathy profile and helical wheel diagram of predicted segments.
10. The Kozak sequence (e.g., ACCACCATG or similar) is not included in the expression vector and needs to be added in the forward primer for proper translation.
11. Refer the article Ye et al. (2012), Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics.* doi:10.1186/1471-2105-13-134, for more information.
12. Custom DNA Oligos can be ordered online from Integrated DNA Technologies (IDT), Invitrogen (Thermo-Fisher Scientific), Sigma-Aldrich, or any similar vendor.
13. Using the reverse primer of the WNV gene of interest is advantageous since it creates gene-specific cDNA. However, random hexamer primers may also be used to prime the complete length of the WNV RNA sequence if the resulting cDNA will be used repeatedly to amplify different WNV genes.
14. Amplification parameters may vary depending on the thermal cycler, the buffers, and the melting temperatures of the primers. If the Forward and Reverse primers have different melting temperatures, use the following formula to quickly calculate the annealing temperature for the PCR as a starting point:

$$\text{Annealing Temperature} = \left[\left(T_{\text{m}} \text{ of Forward Primer} - 5\right) + \left(T_{\text{m}} \text{ of Reverse Primer} - 5\right)\right] / 2$$

Further adjustment of annealing temperatures may be needed to optimize the amplification of the PCR product.

15. The concentration of agarose should be adjusted depending on the expected size of the PCR fragment. Use higher concentrated agarose gels for smaller DNA fragments and lower concentrations (<2 %) for larger fragments >3 kb in size.
16. Adenosine overhangs must be added to the gel extracted PCR products since it may have been lost during the gel extraction process.
17. When setting up the TOPO® cloning reaction for transformation into chemically competent *E. coli*, use as much fresh PCR product as possible. In addition, as little as 0.5 μL of the GFP TOPO® vector can be added. Sterile water is not a necessary

component for the TOPO® reaction and reactions simply with PCR product, salt and vector can yield positive colonies. For larger PCR products >1 kb, incubate the TOPO® reaction at room temperature for 30 min.

18. Incubate the plates for less than 18 h to avoid the formation of satellite colonies.
19. Restriction analysis or sequencing of PCR products can be conducted to verify the sequence and orientation of the inserted gene. Alternatively, positive transformants can be analyzed with colony PCR using, for example, a T7 Forward primer (provided in kit) with a WNV Reverse primer. The PCR cycling conditions to verify the orientation of the insert is generally the same as those used when amplifying the PCR product. However, note that the extension time should be slightly longer to accommodate the extra base pairs between the insert and the priming site on the vector.
20. *Optional*: If only a small amount of plasmid DNA (<20 μg) is needed, the bacteria can be harvested after 16 h. Harvest the bacterial cells in the starter culture by centrifugation at 5000 rpm for 5 min at room temperature. Perform Mini Prep to obtain plasmid DNA, which may be used for subsequent transfection or for sequencing. Plasmid DNA can be quickly isolated using Mini Prep kits, but may not be as pure as those extracted using Maxi Kits.
21. When the insert is larger (>3 kb), shortening the incubation time of the starter culture at 37 °C to 6–8 h before diluting it in the larger culture volume may help prevent overgrowth of culture and inactivation of ampicillin.
22. Because the GFP Fusion TOPO® vector contains a pUC origin of replication which promotes high copy numbers in bacterial cells, any volume between 100 and 500 mL can be used, depending on how much plasmid needs to be purified for downstream applications. Also, use a flask with a volume at least 3–4 times the volume of culture to allow for proper aeration and bacterial growth.
23. Ensure that all of the coverslips do not float to the surface and are completely inundated by the PLL solution. PLL is added to the coverslips to promote attachment of the cells to the coverslips. If the cover slips begin floating to the surface of the PLL solution, use a sterile pipet tip to gently push the coverslips back to the bottom of the well.
24. PLL-coated cover slips in 24-well plates are best when used immediately, but can be stored at 4 °C for approximately 2 months.
25. Add DMEM slowly to the side of the well to avoid dislodging the cells from the cover slip.

26. It is crucial to use DMEM that does not contain any serum, proteins, or antibiotics when diluting the plasmid DNA. The presence of antibiotics and serum in the media at this step will interfere with transfection complex formation and reduce transfection efficiency.
27. Diluting 2 μg of DNA in DMEM for a total volume of 100 μL can be used to transfect four wells of a 24-well plate. If only two wells of a 24-well plate need to be transfected, the total volume of diluted DNA, as well as the volumes for the transfection reagent and DMEM containing serum, should be reduced in half.
28. Changing media containing the transfection complexes is not necessary after 4–6 h.
29. Permeabilizing more than 10 min may lead to over permeabilization of the cells, which can result in fragile cells and broken nuclei.
30. Primary antibody dilution will vary according to the manufacturer's recommendations. The 50 μL antibody solution can be stored at 4 °C for reuse. If expression of the GFP fusion protein is low, incubating with a primary anti-GFP antibody will accentuate and increase the fluorescence of the fusion protein.
31. The cover slip is oriented in this manner so that the cells on the surface of the coverslip will be in complete contact with the primary antibody solution.
32. Secondary antibody dilution will vary according to the manufacturer's recommendations.
33. Store immunostained coverslips on glass slides at 4 °C and protect it from light.

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Chapter 6

Lessons from the Murine Models of West Nile Virus Infection

Brenna McGruder, Vandana Saxena, and Tian Wang

Abstract

West Nile virus (WNV), a mosquito-borne, single positive-stranded RNA virus, has been the leading cause of arboviral encephalitis in the U.S. and other parts of the world over the past decade. Up to 50% of WNV convalescent patients were reported to have long-term neurological sequelae or chronic kidney diseases. However, there are neither antiviral drugs nor vaccines available for humans. The underlying mechanism of the long-term sequelae is not clearly understood either. Animal models have been an effective tool to investigate viral pathogenesis and host immunity in humans. Here, we will review several commonly used murine models of WNV infection.

Key words West Nile virus, Mice, Immunity, Pathogenesis, Infection

1 Introduction

West Nile virus (WNV), a mosquito-borne neurotropic pathogen, belongs to the family of *Flaviviridae*, the genus Flavivirus, a group of plus-sense, single-stranded RNA viruses [1, 2]. It has become a global public health concern. The virus was originally isolated in Africa and later caused epidemics with mainly febrile illness in humans in Europe, Africa, the Middle East, and parts of Asia. In 1999, a more virulent WNV strain was introduced in North America and has since caused significant mortality in humans, horses, and birds. Additionally, it has been the cause of recent outbreaks of viral encephalitis in Europe and Australia [3, 4]. WNV infection of the central nervous system (CNS) commonly presents as encephalitis, meningitis, or acute flaccid paralysis. The overall mortality rate in persons who develop WNV neuroinvasive disease is about 10%, although it increases significantly in the elderly and immunocompromised. Up to 50% of WNV convalescent patients were reported to have long-term neurological

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sequelae or chronic kidney diseases [5–8]. Currently, licensed vaccines are not yet ready for human use, and treatment is mostly nonspecific and supportive.

WNV has been studied in various animal models [9–12]. Among them, mice are generally susceptible to WNV infection, easiest to work with, economic, and are most amenable to immunological manipulation. Thus, several laboratory murine models of WNV infection have been utilized to investigate the host factors involved in protective immunity and viral pathogenesis in humans. Systemic WNV infection has been commonly performed in young adult mice (6–10 weeks old). Following the initial subcutaneous (s.c.) or intraperitoneal (i.p.) inoculation, WNV induces a systemic infection and eventually invades the CNS. The severity and symptoms of lethal infection observed in mice mimic severe cases observed in humans (*see* **Note 1**). The incidence of WNV neuroinvasive disease has been reported to increase with age, with the highest incidence among persons aged over 70 years [13–15]. Systemic WNV infection in old mice (18–22 months old) has been used to define aging as a risk factor in the murine model [16, 17] (*see* **Note 2**). There is increasing evidence that persistent WNV infection contributes to long-term morbidity. WNV antigen or RNA has been detected in the brain and/or urine of WNV patients ranging from a few months to several years after the initial acute illness [18, 19]. We have recently demonstrated that WNV H8912, an isolate previously cultured from the urine of a persistently infected hamster, induces a similar persistent infection phenotype in mice following an i.p. inoculation and preferentially persists in mouse kidneys [20]. Thus, WNV H8912 systemic infection in mice can serve as a tool to study persistent renal infection (*see* **Note 3**). Cutaneous WNV infection either by mosquito feeding or intradermal (i.d.) injection, which presumably mimics natural infection in humans, is an important model for examining host immunity to vector-borne pathogens [21, 22] (*see* **Note 4**). Finally, direct CNS infection by intracranial (i.c.) injection of mouse brain is used to study the neurovirulence of WNV (*see* **Note 5**). In summary, studies from the above well established murine models will provide critical insights into WNV pathogenesis and protective immunity in humans.

2 Materials

1. *Mice*: We purchased 6- to 10-week-old C57BL/6 (B6) mice and 18- to 22-month-old B6 mice from Jackson Laboratories (Bar Harbor, ME) and the National Institute of Aging (Bethesda, MD), respectively. All animal experiments were approved by the Institution Animal Care and Use Committee.

2. *WNV*: The parental strain WNV NY385-99 (WNV NY99, [12]), a kind gift from Dr. R Tesh (UTMB, Galveston, TX), was passaged once in Vero cells and twice in C6/36 cells to make a virus stock (3.5×10^7 PFU/ml) for all acute infection studies. For persistent infection, WNV H8912, which was recovered from hamster urine 274 days post infection after three consecutive passages of a urine isolate from a chronically infected hamster, was used [23].
3. *Phosphate-buffered saline with 5% gelatin (PBSG)*: Dissolve 1.2 g of gelatin type B from bovine skin into 800 ml of PBS. Adjust the pH to 7.4. Autoclave the solution and cool the liquid to 50 °C. Aliquot to 10 ml aliquots until further use.
4. *Syringe needles*: A 1-cc tuberculin syringe with 26-G needle was used for systemic infection studies (acute or persistent, *see* **Notes 1–3**). A 0.5-ml, ultra-fine insulin syringe with 29-G needle was used in cutaneous and direct CNS injections (*see* **Notes 4** and **5**).
5. *Anesthetizing agents*: Isoflurane inhalation and Ketamine/xylazine injection were used for anesthesia of mice before systemic and direct CNS or cutaneous inoculations, respectively.
6. *Facilities*: Biosafety level 3 (BSL3) and animal BL3 (ABLS3) facilities were used for preparation of viral inocula and injection/housing of mice, respectively.

3 Methods

1. *Preparation of viral inocula*: WNV inocula were prepared by serial dilution of viral stocks in phosphate-buffered saline with 5% gelatin (PBSG) at BL3 facilities and were kept on ice before injections. A final volume of 200 and 50 μl of viral inocula was delivered to each mouse for systemic and cutaneous or direct CNS injections, respectively.
2. *Injection procedure*:
   (a) All animal inoculations were performed in ABL3 facilities in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.
   (b) For systemic infection studies, isoflurane inhalation was used first for anesthesia of mice in a small chamber. Next, the tuberculin 1 cc syringe was uncapped, the amount of viral inoculum needed was drawn up into the syringe and needle, and 200 μl of virus inoculum was injected in the left quadrant of the mouse abdomen towards the head at a 30–40° angle (*see* **Notes 1–3**).

(c) For i.d or i.c. inoculation, we first injected 200 μl of Ketamine/xylazine i.p. (administered at 70 mg/7 mg/kg mouse weight). Next, we used a 0.5-ml insulin syringe with a 29 G needle to inject anesthetized mice with WNV isolate in 50 μl PBSG.

(d) For i.d. injection, insert the needle into the dorsal side of the ear, make sure not to insert the needle too far and either go through the ear or into the skull (*see* **Note 4**).

(e) For i.c. injection, carefully insert the needle where the midline of the skull intersects with the midline between the eyes and make sure to use enough force to insert the needle into the skull, but not too deep into the brain to cause damage (*see* **Note 5**).

3. *Post-infection monitor*:

(a) Infected mice were housed in ABL3 facilities and monitored twice daily for 1 month.

(b) When mice showed any sign of morbidity (obvious severe illness, unable to right self when tipped on side or back, obvious extreme weight loss or signs of paralysis), they were immediately collected and euthanized by using CO_2.

4. *Conclusions*: Murine models of WNV infection have provided important insights into host immunity and viral pathogenesis in humans. Results from animal studies will help to identify therapeutic goals to modulate immune functions pharmacologically, as well as antiviral countermeasures, and to develop vaccines that can induce robust memory T cell responses.

4 Notes

1. *Acute systemic infection in young adult mice*: Following the initial i.p. infection, wild-type WNV NY99 strain was found to induce a systemic infection and eventually invaded the CNS [10, 24]. The virus quickly spread to the spleen, kidneys, and other peripheral tissues. Further, WNV crossed the blood brain barrier (BBB) after a brief viremia and entered the CNS (*see* Table 1). An increased viral burden in serum usually correlated with earlier viral entry into the brain [25]. Moreover, systemic WNV replication was reported to induce a pro-inflammatory cytokine response that modulates BBB permeability, which in turn, may enable viral entry into the brain and induce lethal encephalitis [26, 27]. Thus, it is critical to control virus dissemination in the periphery during early stages of WNV infection. WNV-infected mice developed symptoms starting on day 6, and succumbed to infection when encephalitis developed, usually within 1–2 weeks [10, 24, 28]. Similar outcomes have

Table 1
WNV infection models in mice

WNV infection models	Routes of injection, virus dose	Phenotype	Significance
Systemic infection model	i.p. or s.c injection; wild-type or mutant WNV strains; 100 PFU and 1000 PFU are LD_{50} and LD_{100} respectively for wild-type WNV	Following wild-type WNV infection, virus spreads to spleen, kidneys, and other peripheral tissues. Virus enters the CNS following a period of viremia and encephalitis develops	Partially mimics symptoms observed in humans
Old mouse model	i.p. or s.c.; lower LD_{50} compared to young adult mice	Higher viremia and viral loads in the brain and are more susceptible to WNV infection	Study aging as a risk factor for WNV encephalitis
Persistent infection model	i.p. or s.c.; wild-type WNV or WNV H8912	WNV H8912 preferentially persists in the kidneys; the frequency of wild-type WNV persistence is tissue-dependent and was found in the skin, spinal cord, brain, lymphoid tissues, kidneys, and heart	Study persistent infection and possibly the long-term morbidity observed in WNV convalescent patients
Direct CNS infection model	i.c.; lower LD_{50} compared to systemic infection	Infection in CNS tissues	Study neurovirulence of WNV
Cutaneous infection model	i.d. or mosquito feeding 10^5 PFU as LD_{50}	Virus disseminates from the skin to peripheral organs by migration of LCs	Mimic WNV natural infection

been reported following s.c. infection in young adult mice [16, 25]. The LD_{50} and LD_{100} of wild-type WNV NY99 i.p. infections in young adult mice are 100 plaque forming unit (PFU) and 1000 PFU, respectively.

2. *Acute systemic infection in aged mice*: Following either i.p. or s.c. WNV infection, aged mice (18–22 months old) displayed persistently elevated viremia and higher viral loads in the brain (*see* Table 1). As a result, aged mice were much more susceptible to systemic WNV NY99 infection compared to young adult mice (6–10 weeks old). The decline in immunity seen in the elderly is a significant contributor to the increased risk of infection. Impaired innate and adaptive T cell functions in aged mice contribute to an enhanced host susceptibility to WNV encephalitis [16, 17]. Toll-like receptor (TLR)7 provides co-stimulatory signals for γδ T cell activation during WNV infection. An impaired TLR7 signaling led to dysfunction of these cells observed in WNV-infected aged mice [29].

3. *Persistent WNV infection in young adult mice*: WNV antigen or RNA has been detected in the brain or urine of WNV convalescent patients, ranging from a few months to several years after the initial acute illness [18, 19]. To define a murine model of persistent renal infection, we used WNV H8912, an isolate recovered from the urine of chronically infected hamsters. The virus showed a significantly reduced neuroinvasiveness in young adult mice. All mice survived i.p. inoculation of a dose up to 10^6 PFU of WNV H8912, and only about 20% of infected mice developed mild disease symptoms. WNV H8912 induced constitutive interleukin (IL)-10 production and a delayed antiviral response. Viral RNA was detected quickly in blood and spleen but much later in kidneys (*see* Table 1). The virus persisted preferentially in the kidneys with mild renal inflammation, and less frequently in the spleen for up to 2.5 months post infection. This was concurrent with detectable serum WNV-specific IgM and IgG production [20]. In another study, Appler et al. [30] inoculated young adult mice by the s.c. route with a New York (2000) isolate (NY99 genotype) and for 16 months examined their tissues for infectious virus and WNV RNA. Infectious WNV persisted for 1 month in all mice and was found in 12% of mice at 4 months; WNV RNA persisted for up to 6 months in 12% of mice that had subclinical infections. The frequency of persistence was tissue dependent and found in the skin, spinal cord, brain, lymphoid tissues, kidneys, and heart, but the persistence less frequently in the kidney tissues [30, 31]. In summary, these two models share some similarity and discrepancy in symptoms and tissue tropism with the clinical findings in some WNV convalescent patients having long-term morbidity (either chronic kidney diseases or long-term neurological sequelae).

4. *Cutaneous infection model*: WNV infection in mice by i.d. challenge partially mimics natural infection in humans, though it is much less complicated due to a lack of concurrent exposure to components of mosquito saliva [32, 33]. One earlier study [34] revealed that the mean and median doses of WNV inoculated by *Culex tarsalis* mosquitoes as they probe and feed on peripheral tissues of a mouse were $10^{4.3}$ PFU and 10^5 PFU. The same doses of WNV by i.d. infection in mice results in survival rates of 25–40%. Cutaneous infection shares a disease outcome that is similar to those of systemic infection. However, its initial virus dissemination in mice differs from the latter. Following i.d. WNV challenge, the virus is first deposited in the skin cells and is further disseminated to lymph nodes and other peripheral tissues by the migration of the epidermal dendritic cells-Langerhans cells (LCs) to initiate systemic infection [35, 36]. TLR7 innate signaling contributes to host defense mechanisms, which leads to reduced viremia and lethality when WNV infection of mice was initiated by i.p. injection [37]. Nevertheless, the TLR7-induced immune response in mouse skin following cutaneous inoculation plays a role in viral pathogenesis by promoting WNV dissemination from the skin to initiate systemic infection. In particular, WNV infection in epidermal keratinocytes triggers a TLR7-dependent production of IL-1β, IL-6, and IL-12, which promotes LC migration from the skin to other peripheral organs. This process compromises the protective effects of TLR7 signaling during the systemic infection stage.
5. *Direct CNS infection*: It is often used to study the neurovirulence of WNV. The LD_{50} of wild-type WNV NY99 following i.c. inoculation is usually lower than that of systemic infection. Wild-type WNV induces neuroinvasive disease within 2 weeks following i.c. challenge [38]. WNV H8912, which is highly attenuated in neuroinvasiveness, induces no lethality following systemic infection even with a dose up to 10^6 PFU. Compared to wild-type WNV, WNV H8912 also displayed a reduced neurovirulence in mice by i.c. challenge. Mice inoculated with 10^4 and 10^6 PFU of WNV H8912 succumbed to infection; while 41% of mice inoculated i.c. with 10^2 PFU of WNV H8912 survived [20].

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Chapter 7

Exploration of West Nile Virus Infection in Mouse Models

Penghua Wang

Abstract

West Nile virus (WNV) causes neurological diseases by penetrating the central nervous system (CNS)—an immune-privileged system. Although the CNS residential cells can produce antiviral immune responses, the blood leukocytes are required to contain virus spread. However, infiltrating leukocytes may also contribute to immunopathology if they overreact. Thus analyses of WNV infectivity and leukocyte numbers in the CNS are critical for understanding of WNV pathogenesis in experimental mouse models. Here I describe two basic assays for quantification of viral titers and infiltrating leukocytes in the mouse brain after WNV infection.

Key words West Nile virus, Central nervous system, Infiltrating leukocytes, Plaque assay, Viral titration

1 Introduction

West Nile virus (WNV) is the most significant cause of viral encephalitis in the United States of America, responsible for 1753 lethal human meningoencephalitis cases since 1999. Addressing the fundamental questions regarding host–WNV interactions is an integrated and indispensable part of the effort towards development of vaccines and therapeutics. The murine model of WNV infection reproduces all the essential elements of human infection and allows for investigation of infection in genetically altered animals. There is a large body of literature showing that WNV infection in mice is an effective in vivo model to investigate viral pathogenesis and host immune responses to infection [1–4]. We have successfully used several different backgrounds of mice including C57BL/6, C3H/He, FVB/N, and BALBc for WNV studies, and these strains show differential susceptibility [5–10]. WNV is a neurotropic virus that disseminates from the blood circulation to the central nervous system and causes severe conditions such as meningitis, meningoencephalitis, or poliomyelitis. The brain is an immune-privileged organ that excludes blood leukocytes under healthy conditions,

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but becomes permissive to leukocytes after WNV infiltration. The influx of leukocytes is essential for control of WNV dissemination in the brain, but elevated immune responses elicited by leukocytes may contribute to pathogenesis of encephalitis [11]. Here we describe the methods for quantification of viral loads and immune responses in the brain of C57BL/6 mice.

2 Materials

Prepare all reagents with deionized water (18 MΩ cm at 25 °C), and store at room temperature unless indicated otherwise.

2.1 Viral Quantification

1. Sterile phosphate buffered saline (PBS): 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, adjust pH 7.4 with 6 N HCl and NaOH, autoclaved at 121 °C for 15 min.
2. Virus suspension buffer: 1% gelatin prepared in PBS, autoclaved at 115 °C for 15 min.
3. Vero cells: kidney epithelial cells originated from an African green monkey, deficient in type I interferon expression and thus suitable for virus production and titration.
4. Complete Dulbecco's Modified Eagle's Medium (DMEM) (DMEM medium, 10% fetal bovine serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B, filter-sterilized through a 0.2 μM filter bottle) (*see* Table 1).
5. Neutral red (3.3 g/L in PBS, filter-sterilized through a 0.2 μM filter syringe).
6. Bradford protein assay kit: a coomassie blue-based protein quantification assay commercially available.
7. Inhalational anesthetics: 30% isoflurane diluted in propylene glycol, valid for 3 months.
8. Injectable anesthetics: ketamine/xylazine mixture (up to 80 mg/kg body weight ketamine and 10 mg/kg body weight xylazine).
9. 5% agarose: 5 g low-melting (melting temperature 65 ± 1.5 °C) agarose in 100 mL PBS, autoclaved at 121 °C for 15 min.
10. 0.4% (w/v) Trypan Blue solution.

2.2 Immunoassays

1. Prepare 70, 37 and 30% (v/v) Percoll by diluting sterile isotonic Percoll into RPMI complete media (RPMI 1640 with 10% fetal bovine serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B, filter-sterilized through a 0.2 μM filter bottle (*see* Table 1). Store at 4 °C.

Table 1
Components of base DMEM/RPMI1640 medium

Components	DMEM, mg/L	RPMI1640, mg/L
Glycine	30.0	10.0
L-Arginine hydrochloride	84.0	200.0
L-Aspartic acid	0.0	20.0
L-Asparagine	0.0	50.0
L-Cystine·2HCl	63.0	63.0
L-Glutamine	584.0	300.0
L-Glutamic acid	0.0	20.0
L-Histidine hydrochloride-H_2O	42.0	15.0
L-Isoleucine	105.0	50.0
L-Leucine	105.0	50.0
L-Lysine hydrochloride	146.0	40.0
Hydroxyl-L-proline	0.0	20.0
L-Methionine	30.0	15.0
L-Phenylalanine	66.0	15.0
L-Serine	42.0	30.0
L-Threonine	95.0	20.0
L-Tryptophan	16.0	5.0
L-Tyrosine disodium salt dihydrate	104.0	28.8
L-Valine	94.0	20.0
L-Proline	0.0	20.0
Choline chloride	4.0	3.0
D-Calcium pantothenate	4.0	0.0
Calcium nitrate·$4H_2O$	0	100
Folic acid	4.0	1.0
D-Biotin	0.0	0.2
Niacinamide	4.0	1.0
p-Aminobenzoic acid	0.0	1.0
D-Pantothenic acid	0.0	0.25
Vitamin B_{12}	0.0	0.005
Pyridoxine hydrochloride	4.0	1.0
Riboflavin	0.4	0.2

(continued)

Table 1 (continued)

Components	DMEM, mg/L	RPMI1640, mg/L
Thiamine hydrochloride	4.0	1.0
i-Inositol	7.2	35.0
Calcium chloride ($CaCl_2$) (anhyd.)	200.0	0.0
Ferric nitrate (Fe(NO3)3″ $9H_2O$)	0.1	0.0
Magnesium sulfate ($MgSO_4$) (anhyd.)	97.67	49.0
Potassium chloride (KCl)	400.0	400.0
Sodium bicarbonate ($NaHCO_3$)	3700.0	2000.0
Sodium chloride (NaCl)	6400.0	6000.0
Sodium phosphate monobasic	125.0	0.0
Sodium phosphate dibasic	0.0	800.0
D-Glucose (dextrose)	4500.0	2000.0
Phenol red	15.0	5.3
Glutathione reduced	0.0	1.0

2. Flow cytometry (FACS) staining buffer: make 1% bovine serum albumin, 0.1% w/v NaN_3 in PBS, store at 4 °C.
3. Fixation solution: 8% paraformaldehyde (PFA) prepared in PBS.
4. Cell storage buffer: 10% Dimethyl sulfoxide (DMSO) and 40% FBS in PBS.
5. Red blood cell lysis buffer: 0.2% (w/v) NaCl, autoclaved at 121 °C for 15 min.
6. Neutralizing buffer: 1.6% (w/v) NaCl, autoclaved at 121 °C for 15 min.

2.3 General Equipment and Materials

CO_2 incubator, 42 °C water bath, microwave oven, white light transilluminator, Eppendorf 5810 centrifuge with a swing bucket rotor and sealed buckets (for 1.5, 15 and 50 mL tubes), light microscope, inverted light microscope, tube stands (1.5, 15, 50 mL), balance, water-proof tapes, syringes (5, 10 and 60 mL), needles (20, 25 G), 1.5 mL microfuge tubes (autoclaved at 121 °C for 15 min), 20-G olive-tipped perfusion needle, absorbance microplate reader, 1.8 mL sterile Cryotube vials (Nunc product), surgical scissors/forceps, pipettes and sterile tips (10, 20, 200, 1000 μL),

electronic pipette and sterile serological pipettes (10, 25 mL), sterile 40 μM cell strainers, crushed ice and box, a stainless still tray, Petri dishes (60 mm in diameter), hemocytometer, sterile conical tubes (15, 50 mL), 70% ethanol pads, 24-well culture plates, 75 mL culture flasks, 0.2 μM syringe and bottle filters, a glass jar.

3 Methods

Perform all procedures in certified biosafety level 3 facilities when working with live WNV and follow institutional BSL3 biosafety and animal care regulations.

3.1 Mouse Infection and Tissue Collection

1. Infection. Prepare 200 plaque forming units (p.f.u.) of WNV NY99 strain in 50 μL of virus suspension buffer for each mouse (*see* **Note 1**). Add 1 mL of inhalational anesthetics to a wide mouth glass jar; place an 8–12 week old C57BL/6 mouse. Wait until the mouse falls in deep sleep (*see* **Note 2**), disinfect one hind foot with 70% ethanol, use forceps to hold the foot, inject virus with an 25-G or smaller needle (we usually use a 0.5 mL insulin syringe) to its ventral aspect. Wipe the footpad with a 70% ethanol pad after injection.
2. Perfusion. On day 6 (or the time points appropriate for your study), anesthetize and perfuse mice as the followings [12]. Before surgery, anesthetics will be injected to the intra-peritonea using a 25-G needle. Additional administration of anesthetic may be needed during the course of each perfusion in order to maintain a surgical plane of anesthesia. Once the animal is in a surgical plane of anesthesia (*see* **Note 1**); place the mouse with its face-up on a tray on crushed ice, spread out the mouse and tape its four feet firmly to the tray. Open the diaphragm to expose the heart. Make a small incision to the posterior end of the left ventricle. Insert a 15-G olive-tipped perfusion needle through the incision into the ascending aorta. Finally, make a large incision to the animal's right atrium without damaging the descending aorta. Fill a 60 mL of syringe with ice-cold PBS, push through the perfusion needle. Try twice until the flow-through is colorless. Now flip the mouse with its face down; cut along the coronal suture and sagittal suture then pull off both sides of parietal bone and interparietal bone; carefully remove the whole brain using a pair of blunt tip forceps. Figure 1 shows that a well-perfused brain should be free of blood.

3.2 Plaque Forming Assay

1. Prepare Vero cell monolayer a day before processing the brain. In a 24-well culture plate, seed 2×10^5 viable cells (*see* **Note 3**) in 0.5 mL of DMEM complete medium, and grow it in an incubator at 37 °C, 5% CO_2.

Fig. 1 A thoroughly perfused mouse brain

2. Take a half well-perfused brain from Subheading 3.1 **step 2** and pipet up-down with a P1000 pipette several times gently to homogenize the brain on ice.
3. Add 1 mL of ice-cold PBS; re-suspend the brain homogenate. Centrifuge at 6000 × *g*, 4 °C for 10 min; transfer the supernatant to a sterile 1.5 mL microfuge tube. When transfer, try to avoid the white fats on the top. Keep samples on ice.
4. Determine the protein concentrations of the brain lysates using a Bradford protein assay (*see* **Note 4**).
5. Remove 300 μg of protein-equivalent brain lysate to a sterile microfuge tube, and add ice-cold sterile PBS to a final volume of 300 μL.
6. Change **step 1** Vero culture medium of the 24-well plate with 0.5 mL of pre-warmed fresh one and then add 100 μL of brain lysate from **step 5** to the cells (duplicate each brain sample). Swirl the plate gently to spread the brain lysate evenly. Incubate the 24-well plate at 37 °C, 5 % CO_2 for 2 h, allowing virions to sediment and enter Vero cells.
7. During the incubation, melt 5 % agarose (0.15 mL per well) in a microwave oven and cool it in a 42 °C water bath. Also warm the complete DMEM medium (0.6 mL per well) in the water bath. When the agarose and DMEM medium temperature equilibrates to 42 °C, transfer the medium to agarose (4:1 ratio) and mix well. Keep the mixture in the water bath at 42 °C.

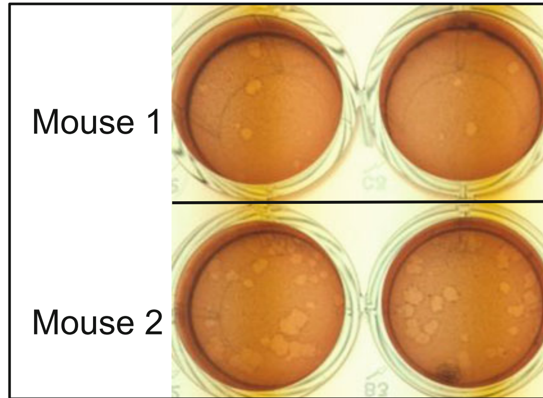

Fig. 2 Representative results of two mice

8. When 2 h-incubation (**step 6**) is over, carefully remove the culture medium from each well, and then add 0.75 mL of agarose-medium mixture (**step 7**) along the wall of the culture well. Leave the plate in the biosafety cabinet for 15 min, allowing the agarose to solidify.
9. Incubate the plate at 37 °C, 5% CO_2 for 72 h. Mix 1 mL of neutral red with 4 mL of DMEM complete medium, add 100 μL to each well. Incubate the plate at 37 °C, 5% CO_2 for 2 h. Remove the unabsorbed neutral red from each well and then count plaques with a white light transilluminator. Viable cells can take up neutral red, while dead cells will exclude it and thus forms a white spot (Fig. 2). Based on our experience, the number of plaques per 100 μg of proteins may be between 0 and 100. Reduce the amount of brain lysates if the plaque numbers are too large.

3.3 Isolation of Brain Immune Cells

1. Place two individual perfused brains from Subheading 3.1 in a 40 μM cell strainer (Corning product #354320, other comparable products are acceptable) which is sitting in a 60 mm Petri dish containing 10 mL of complete DMEM media on ice.
2. Use the rubber stopper of a 10 mL syringe to crush gently the brains in the cell strainer. Make sure that just the single-cell suspensions, not the large clumps pass through the cell strainer.
3. Transfer the cell suspension to a 15 mL conical tube and centrifuge the cell suspensions at 500 × *g* for 10 min at 4 °C. Discard the supernatants.
4. Add 4.5 mL of 70% Percoll to the cell pellet; resuspend the pellet by pipetting up and down gently; and then transfer the suspension to a 15 mL conical tube on ice.
5. Use a 5 mL syringe with 20-G needle to transfer very gently 4.5 mL of 37% Percoll to the top of the 70% Percoll solution

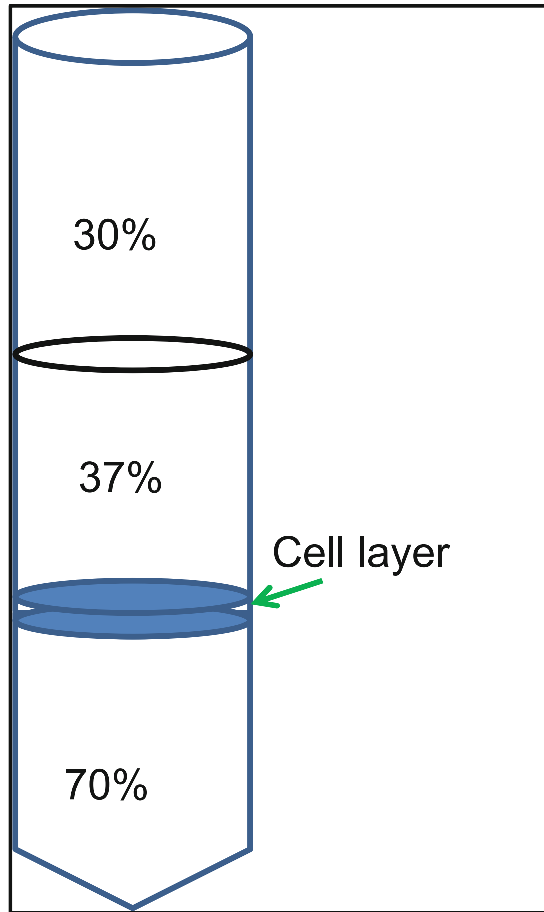

Fig. 3 Illustration of Percoll gradient

on ice. Be very careful not to disturb the 70% Percoll phase. Next gently overlay 4.5 mL of 30% Percoll (*see* **Note 5**).

6. Set the brake to 0 on an Eppendorf 5810 centrifuge (with a swing bucket rotor and a basket for 15 mL tubes). Centrifuge the discontinuous Percoll gradient at 500 × *g* for 20 min at room temperature.
7. After centrifuge, you will notice a small ring of cells at the 37:70% Percoll Interface (Fig. 3). Using a P1000 pipette, carefully remove the ring (in between 4 and 5 mL marks of a 15 mL Falcon tube) and place it in another tube (~2–2.5 mL). Add 10 mL complete RPMI 1640 medium to dilute the Percoll solution (*see* **Note 6**), and spin-down cells at 1000 × *g* for 10 min. Remove most medium and leave ~1 mL, resuspend cells and transfer to a 1.5 mL microfuge tube. Spin again at 1000 × *g* for 5 min at 4 °C, and resuspend in 200 μL of ice-cold

FACS staining buffer. Now these cells are ready for cell surface antibody labeling and FACS.

8. Count cells. Because WNV-positive cells must be inactivated before being counted using a microscope outside the biosafety cabinet, manual counting of viable cells is very challenging. We have to fix cells first with 4% PFA (final concentration) for 15 min, and then count the intact cells using a hemocytometer. But generally we will have enough viable cells from two brains to perform two "cocktails" of markers.
9. Divide the cells equally into two tubes-100 μL each (<1 million cells). First block Fc receptors by incubating the cells with 100 μg/mL of rat anti-mouse CD16/CD32 Ig (2.4G2 clone) for 30 min at 4 °C. Add fluorochrome-conjugated antibody (*see* **Note 7**) cocktails of interest and incubate at 4 °C in dark for 30 min. Add 120 μL of 8% PFA and fix cells for 15 min at room temperature. Spin down cells at 1000 × *g* for 5 min, and wash cells with 0.5 mL of FACS buffer twice. Resuspend cells in 200 μL of cell storage buffer. Use the cells from one brain for unstained and isotope IgG controls. Include immune cells from one spleen for single color positive controls.
10. Isolate splenocytes for positive staining controls. Splenocytes consist of a variety of cell populations such as T and B lymphocytes, dendritic cells and macrophages. These cells are positive for the surface markers of brain leukocytes. Place a spleen of uninfected mouse in a Petri dish on ice, hold a razor blade with forceps and mince the spleen. Suspend cells in 2 mL of ice-cold RPMI complete medium, and pass the cell suspension through a 40 μM strainer to a 50 mL conical tube. Add 20 mL of ice-cold PBS and centrifuge it at 1000 × *g* at 4 °C for 5 min. Resuspend the cell pellet in 1 mL of ice-cold red blood cell lysis buffer for 1 min, and add 1 mL of ice-cold neutralizing buffer followed by 20 mL of PBS. Spin down cells at 1000 × *g* at 4 °C for 5 min. Resuspend cells in 5 mL of ice-cold FACS staining buffer.
11. The most informative FACS is done by gating on intact cells and then $CD45^{+}$ cells ($CD45^{hi}$ cells are infiltrating leukocytes; $CD45^{int}$ cells are brain resident microglia), and combining this with other markers such as F4/80 Ag, Ly6G/C, CD11b, CD11c, CD4, CD8, and B220 or CD19.
12. The discontinuous Percoll gradient centrifugation is an easy and fast method for isolation of brain immune cells, particularly in the tedious BSL3 environment (*see* **Note 8**). This method when coupled with a well-defined FACS protocol may quantify different immune cell types, distinguish residential microglia from infiltrating leukocytes, and help us define their roles in antiviral immune responses and pathogenesis of WNV encephalitis [13].

4 Notes

1. The titer of WNV stock is generally high, ~ 10^6–10^7 plaque forming units/mL. Viral stock is made with C6/36 cell, a mosquito cell line. To make virus suspension for ten mice, dilute the stock to 1×10^5 p.f.u./mL with sterile PBS, take 30 μL to a sterile 1.8 mL cryotube, add 720 μL of virus suspension buffer and mix well by pipetting ten times gently.
2. After administration of anesthetics, the mouse heart beats from normal, fast to slow. Pinch the toes and make sure that animals are unresponsive before continuing.
3. Vero cells should be maintained in complete DMEM medium in a 75 mL-cell culture flask (tissue culture treated) with a cell density of ~70–90 % confluence. Dislodge the cells with 0.05 % trypsin and count the viable cells using a hemocytometer. We use trypan blue to stain dead cells. Healthy cells are crucial for the success of plaque forming assays.
4. To measure a protein concentration with Bradford Assay Kit, first prepare bovine serum albumin standards: 100, 200, 400, 800, 1600 μg/mL. Second, pipette 10 μL of each standard (BSA), known sample or three blank controls (which is PBS in this case) into microplate wells (we use Nunc Apogent MaxiSorp 96-Well Clear Polystyrene Non-Sterile Micro Plates). Third, add 300 μL of Pierce Detergent Compatible Bradford Assay Reagent to each well, preferably with a multichannel pipettor, and pipette up and down 4–5 times to mix sample with reagent. Fourth, incubate plate for 10 min at room temperature. Fifth, measure the absorbance at 595 nm ($O.D_{595}$) with a plate reader (make sure the reading is between 0.2 and 0.8. Sample dilution is needed if its $O.D_{595}$ is above 0.8). Sixth, subtract the average readings of blank controls from those of each standard/sample. Use the blank-adjusted standards to plot a linear curve vs. their concentrations in μg/mL. An ideal curve would be $y = Ax + B$, where y is the concentration in μg/mL; x stands for OD_{595}; A is the slope of the standard curve; B is y-intercept (ideally $B = 0$). Calculate each sample concentration using the standard curve.
5. We use a 5 mL syringe with a 20-G needle to overlay 37 or 30 % Percoll. Push out the solution slowly against the conical tube wall. Handle the Percoll gradient carefully at all steps.
6. This dilution step is critical; otherwise cells are not easily pelleted down in the following step.
7. Choose the fluorochromes that are compatible with your FACS machine.

8. BSL3 practice is much more time-consuming than BSL1/2 work. In order to get as many viable cells as possible, efficient work-flow is essential. Get all materials, reagents, tools, and equipment ready before starting work. Work on no more than four mice at a time.

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Chapter 8

Examination of West Nile Virus Neuroinvasion and Neuropathogenesis in the Central Nervous System of a Murine Model

Hameeda Sultana

Abstract

West Nile virus (WNV) is a neurotropic virus that causes inflammation and neuronal loss in the Central Nervous System leading to encephalitis and death. In this chapter, detailed methods to detect WNV in the murine brain tissue by quantitative real-time polymerase chain reaction and viral plaque assays are described. Determination of WNV neuropathogenesis by Hematoxylin and Eosin staining and immunohistochemical procedures are provided. In addition, TUNEL assays to determine neuronal loss during WNV neuropathogenesis are discussed in detail. Collectively, the methods mentioned in this chapter provide an overview to understand neuroinvasion and neuropathogenesis in a murine model of WNV infection.

Key words West Nile virus, Blood–brain barrier, Neuroinvasion, Neuropathogenesis, Neuronal death

1 Introduction

West Nile virus (WNV) a mosquito-borne, positive-sense, single-stranded RNA virus is a neurotrophic virus with tropism for nervous tissue [1]. WNV invades the Central Nervous System (CNS) to cause inflammation, neuropathogenesis, and neuronal loss leading to encephalitis and death [1–5]. In the United States, WNV is responsible for the increasingly severe encephalitis outbreaks that are reported from horses and humans [2, 5]. Although, WNV is considered as a neurotropic virus, the molecular mechanisms to understand viral entry or neuroinvasion into the brain are poorly understood. The availability of the current murine model serves as a good example to understand the mechanisms of blood–brain barrier (BBB) permeability, neuroinvasion, and neuropathogenesis. The viremia and mechanisms of CNS infections with WNV are not similar in the most common target hosts such as horses and humans [2, 5]. When viremia is high in the blood and other peripheral tissues, WNV has an ability to overcome and cross the extraneural

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and neural barriers by breaching the BBB, the blood–cerebrospinal fluid (CSF) barrier, the CSF–brain barrier, and the blood–spinal cord barrier [1]. Several studies have shown the virological and immunological molecular mechanisms compromise these barriers and allow neuroinvasion of WNV into the brain [6–11]. An overview of the important molecular mechanisms that validate the current mouse model relevant for WNV neuroinvasion needs to be still investigated.

In the murine model, WNV that replicates in the peripheral organs (days 2–4 postinfection p.i.), crosses the barriers (day 5 p.i.) and infects the CNS leading to death of the animals within 2 weeks postinfection [9, 10] (Fig. 1). Detailed in vivo and in vitro BBB models including the Evan's blue staining procedure have been described in the earlier chapter. Also, mice inoculations, perfusions, and isolation of mouse brain procedures have been discussed in the earlier chapter. Here, I have demonstrated the methodology to determine neuroinvasion and neuropathogenesis in murine brains infected with WNV (Fig. 1). This chapter highlights the methodologies for the detection of WNV viral loads by Quantitative real-time PCR (QPCR). Western Blotting using antibodies against the WNV envelope or nonstructural proteins can also be used to detect WNV in the brain. In addition, detection of the higher level of IgG also determines the neuroinvasion of WNV. This chapter does not discuss these other alternative approaches. However, the classical viral plaque assay recommended to determine the virus integrity, viability, and virulence by revealing the virus titers is discussed in detail. The recruitment of immune cells highlighting the process of neuroinflammation and cytokine storms produced by these infiltrated cells suggests the major cause for neuropathogenesis. This chapter discusses the Hematoxylin & Eosin (H&E) staining and immunohistochemical (IHC) methods to determine neuropathogenesis. The neuronal cell death determined by TUNEL assays is also discussed.

2 Materials

Any procedures with WNV-infected mice or brain tissues require the use of a biosafety level III laboratory containment and work practices. Hence it is highly recommended that individuals acquire proper trainings, orientations, and require authorizations to work with WNV. Please follow all relevant regulations and procedures accordingly.

2.1 Neuroinvasion

2.1.1 Mice Inoculation and Brain Collection

1. BD™ U-100 insulin syringe with 27 G × 5/8 in. self-contained BD Micro-Fine™ IV permanently attached needle, 1 ml, regular bevel, regular wall. (Orange) (100/sp, 500/ca), Sterile, VWR (Catalog number BD329412).

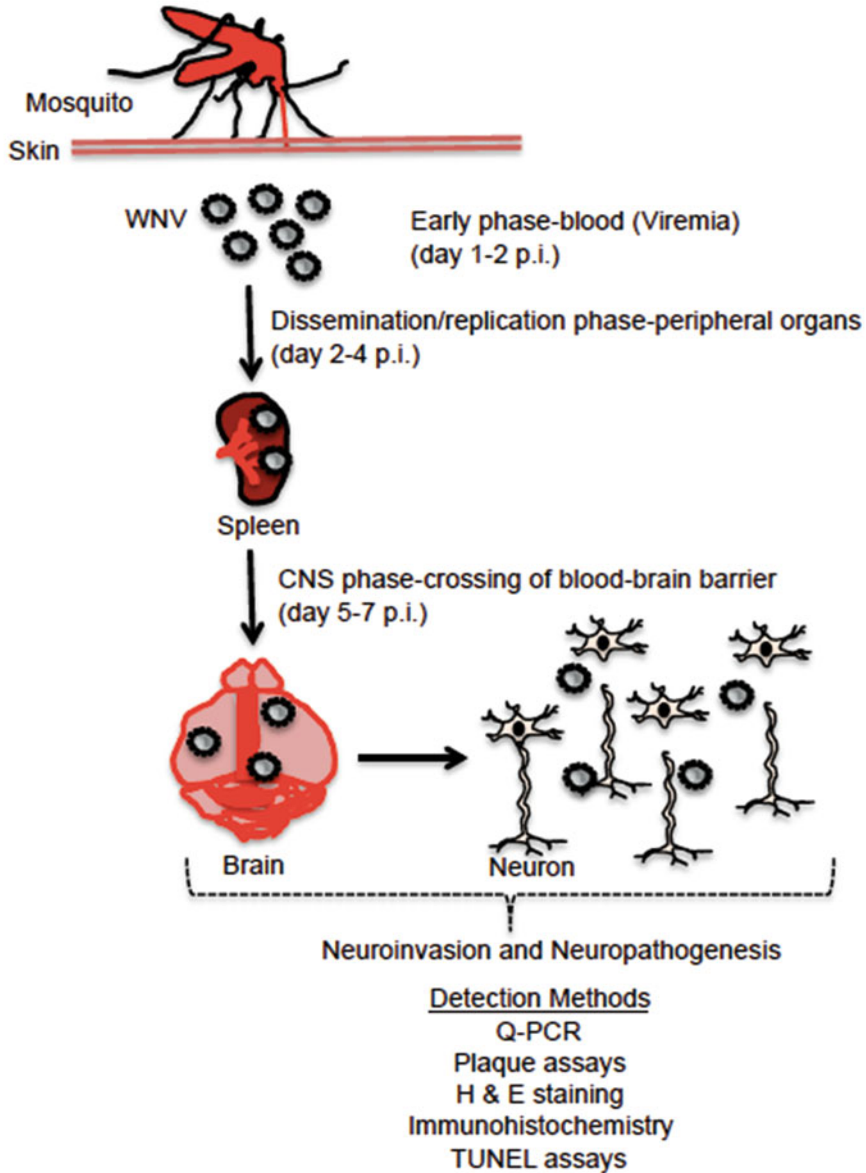

Fig. 1 Schematic representation of WNV pathogenesis and methods used to detect neuroinvasion and neuropathogenesis in a murine model. Overview of the WNV infection kinetics in a murine model and methods used to detect neuroinvasion and neuropathogenesis is shown. In a murine model, high viremia is seen in the blood between day 1 and 2 p.i., followed by dissemination and replication of WNV to peripheral organs such as spleen between days 2 and 4 p.i and infection of CNS between day 5 and 7 p.i. Entry of WNV into the brain causes neuropathogenesis leading to neuronal death. Several methods including but not limited to Q-PCR, H&E staining, immunohistochemistry, and TUNEL assays are used to determine WNV neuroinvasion and neuropathogenesis in the murine brain tissue

2. Phosphate buffered saline (PBS) with 5% Gelatin (Sigma).
3. High-strength autoclavable polypropylene Biohazard bags (VWR).
4. Anesthesia (Forane or Isoflurane), Baxter.
5. Mouse dissection kit with storage case. Includes the following components: Dumont Forcep #5, Vannas scissor, dissecting scissors, wire retractor, needle holder, and blunt probe (Braintree Scientific, Inc; Lab research products) (catalog number MDK-M).
6. Sterile microcentrifuge tubes (any brand).

2.1.2 RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR (QPCR)

1. RNeasy mini kit (50), Includes 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 and 2 ml), RNase-free Reagents, and Buffers (Qiagen; catalog number 74104).
2. RNase-free DNase I (Qiagen) (for on-column DNA digestion).
3. Bench-top Centrifuges (any brand, e.g., Beckman Coulter or Eppendorf).
4. Thermal Cycler (PCR machine) (BioRad or any other).
5. iScript cDNA Synthesis Kit, 100×20 μl reactions, includes 5× reverse-transcription reaction mix, iScript reverse transcriptase, nuclease-free water (BioRad: catalog number 1708891).
6. Real-Time PCR detection System (e.g., CFX96 Touch Real-Time PCR Detection System, BioRad or other).
7. Supermixes for Real-Time PCR (e.g., iTaq Universal Supermixes; advanced master mixes that are compatible with any real-time PCR instrument, BioRad).
8. Primers for WNV detection (e.g., forward and reverse primers specific for WNV Envelope or Capsid gene), IDT technologies.
9. Kontes pellet Pestle and microcentrifuge tubes, 1.5 ml, 70 mm length, pack of 100, Kimble-Chase.
10. Kontes Pellet Pestle Grinders, Kimble-Chase.
11. Sterile microcentrifuge tubes (any brand).
12. Brain tissue or lysates in RNA lysis buffer (e.g., RLT buffer from Qiagen kit).

2.1.3 Viral Plaque Assay (Requires a BSL-3 Cell Culture Laboratory)

1. Cell culture growth medium (DMEM high glucose with 4–6 mM glutamine and 10% fetal bovine serum), penicillin and streptomycin (fungizone or antibiotic/antimycotic solutions can be added if required), Life technologies/Thermo-Fisher Scientific.

2. Ultrapure 2% and 1% SeaPlaque agarose (original low melting temperature agarose ideal for viral plaque assays) (Lonza; catalog number: 50101, 25 g).
3. Plaquing medium (DMEM high glucose with 4–6 mM glutamine and 2% fetal bovine serum and SeaPlaque agarose).
4. 6-well plates (any brand works; e.g. Corning, BD, Co-star, GeneMates).
5. Vero host cells (ATCC).
6. Phosphate buffered saline (PBS).
7. Brains homogenized in PBS.
8. Tissue culture grade sterile water (100 ml).
9. Staining dye for plaque (0.03% Neutral Red) (Sigma).
10. Waterbath (ThermoScientific or any vendor).
11. Standard microwave oven (any brand, domestic).
12. Inverted microscope (any with phase contrast channel).
13. Biosafety cabinet (in a regulated BSL-3 lab) (NuAire or other).
14. CO_2 incubator, set at 37 °C (NuAire or other).
15. Microcentrifuge tubes (any brand).
16. Sterile Pasteur pipettes, serological pipettes, and barrier tips (any brand).
17. Aluminium Foil.
18. Millipore autoclaved water.
19. Sterile glass bottles (Hybex).

2.2 Neuro-pathogenesis and Neuronal Death

2.2.1 Histology and Immunohistochemistry

1. Phosphate buffered saline (PBS).
2. Fixatives; Preferred standard fixative solution is 10% neutral buffered formalin (100 ml of 37–40% Formaldehyde, 900 ml of distilled Water, 4 g NaH_2PO_4 and 6.5 g of anahydrous Na_2HPO_4); for immunoperoxidase techniques 4% buffered neutral Paraformaldehyde is recommended (8% paraformaldehyde (PFA): 40 g PFA in 500 ml distilled water and 0.2 M Phosphate buffer, pH 7.4 (10.9 g of Na_2HPO_4 and 3.2 g of NaH_2PO_4 in 500 ml distilled water) can be used. While stirring heat 8% PFA solution strictly at 60 °C, once the solution is at 60 °C the PFA is dissolved and 500 ml of 0.2 M phosphate buffer is added to bring the solution to 4% PFA in 0.1 M phosphate. Add two pellets of solid NaOH in 1–2 L of solution or alternatively, add few drops of 1 N NaOH solution, until 4% PFA solution is clear. It is highly recommended to make fresh PFA solution on the same day of fixation, otherwise the 4% PFA can be frozen at −20 °C, but always use as freshly thawed solution. For Bouin fixative Solution add 75 ml of saturated picric acid, 25 ml of 37–40% formaldehyde and 5 ml of glacial acetic acid and mix well (*see* **Note 10**).

3. Mouse brain tissues (frozen or fixed and embedded in paraffin).
4. Histology cassettes (VWR).
5. Hematoxylin Solution (Harris Modified, Sigma).
6. Eosin Y Solution, Alcoholic (Sigma).
7. Resinous mounting medium or Permount histological mounting media (ThermoScientific).
8. Paraffin (ThermoScientific) (*See* **Note 13**).
9. Cryostat containing microtome with sharp knife (Leica or any vendor) (*See* **Notes 13** and **14**).
10. APES (amino-propyl-tri-ethoxy-silane) coated slides (alternative Poly-L-Lysine treatment for slides) (Sigma).
11. Xylene.
12. 100 and 95 % Ethanol solutions.
13. Tris/EDTA buffer, pH 9.0 (10 mM Tris Base, 1 mM EDTA solution, 0.05 % Tween 20).
14. Sodium citrate buffer, pH 6.0 (10 mM Sodium Citrate, 0.05 % Tween 20).
15. 1 mM EDTA, adjusted to pH 8.0.
16. Antigen retrieval buffer (e.g. Tris/EDTA pH 9.0, Sodium Citrate pH 6.0).
17. Vegetable Steamer or waterbath (set to 100 °C).
18. Container/vessel for slide rack (the vessel should hold roughly 400–500 ml of buffer).
19. Microwave (domestic).
20. Waterbath (set at 37 °C).
21. Slide racks (metal and/or plastic).
22. Humidified chamber (shallow plastic box with a sealed white lid, Nalgene).
23. Wet tissue papers.
24. Wash buffer (TBS and TBS with 0.025 % Triton X-100).
25. Block buffer (10 % Normal serum with 1 % BSA in TBS).
26. Primary antibodies, matching isotype control antibodies and fluorophore-conjugated secondary antibodies and counter nuclear stains (diluted in TBS with 1 % BSA) (*see* **Notes 17–19**).
27. Refrigerator.
28. Mounting medium (aqueous-based permanent mounting media).
29. CO_2 incubator (set at 37 °C).
30. Fluorescent or confocal microscope (Olympus or Leica or Zeiss).

2.2.2 Apoptosis (TUNEL) Assay

1. Formaldehyde (4% buffered solution; freshly prepared, prior to use).
2. Phosphate buffered saline (PBS), pH 7.4.
3. Paraffin.
4. Glass slides (pretreated with 0.01% aqueous solution of Poly-L-Lysine from Sigma).
5. Coupling jars.
6. Water or heat baths (set to 60 °C).
7. Xylene bath.
8. Ethanol in coupling jars (96, 90, 80, and 70%).
9. Double distilled or millipore water.
10. Proteinase K stock solution (20 μg/ml; Store for long term at −20 °C) (Sigma).
11. TdT equilibration buffer (2.5 mM Tris–HCl; pH 6.6, 0.2 M potassium cacodylate, 2.5 mM $CoCl_2$, BSA (0.25 mg/ml) (Long-term storage at −20 °C) (*see* **Note 21**).
12. TdT reaction buffer (0.5 U/μl of TdT enzyme and 40 pmol/μl biotinylated-dUTP in TdT equilibration buffer; freshly prepared) (*see* **Note 21**).
13. Humidified chamber (Nalgene; and prepared as described above).
14. Glass coverslips.
15. SSC (2×; 300 mM NaCl, 30 mM sodium citrate, long-term storage at room temperature).
16. BSA solution (2% in PBS) (filter sterilized).
17. TdT staining solution (4× Saline Sodium Citrate Solution; containing 0.6 M NaCl, 60 mM sodium citrate), 2.5 μg/ml fluorescein isothiocyanate-conjugated avidin (Sigma, ThermoScientific), 0.1 %Triton X-100, 1% BSA, prepared freshly prior to use (*see* **Note 21**).
18. Hematoxylin (Sigma) (*see* **Note 11**).
19. Hoechst 33342 (2 μg/ml in PBS; Molecular Probes or Cell Signaling Technologies) (Stored in dark at 4 °C for several weeks) (*see* **Note 22**).
20. Vectashield antifade mounting medium (Vector Laboratories).
21. Fluorescent or confocal microscope (Olympus or Leica or Zeiss).

3 Methods

3.1 Neuroinvasion

3.1.1 Mice Inoculation and Brain Collection

All procedures in this section should be carried out at authorized BSL-3 laboratory and in biosafety containment facilities or cabinets. Researchers should follow all the Biosafety regulations according to their University biosafety office policies. *See* **Note 1**. The previous chapter describes the inoculation of mice and collection of intact brains in detail. Here, I have described these procedures in brief.

1. Age appropriate female C57BL/6 mice (6–8 weeks old) were housed in the ABSL-3 mice facility for 2 days to acclimatize to the new conditions.
2. Biosafety cabinets are divided into two areas as clean or dirty, the diluted virus for inoculation and all supplies in contact with virus should be kept at the dirty side of the cabinet. Supply the biosafety cabinet with container for sharps, biohazard red bags (one bag inside the cabinet and one outside for later use as double bagging) and other required materials.
3. Before inoculation, mice bellies were sterilized with 70 % ethanol pads to clear any microbes on the mice or on mice fur.
4. In biosafety cabinets, mice were inoculated intraperitoneal (i.p.) with 1000 Plaque forming units (pfu) of WNV (example: CT2741 or NY99 wild-type parental strains) in PBS with 5 % Gelatin, using BD™ U-100 insulin syringe with 27 G × 5/8 in. self-contained BD Micro-Fine™ IV permanently attached needle (*See* **Notes 2** and **3**).
5. Needles are immediately disposed in sharps container without capping.
6. Mice were observed for 5–10 min for behavior changes or immediate lethargy or other conditions and cages were decontaminated with 10 % bleach and moved to the respective racks.
7. Remaining diluted virus stocks are thoroughly mixed with 10 % bleach and discarded along with the original vial (closed) into the Biohazard red bag (inside), double bagged with outside bag and disposed into the regulated waste that gets autoclaved by the ABSL3-regulated facility.
8. Day seven postinfection, mice brains were collected from WNV-infected and control (uninfected) mice by cervical dislocation. Briefly, mice were anesthetized with isoflurane followed by cervical dislocation and brains were isolated using the dissection kit. Brain tissues were collected for RNA isolation in Qiagen RLT buffer (in microcentrifuge tubes) and homogenized (*see* **Notes 4** and **5**) or homogenized in PBS for viral plaque assays (*see* **Notes 6–9**) or otherwise quickly embedded

in histological cassettes and fixed (by completely immersing) in either 10% neutral buffered formalin solution or 4% PFA for immunohistochemistry (*See* **Note 10**).

3.1.2 RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR (QPCR)

Other than proper tissue homogenization (*see* **Notes 4** and **5**), the above procedures are performed with usual general guidelines and as per the manufacturer's instructions.

1. For RNA extraction, WNV-infected and control brain samples (half brain tissue is sufficient for higher RNA yields) were homogenized in 350 μl of RLT buffer containing beta-mercaptaethanol (BME) (according to the manufacturer's instructions). Brains were first homogenized with Kontes Pellet Pestles and Grinders until homogenous solutions has been achieved, further homogenization is performed with passages through 18, followed by 25 G needle and syringes to lyse individual cells (*See* **Notes 4** and **5**). Another 350 μl of RLT buffer with BME is added and the tissue homogenates were kept overnight at −20 °C for lysis to complete.
2. Homogenous brain lysates in RLT buffer were mixed with equal volumes of 70% ethanol and loaded onto RNA columns provided (Qiagen kit). Further steps were performed according to the manufacturer's instructions.
3. The optional step of on-column DNase digestion using DNase I is highly recommended to get pure RNA for sensitive downstream applications such as QPCR. RNA extracted from half of the brain tissue is eluted in 75 μl of RNase-free water. The elution step is repeated with another 50 μl of RNase-free water. Nearly 30–40 μg of RNA is extracted from every 10 mg of homogenized brain tissue.
4. Double-stranded complementary DNA (cDNA) is prepared from 1 μg RNA using iscript cDNA synthesis kit from BioRad (following manufacturer's instructions).
5. Quantitative real-time PCR can be performed with 3–5 μl of cDNA, using BioRad SYBR Green, Primer mix for WNV *Envelope* or *capsid* gene (Forward and Reverse; 1 pmol/μl of each) and nuclease-free water (Sigma).
6. Primers for beta-actin were used in parallel with the primers for Q-RT-PCR normalization. Equal amounts of cDNA samples were used in parallel for beta-actin and WNV *E* gene or capsid gene.
7. Ratio of WNV-*E* or *capsid* gene copy/*beta-actin* gene copy is used as an index to determine the infection rate of each WNV-infected brain samples. QPCR data is representative of results performed in triplicate.

8. Standard curve was prepared using 10-fold serial dilutions starting from 1 ng to 0.00001 ng of known quantities of *WNV-E or beta-actin* fragments as standards.

3.1.3 Viral Plaque Assay (Requires a BSL-3 Cell Culture Laboratory)

1. Vero host cells are plated at a cell density of $1–2 \times 10^6$ per well of a 6-well plate in DMEM high glucose medium with 4–6 mM glutamine and 10 % fetal bovine serum, penicillin, and streptomycin. Cells are allowed to grow overnight, in order to reach 65–85 % confluency and formation of complete monolayers before infection (*See* **Note 6**).
2. Brain lysate homogenized in PBS is used to determine the WNV titers and virus integrity, viability, and virulence. First six serial dilution of this virus stock is made in labeled sterile microcentrifuge tubes (e.g., add 60 μl of brain homogenate to the first stock tube containing 540 μl of DMEM medium, mix (gently vortex), avoid pipetting as brain homogenates tend to stick to the pipettetip walls. Transfer 60 μl of brain homogenate from first tube to the second tube containing 540 μl of DMEM medium, repeat this to other tubes and make a serial 1:10 dilution of the virus stock.
3. Confluency of Vero host cells is analyzed and DMEM medium is removed using a sterile Pasteur pipette (3 ml). Use of other pipettes may dislodge the cell monolayer (*See* **Note 6**).
4. To each of the dilution well add 500 μl of the $10^{-1}–10^{-6}$ brain homogenate. If plaque assay is performed in duplicate or triplicate, repeat the same for second and third plate.
5. Incubate the plates in a CO_2 incubator, set at 37 °C, for 1–2 h to allow the virus to infect Vero host cells.
6. Microwave or melt 10 ml of sterile (autoclaved) 2 % SeaPlaque agarose in water (100 ml glass bottle) and the molten agarose is kept warm at 37 °C waterbath. The culture media is also kept prewarmed to 37 °C before next step (*See* **Notes 7** and **8**).
7. Carefully remove the virus from the host Vero cells using a sterile Pasteur pipette (1 ml) and without dislodging the cell monolayer (*See* **Note 7**).
8. Quickly mix the warm DMEM media and 2 % agarose in 1:1 ratio, 1.5 ml of this mix is gently added to make an overlay on the host Vero cells in each well. The plates were incubated for 20 min at room temperature (In BSL3 certified hood) to allow solidification of agarose (*See* **Note 8**).
9. After 20 min, add 1.5 ml of warm DMEM media to each well (Fungizone or antibiotic/antimycotic solutions can be added if required) and transfer the plates carefully to the CO_2 incubator, set at 37 °C (*See* **Note 7**).

10. Incubate plates for 5–6 days, to allow virus replication and infection of the complete monolayer (*See* **Note 9**).
11. After day 6, remove the DMEM media using sterile Pasteur pipettes, add 1 ml of 0.03 % Neutral Red in PBS to each well, incubate plates for 2–4 h in a CO2 incubator, set at 37 °C.
12. Remove Neutral Red solution from plates, invert the plates and wrap in Aluminium Foil (dark) and incubate the plates in CO_2 incubator, set at 37 °C, overnight (*See* **Note 9**).
13. Vero cells infected with WNV die and do not take up the Neutral Red stain, however, uninfected cells take up stain and allow to distinguish the plaques as small clear areas against the red and pink backgrounds.
14. Count viral plaques and determine the viral titers on each well: Virus Titer (pfu/ml) = number of plaque × (1 ml/0.1 ml)/fold of dilution.

3.2 Neuropathogenesis and Neuronal Death

3.2.1 H&E Staining

To generate frozen sections, pieces of brain tissue are snap frozen in a cold liquid (liquid Nitrogen) or in cold environments such as −20 or −80 °C freezers. Freezing makes the tissue solid enough to section with a microtome sharp knife. Detailed tissue sectioning has been described in Subheadings 3.2.2 and **Notes 4**. Briefly, frozen sections can be obtained with an instrument Cryostat containing microtome. Make sure the temperature inside the cryostat is about −20 to −30 °C. The tissue sections are collected on a glass slide and processed for immediate staining or embedded in paraffin and kept frozen at −80 °C until use (*See* **Notes 10–15**).

1. For H&E staining, either paraffin or frozen brain sections adhered on slides were first hydrated or deparaffinized. To hydrate the frozen sections (collected freshly) incubate them in three changes of distilled water (3 × 2 min each). If the tissues have to be deparaffinized, sections are incubated for 2 min in each of the following solutions- Xylene (2×), 100 % Ethanol (1×), 95 % Ethanol (1×), 70 % Ethanol (1×) and water (2×). No staining can be performed on tissues containing paraffin, hence the tissues have to be first deparaffinized before processing (*See* **Note 15**).
2. Stain slides with Mayer's Hematoxylin Solution (1 min; if sections were fixed in Bouin's solution, stain for 2 min). Remove excessive water by blotting carefully.
3. Sections are rinsed in warm running tap water for 15 min, avoid the water to run directly on to the slides. Incubate slides in distilled water for 30 s followed by incubation in 95 % reagent alcohol for another 30 s.
4. Counterstain the sections with Eosin Y solution (1 min; 2 min for Bouin's fixed sections).

5. Before processing for mounting, sections are dehydrated and cleared in following solutions for 2 min in each; 95% reagent alcohol (2×), 100% absolute reagent alcohol (2×), Xylene (2×). *Dehydration before placing coverslips is discussed in the Notes* (*See* **Note 15**).
6. Mount with resinous mounting medium or Permount histological mounting media.
7. Dry slides overnight and analyze tissues using a microscope.

3.2.2 Immunohistochemistry (IHC)

Tissue samples are too thick for light to penetrate and be transmitted through them. Hence, fixed brain tissues are sliced into very thin sections. Processing of tissues involves several steps: fixation, dehydration, embedment, and subsequent sectioning with a Microtome or a modified microtome, the cryostat device (*See* **Notes 10**, **13–16**). To demonstrate the presence and location of a target protein or antigen in WNV-infected brains, this protocol uses antibodies that recognizes the target protein in fixed tissues [12–15]. Before proceeding with examination and conclusions it is important to optimize a new antibody for antigen retrieval, correct concentrations, and detection. Tips on optimizing a new antibody for IHC are discussed (*See* **Notes 17** and **19**). Although, IHC is less sensitive in comparison to Western Blotting and ELISA, but it provides enormous amount of information on the progression of neuropathogenesis as an intact tissue. In combination with microscopic procedures, IHC on human tissues would provide a complete set of information for pathologist and clinicians. Immunohistochemical staining discussed here uses fluorophore conjugated to the antibody that can be visualized using fluorescence or confocal microscopy.

1. Collect WNV-infected brains and controls in histological cassettes and gently close them. Label the sides of cassettes with pencil and not with pen or permanent markers.
2. Fix the brain tissues in 10% Neutral buffered Formalin solution (preferred fixative over 4% paraformaldehyde) for 24 h. For a successful IHC, proper fixation is highly recommended (*See* **Note 10**).
3. Tissue blocks are embedded in paraffin, and sections are cut to the desired thickness using a microtome. Sections that are five microns are highly recommended for IHC (the thicker ones will raise issues with penetration). Mount the sections on the positively charged amino-propyl-tri-ethoxy-saline coated glass slides or slides treated with Poly-L-Lysine. Dry slides to remove excess water trapped between the section and the slide. Keep slides at room temperature for overnight drying. Alternatively, slides can be kept at 60 °C incubator or oven to allow sections to tightly adhere on to slides (*See* **Notes 13** and **14**).

4. Rehydrate or deparaffinize the slides in a rack by placing them in coupling jars containing series of following solutions; Xylene (2×, 3 min), Xylene 1:1 with 100% ethanol (1×, 3 min), 100% ethanol (2×, 3 min), 95% ethanol (1×, 3 min), 70% ethanol (1×, 3 min), 50% ethanol (1×, 3 min). Run the cold tap water to rinse the slides. Do not dry the slides, but keep them in tap water until ready for antigen retrieval step (*See* **Note 15**).
5. Perform antigen Retrieval by heat mediated (heat-induced epitope retrieval) method, and by using pressure cooker, a microwave, or a vegetable steamer/Rice Cooker/Waterbath set at 100 °C (preferred over others). The enzymatic method is NOT recommended for WNV-infected brain tissue samples, due to higher damage of morphology of the sections (*See* **Notes 16–18**).
6. Set up the Vegetable streamer according to the manufacturer's instructions.
7. Preheat the antigen retrieval buffer (Tris/EDTA pH 9.0, Sodium Citrate pH 6.0) to boil (95–100 °C) in a flask (use domestic microwave as a handy tool to boil the antigen retrieval buffer).
8. Add the hot boiling antigen retrieval buffer to the vegetable steamer container/Vessel and slowly place the slides rack (Metal or plastic) into the vessel, close the vessel lid, and keep the vessel with slides rack inside the steamer and close the steamer lid. Incubate slides in a vessel kept in a steamer for 20 min (*See* **Notes 17–19**).
9. Remove the vessel and run in cold tap water for 10 min (be cautious with the hot solution!).
10. Before immunohistochemical staining, prepare a humidified chamber to avoid the drying of the tissue that may lead to nonspecific binding and high backgrounds. White sealer lid, shallow plastic boxes from Nalgene serves as the best for this purpose. Place wet tissue paper in the bottom and place a square cut parafilm wrap over the tissue paper to avoid the slides touching the wet tissue (*See* **Note 19**).
11. Wash slides (2×, 5 min each) in TBS with 0.025% Triton X-100 with gentle agitation.
12. Block slides in 10% normal serum with 2% BSA in TBS for 2 h at room temperature or overnight at 4 °C.
13. Drain slides and remove excess solution around sections with Kim wipes.
14. Incubate slides with primary antibody (diluted in TBS with 1% BSA), overnight at 4 °C (in humidified chamber to avoid drying) (*See* **Note 19**).
15. Wash slides with TBS containing 0.025% Triton (2×, 5 min).

16. Incubate the sections with fluorophore-conjugated secondary antibody diluted in TBS with 1% BSA at room temperature for 1 h (in humidified chamber to avoid photobleaching).
17. Rinse with TBS (3×, 5 min), gently overlay coverslips with aqueous-based permanent mounting medium.
18. Dry slides overnight at room temperature and observe under a fluorescent or confocal microscope.

3.2.3 Apoptosis (TUNEL) Assay

Severe neuronal loss and cell death or DNA damage/fragmentation during WNV infection is analyzed by Apoptosis (TUNEL) assays. TUNEL staining is not just restricted for the detection of apoptotic cells, but it can also detect DNA damage associated with nonapoptotic events or undergoing active DNA repair [16, 17]. In situ TUNEL staining for nuclear DNA fragmentation in WNV-infected and control brain tissue sections is described in detail. Sections should be permeabilized with ethanol to allow the TUNEL reaction reagents to penetrate into the cell nucleus. The colorimetric method using Light Microscopy is discussed elsewhere. This chapter describes the TUNEL assay that uses fluorescence staining of tissues (*See* **Notes 20** and **21**).

1. Brain tissues are fixed in 4% Paraformaldehyde (in PBS) for 24 h and embedded in paraffin. Paraffin sections (4–6 μM) were obtained using Cryostat (as described in Subheading 3.2.2) and adhered on to clean glass slides that were pretreated with 0.01% aqueous solution of Poly-L-Lysine coating material (*See* **Notes 10** and **20**).
2. Sections are deparaffinized by heating the slides for 30 min at 60 °C, followed by incubation in a xylene bath for 5 min at room temperature. All procedures were performed in coupling jars unless otherwise stated (*See* **Note 15**).
3. Rehydrate tissue sections by incubating the slides in series of graded ethanol solutions; 96% ethanol (2×, for 3 min each), 90% ethanol (1×, 3 min), 80% ethanol (1×, 3 min), 70% ethanol (1×, 3 min) and lastly sections were rehydrated with double distilled water (1×, 3 min). Excessive water is carefully removed by blotting.
4. Incubate slides with 20 μg/ml of Proteinase K solution for 15 min at room temperature, then wash slides with double distilled water (3×, 5 min each). Excessive water is carefully removed by blotting.
5. Incubate slides with TdT equilibration buffer for 10 min at room temperature, and then remove buffer. *Lower volumes of TdT buffers can be used if desired* (*See* **Note 21**).
6. Carefully cover sections with TdT reaction buffer, incubate slides in a prepared humidified chamber for 30 min at 37 °C.

7. TdT reaction is stopped by incubating the slides in 2× SSC (2×, 10 min each), slides were rinsed in 1× PBS.
8. Block the nonspecific binding by incubating slides with 2% BSA solution for 60 min at room temperature or overnight at 4 °C.
9. Wash slides with 1× PBS (2×, 5 min each), cover the sections in TdT Staining buffer, and incubate at room temperature for 30 min in dark humidified chamber. Wash Slides again with 1× PBS (2×, 5 min each) (*See* **Note 21**).
10. Stain sections with hematoxylin, Hoechst 33342, or other appropriate counterstains. *See* **Note 22** *on the benefits of Hoechst counterstaining*.
11. Wash slides with 1× PBS and gently adhere coverslip using Vectashield antifade mounting medium. Air dry slides (room temperature) in dark humidified container for overnight.
12. Keep slides at 4 °C, until ready for use. Examine the brain tissue sections using fluorescent or confocal Microscope.

4 Notes

1. As mentioned before, all procedures with WNV-infected brain tissues should be carried out at an authorized and regulated BSL-3 or ABSL-3 laboratory and in Biosafety cabinets designated separately for cell culture or animal procedures.
2. It is required to determine the virus titers and also test different mice backgrounds and different WNV strains for neuroinvasion and neuropathogenesis.
3. *Mice/brain tissues*: Researchers can anesthetize mice with recommended amounts of Isoflurane before inoculation of WNV, although, it is preferred to inoculate mice without anesthesia for quick recovery after infection.
4. All virus dilutions are done in a BSL-3 cell culture laboratory and the diluted virus stocks are carried to the ABSL-3 facility in a safe double container. WNV-infected brain samples can be transferred to the BSL-2 laboratory in RLT buffer that contains high concentration of guanidine isothiocyanate, which completely inactivates the virus.
5. For complete homogenization of brain lysates, it is strongly recommended to lyse brains by both Pestle-grinder and needle-syringe passages. Extreme care should be taken during the needle-syringe passages. After use, throw the needles in sharps containers without capping.
6. *Viral plaque assays*: Vero cells are recommended. But upon unavailability baby hamster kidney (BHK) cells can be used.

Do not overgrow host cells above 85 % confluency, as cells overgrown tend to dislodge monolayers easily. Also, it is recommended not to swirl the cells as this would lead to lower cell numbers in the plate center. For use on same day it is suggested to plate $2–3 \times 10^6$ cells per well, to allow the formation of a complete monolayer.

7. Fungizone or antibiotic/antimycotic solutions can be added if required, as plaque assay plates usually gets fungus growth within 5–6 days of incubation.
8. Keep the culture media and agarose at 37 °C waterbath and check the appropriate temperature with a thermometer, slight increase in temperature would dislodge the Vero host cell monolayer. Culture media is also prewarmed to 37 °C, in order to keep agarose in molten state and not to solidify.
9. It is not advisable to keep plaque assay plates for more than 4 h in staining with Neutral Red, as living cells would take up more dye and give hard time to distinguish the lighter areas. Also, using more Neutral Red would result in clear identification of plaques. If desired, plaque assay plates can be kept for 5–8 days (if fungizone is added to avoid fungus growth).
10. *H&E staining*: Use the "Progressive" Hematoxylin stain that gives a desired intensity of staining for frozen sections when dipped in the stain solution. Try to avoid over staining with Eosin. Also avoid excessive water carry over by timely blotting the sections.
11. If tissues are kept in reusable cassettes, make sure that there are no carried-over tissues to avoid "floaters" problems that may occur during and after staining of sections. It is very important to identify any remaining tissue and wash the cassettes thoroughly with running tap water.
12. *Fixation*: Immediately after removing the brain tissue, keep in histological cassettes and completely immerse in the fixative solution to preserve the tissue. The volume of fixative should be 10:1 ratio of fixative to tissue. Although, 10 % neutral buffered formalin solution is relatively slow, but it penetrates the tissue well and prevents acidity that would promote formation of formalin-heme pigments that appear as black-polarized structures/deposits in tissues and upon tissue damage and autolysis. Also, fixation is carried out best at neutral pH, in the range of 6–8. Commercial formalin solutions are preferred as their are buffered at a pH of 7 with phosphate. The 4 % paraformaldehyde is good for immunoperoxidase staining. To avoid the exhaustion of the fixative, it can be changed at intervals. Avoid too high concentrations of fixatives and drying of tissues (always keep tissues moist with PBS) that may give adverse effects and artifacts (cellular organelle loss, nuclear shrinkage, and clumping).

13. *Sectioning*: Using a sharp knife microtome, cut thin microscopic sections (2–3 mm), to allow best penetration of fixative solution into thin sections. Paraffin is highly recommended for tissue embedding in comparison to other alternatives, such as less expensive plastics (that may allow much thinner sections). But, plastic requires special reagents for dehydration and clearing and a special microtome with a glass or diamond knife. Tissues embedded in paraffin will result in 8–10 thin sections. Before embedding or infiltration in to paraffin, tissues are dehydrated in a series of alcohols. After dehydration, use less expensive Xylene as the best clearing agent to remove the dehydrants. Depending on the climate and season, paraffin can be obtained with different melting points.
14. Sectioning needs time, patience, and real skills that comes over with practice. When sections are done carelessly, several artifacts such as folding, ripping, holes, and blinds are generated. To help remove the folds and wrinkles, sections are floated in warm waterbaths and picked on microscopic glass slides. Slides are incubated in warm oven for 15 min to allow adherence of the sections. If heat can harm the antigens for immunostaining, use glue-coated slides to pick up the sections and skip the oven incubation step.
15. Before staining, *deparaffinize* or dehydrate the sections by running through the recommended solution of Xylene, alcohols, and water. If paraffin is not completely removed, staining will not work on tissues containing paraffin.
16. *Antigen retrieval*: During tissue fixation, methylene bridges are formed, that may cross-link the proteins and hide/mask the antigenic sites. To expose the antigen binding sites, it is highly recommended to perform antigen retrieval procedures.
17. Optimize the new antibodies for *antigen retrieval*, concentration/dilution, and detection, as each new antibody will have its preferred method. Detailed protocol for heat-induced antigen retrieval using vegetable steamer is provided in this chapter, however, there are other methods (described elsewhere) to perform antigen retrieval using a pressure cooker or microwave or waterbath set at 60 °C to incubate slides for overnight in antigen retrieval solution. If the antibody datasheet does not describe the method for antigen retrieval, it is advised to test for antigen retrieval using different methods. Also, the enzymatic methods using horseradish peroxidase (HRP) or alkaline phosphatase (AP) and chromogens for immunoperoxidase staining are described elsewhere.
18. For heat-induced antigen retrieval using vegetable steamer, 20 min of retrieval time is suggested. However, it is recommended to perform a test experiment with 5, 10, 15, 20, 25,

and 30 min to find out the correct retrieval time for any particular or new antibody. Less than 20 min may result in under-retrieval of antigens and weak immunostaining and over 20 min may cause over-retrieval, nonspecific background staining and damage of the tissue sections and disassociation from the slides. Also, it is highly recommended to cool the slides after 20 min of incubation, for easy handling and allowing the antigenic sites to re-form after being heated at high temperatures.

19. *Optimization of antibodies*: Use appropriate antigen retrieval method and correct optimized amounts of the primary and secondary antibodies for detection. Dilutions of the antibodies are sometimes provided on the datasheets (by the manufacturer), otherwise determined by the end-user by testing a range. Most of the primary antibodies work great between 0.5 and 10 μg/ml concentrations. It is highly recommended to check that the primary antibody is not raised in a mouse species similar to the tissue sections being stained, otherwise anti-mouse IgG secondary antibody would bind to all the endogenous IgG in the mouse tissue, leading to high nonspecific staining. In case of lower antibody titer or targets with less affinity, longer incubation times with the primary antibody is recommended to allow sufficient time for binding. Using Isotype control antibodies directed to an irrelevant target or antigen or negative or positive control antibodies are highly recommended to ensure the immunostaining of the experimental antibodies. Also, a control tissue with no expression of target protein can be used as a negative tissue control.

20. *TUNEL assay*: Optimization of formaldehyde fixation is required, it can be resolved with shorter fixations or reducing the concentration of formaldehyde.

21. False positive staining in the negative controls may arise due to nonspecific binding of the FITC-conjugated reagent used for fluorescence detection. Reduce the fluorophores and incubation times to avoid the background fluorescence. Optimize the staining conditions for controls prior to experimental set up. The control panels are highly recommended in TUNEL staining to help interpret the data. Positive controls can be treated with DNAse I (1 μg/ml in 30 mM Tris–HCL (pH 7.2), 140 mM Potassium cacodylate, 4 mM $MgCl_2$, 0.1 mM DTT) for 10 min at room temperature. Wash samples for 3× (2 min each) with double distilled water and then proceed with TUNEL. Omitting TdT enzyme from TdT reaction can serve as negative control. If lower volumes of TdT buffers are preferred, carefully cover the buffers using a glass coverslip. Care should be taken to avoid trapping air bubbles that may lead to staining and fluorescence artifacts.

22. Hoechst 33342 staining enables comparison of TUNEL positive nuclei and changes in nuclear size and morphology. Also, it can serve as counter stain for the visualization of gross anatomical changes in TUNEL positive and negative tissues.

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Chapter 9

In Vitro and In Vivo Blood–Brain Barrier Models to Study West Nile Virus Pathogenesis

Mukesh Kumar and Vivek R. Nerurkar

Abstract

The blood–brain barrier (BBB), a specialized interface between the peripheral blood circulation and the central nervous system, specifically regulates molecular and cellular flux between the two. It plays a critical role in the maintenance of brain hemostasis. The BBB restricts the entry of pathogens into the brain, and thus its permeability is a critical factor that determines their central effects. Once the permeability of BBB is compromised, it has serious implications in the etiology of many brain pathologies including West Nile virus (WNV) disease. In this chapter, we describe protocols for preparation, maintenance, infection and permeability measurement of monolayer and bilayer in vitro BBB models to study WNV pathogenesis. We also describe Evans blue dye assay, a well-established method to test vascular permeability in vivo after WNV infection.

Key words West Nile virus encephalitis, Blood–brain barrier, Transmigration

1 Introduction

West Nile virus (WNV), a mosquito-borne flavivirus that causes lethal encephalitis has emerged as a leading cause of arboviral encephalitis in the United States. Since its introduction to North America in 1999, outbreaks of West Nile fever (WNF) and West Nile virus associated encephalitis (WNVE) have occurred in all 48 contiguous states [1]. The fatality rate is approximately 10% for hospitalized encephalitic cases with increased risk in patients with compromised immune systems, older age and having underlying conditions such as diabetes mellitus [2]. Recent outbreaks of highly virulent WNV strains have also been reported in the Mediterranean basin, southern Europe and Russia [3]. Although the worldwide incidence of WNV infection is increasing, there is no specific treatment or vaccine available for use in humans.

Following peripheral infection, WNV replication is first thought to occur in skin Langerhans dendritic cells. These cells migrate to draining lymph nodes, resulting in primary viremia. By

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the end of the first week, the virus is largely cleared from the peripheral organs, but in a subset of patients, WNV enters the brain and causes a spectrum of neurological disorders. WNVE is characterized by disruption of the blood–brain barrier (BBB), enhanced infiltration of immune cells into the central nervous system (CNS), microglia activation, inflammation and eventual loss of neurons [2, 4]. Since high viremia is directly correlated with early WNV entry into the CNS [2], it is suggested that WNV in the periphery enters the CNS by crossing the BBB. Increased production of pro-inflammatory mediators facilitate WNV neuroinvasion by compromising the BBB integrity and are associated with high virus titers in the brain and increased mortality in WNV mouse models [5–7]. The BBB, which consists of microvascular endothelial cells, perivascular astrocytes, basement membrane, and pericytes, is a highly regulated interface which separates blood-borne entities from the CNS [8]. The tight junction proteins (TJPs) such as zona occludens, claudins and occludin, the main structural basis of BBB integrity, play a key role in the physiology of the BBB. However, our understanding of the cellular mechanisms associated with WNV-induced BBB disruption, specifically the contribution of BBB-associated cells, is limited. Using an in vitro BBB model comprised of primary human brain microvascular endothelial (HBMVE) cells, we have demonstrated that cell-free WNV can cross the BBB, without compromising the BBB integrity [9]. Further, we have demonstrated that inflammatory mediators secreted from WNV-infected astrocytes degrade key TJPs of HBMVE cells and compromise the integrity of the BBB model thereby contributing to WNV-associated neuropathogenesis [10].

2 Materials

2.1 Cell Culture

Primary human brain microvascular endothelial (HBMVE) cells

1. HBMVE cells are cerebral microvascular endothelium cells from normal human brain cortex tissue (ACBRI 376).
2. Culture medium: For 1 L, add 965 mL Endothelial Cell Media (ECM; Science Cell Research Laboratories, Cat. 1001), 25 mL fetal bovine serum (FBS) (Science Cell Research Laboratories, Cat. 0025), 5 mL 100× Penicillin/Streptomycin (P/S) solution (Science Cell Research Laboratories, Cat. 0503), 5 mL 100× Endothelial Cell Growth Supplement (ECGS; Science Cell Research Laboratories, Cat. 1052).
3. Cell passage reagents: CSC certified Passage Reagent Group [(PRG; Cell Systems Corporation, Cat. 4Z0-800): PRG-1 (EDTA solution), PRG-2 (Trypsin/EDTA solution), PRG-3 (Trypsin Inhibitor solution)], 1× CSC certified attachment factor (Cell Systems Corporation, Cat. 4Z0-210).

4. Culture conditions: HBMVE cells are cultured in a 37 °C and 5% CO_2 incubator. These cells require attachment factor to attach to the bottom of the culture flask (*see* **Note 1**).

Primary human brain cortical astrocyte (HBCA) cells

1. HBCA cells are astrocyte cells from normal human brain cortex tissue (ACBRI 376).
2. Culture Medium: For 1 L, add 980 mL Astrocytic Cell Media (ACM; Science Cell Research Laboratories, Cat. 1801), 10 mL FBS, 5 mL 100× P/S solution, 5 mL 100× Astrocytic Cell Growth Supplement (ACGS; Science Cell Research Laboratories, Cat. 1852).
3. Cell passage reagents: Same as employed for aforementioned HBMVE cells.
4. Culture conditions: HBCA cells are cultured in a 37 °C and 5% CO_2 incubator. These cells require attachment factor to attach to the bottom of the culture flask (*see* **Note 1**).

2.2 In Vitro BBB Model

1. Inserts: BioCoat® Cell Environment™ Human Fibronectin PET (polyethylene terephthalate) inserts with 3.0 μm pore size (BD Bioscience, Cat. 354543).
2. Instruments: EVOM meter and End Ohm (World Precision Instruments Inc.), Victor™ 1420 fluorescence microplate reader (Perkin Elmer), humidified incubator.
3. Reagents: 1× phosphate buffered saline (PBS), fluorescein isothiocyante (FITC)-dextran (4 kDa molecular weight; Sigma), 24-well plates, 96-well plates, forceps.

2.3 In Vivo BBB Model

1. Animals: 8–12 weeks old C57BL/6 mice.
2. Evans blue dye (*see* **Note 2**).
3. Reagents: 1× PBS, formamide, isoflurane anesthesia, 70% ethanol, 29-G 3/8″ needle, syringes (1 mL, 10 mL), needles (26 G), 1.5 mL tubes, dissection board, surgical scissors, forceps.

3 Methods

WNV is a bio-safety level 3 (BSL-3) agent. All procedures involving WNV infection should be restricted to a bio-safety cabinet in an authorized A/BSL-3 laboratory.

3.1 In Vitro Monolayer and Bilayer BBB Model

Cell culture (3–7 days prior to BBB assembly) and maintenance

1. Coat the tissue culture flasks with 1× CSC certified attachment factor (2 mL for T-75 flasks) before adding any media or plating HBMVE and HBCA cells (*see* **Note 1**).

2. Maintain low passage (2–8) HBMVE and HBCA cells in ECM and ACM complete media (described in Subheading 2), respectively, at 37 °C, 5 % CO2 and 100 % humidity.
3. Split confluent HBMVE and HBCA cells at a ratio of 1:3–1:6. Wash the cells with 2 mL of PRG-1. Add 2 mL of PRG-2 and incubate for 2 min. Add 2 mL of PRG-3 to neutralize the trypsin.
4. Collect HBMVE and HBCA cells by centrifugation and count with 1:2 dilution.
5. Resuspend the cell pellet in ECM and ACM complete media to get 1×10^5 HBMVE cells/mL and 1×10^6 HBCA cells/mL, respectively.

Monolayer HBMVE BBB model

1. Place tissue culture inserts in the wells of a 24-well plate (Fig. 1) and hydrate in ECM at 37 °C, 5 % CO_2 and 100 % humidity for 1 h.
2. Wash the inserts once with 1× PBS to remove the residual media.
3. Add 100 μL 1× CSC certified attachment factor in each insert (*see* **Note 1**).
4. To bottom of the well add 500 μL of ECM complete media.
5. To top of inserts (luminal membrane surface) add 500 μL of ECM complete media with cells (5×10^4 HBMVE cells). In 1–2 inserts, add only ECM complete media to be used as controls (*see* **Note 3**).
6. Incubate the inserts at 37 °C, 5 % CO_2 and 100 % humidity.
7. Change fresh media every 2–3 days.

Bilayer HBMVE-HBCA BBB model

1. Place tissue culture inserts in the wells of a 24-well plate and hydrate in ECM or ACM at 37 °C, 5 % CO_2 and 100 % humidity for 1 h.
2. Wash the inserts once with 1× PBS to remove the residual media.
3. Add 100 μL 1× CSC certified attachment factor to each insert from the abluminal and luminal sides (*see* **Note 1**).
4. Invert the inserts and to bottom of inserts (abluminal membrane surface) add 100 μL of ACM complete media with cells (1×10^5 HBCA cells). In 1–2 inserts, add only ACM complete media to be used as controls.
5. Incubate the inserts at 37 °C, 5 % CO_2 and 100 % humidity for 4 h. Rehydrate the inserts with ACM complete media periodically to keep the cells wet.

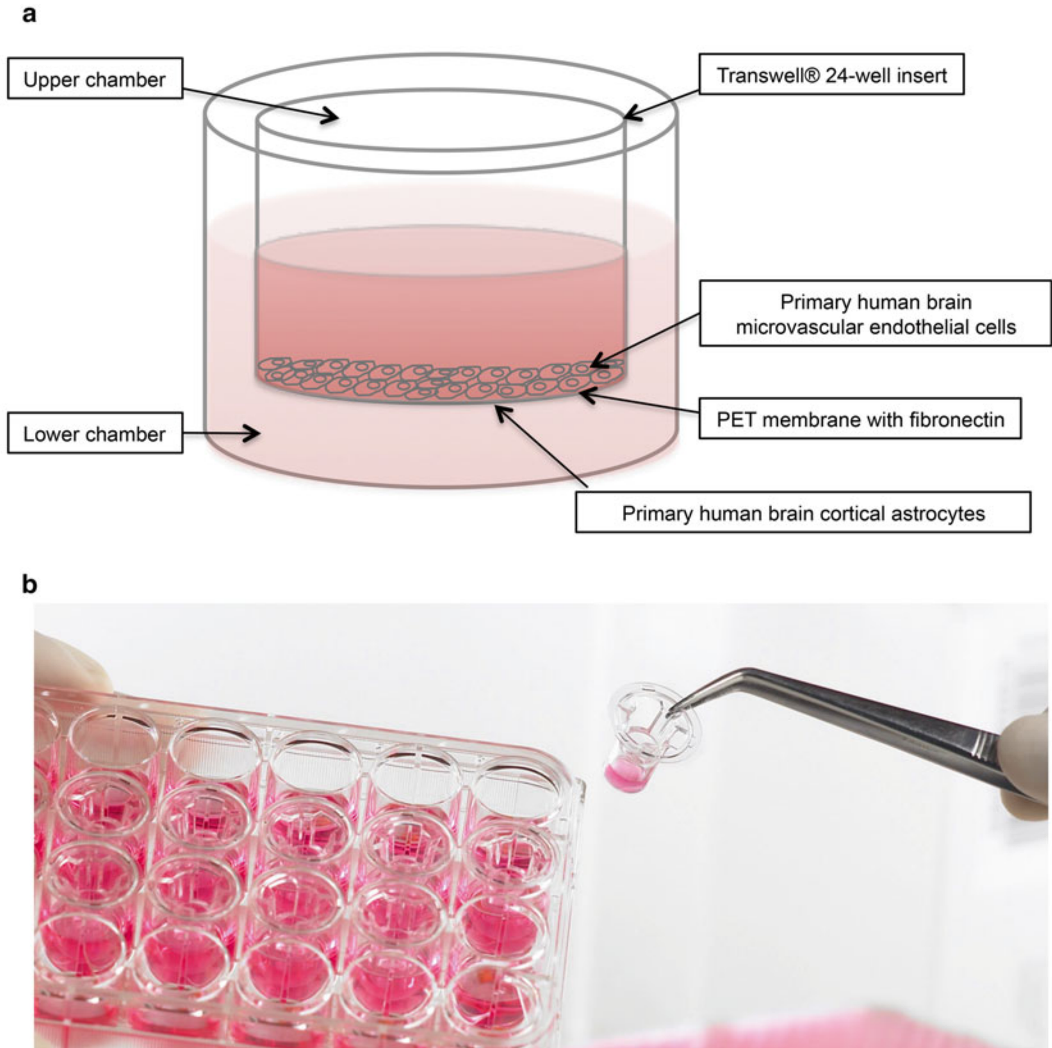

Fig. 1 In vitro BBB model. (**a**) A BBB insert and (**b**) demonstration of how the inserts are situated in the plate containing wells

6. To bottom of the well add 500 μL of ECM complete media.
7. After 4 h incubation, transfer the inserts to a plate and to the top of inserts (luminal membrane surface) add 500 μL of ECM complete media with 5×10^4 HBMVE cells. In controls, add only ECM complete media.
8. Incubate the inserts at 37 °C, 5 % CO_2 and 100 % humidity.
9. Change fresh media every 2 days.

3.2 Integrity of the BBB Model

It generally takes between 5 and 6 days to form a tight BBB model (*see* **Note 4**). Check tightness by measuring transendothelial electric resistance (TEER) and FITC-dextran transmigration starting day 3 after putting cells onto inserts [9–12].

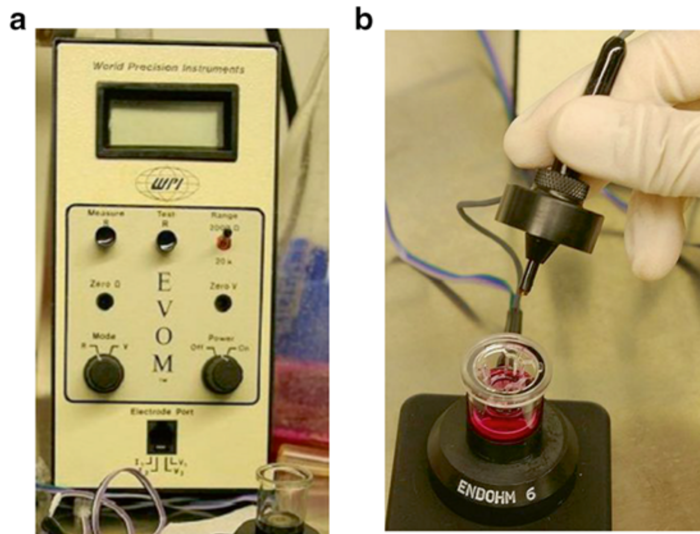

Fig. 2 EVOM meter and End Ohm for the measurement of TEER. (**a**) EVOM meter. (**b**) End Ohm TEER measurement chamber with BBB insert

TEER measurement

1. Remove the old ECM complete media from the upper chamber.
2. Add 500 μL of fresh warmed ECM complete media to upper chamber of cell insert.
3. Add 1.5 mL fresh warmed media to End Ohm connected to the EVOM meter (TEER measuring instrument) (Fig. 2). For monolayer BBB, add ECM media. For bilayer BBB, add ECM or ACM media.
4. Transfer insert (with media) carefully into End Ohm using forceps.
5. Turn machine on after putting measuring tip of End Ohm into the insert and toggle the switch to measure resistance (*see* **Notes 5** and **6**). Make sure machine reads 0 before toggling measuring switch.
6. After taking reading, transfer insert (with media) back to the well in the 24-well plate.

FITC-dextran transmigration assay (All steps must be done in the dark)

1. Add 750 μL ECM to bottom chamber of fresh 24-well plate.
2. Decant media from upper chamber of cell inserts.
3. Transfer cell inserts into new 24-well plate with 750 μL ECM in corresponding wells.

4. Add 150 μL of FITC-dextran (with ECM at 100 μg/mL concentration) to upper chamber of the insert.
5. Incubate plate for 2 h in dark at 37 °C.
6. Remove 150 μL samples from lower chamber of each cell insert.
7. Place samples into 96-well plate (add samples in the second row).
8. Make standards in serial dilution from the stock of FITC-dextran (100–0.098 μg/mL) in a 96-well plate.
9. Read 96-well plate at 485 nm excitation and 535 nm emission wave lengths using the plate reader (Victor™ 1420 fluorescence microplate reader, Perkin-Elmer Wallance Inc).
10. The FITC-dextran transmigration across the inserts is calculated as percentage of the total amount added in the upper well.

3.3 Infection of In Vitro BBB Models

1. Decant media from upper chamber of cell inserts.
2. Make WNV working dilution in serum-free ECM from the stock. Bring the titer to 50,000 plaque-forming units per 100 μL of serum-free ECM to infect the inserts with multiplicity of infection 1.
3. Add this 100 μL of serum-free ECM containing WNV to each insert.
4. Add 100 μL of serum-free ECM without virus for the controls.
5. After adsorption for 1 h at 37 °C, aspirate the media and wash the inserts two times with 1× PBS and then add 500 μL of fresh ECM.
6. Incubate the inserts at 37 °C, 5 % CO_2 and 100 % humidity.
7. At 6, 12, 24, 48, and 72 h after incubation, remove the media from the upper chamber of the inserts and wash the inserts twice with 1× PBS and assay the BBB integrity by TEER and FITC-dextran transmigration assay as described above.

3.4 In Vivo BBB Model

West Nile virus inoculation

1. Dilute the WNV NY99 stock to 100 (LD_{50} dose) or 1000 (LD_{100} dose) plaque-forming units (PFU) per 20 μL in 1× PBS and keep it on ice (*see* **Note 7**). Appropriately diluted WNV should be drawn into the syringe and kept ready for inoculation.
2. Anesthetize mice in an induction chamber using 2–5 % Isoflurane at a rate of 1–2 L/min of O_2. After 30–40 s, observe the mice to ensure complete anesthesia. Carefully monitor the

following symptoms during this time: (a) mouse is still but breathing and (b) ears are still pink.

3. Once anesthetized, place the mouse on a warming table and hold the hind limb with a flat end forceps.
4. Use the forceps to extend the left foot and wipe the foot with 70% alcohol to remove debris before injection.
5. Inject 20 μL of WNV subcutaneously into the center of hind foot forming a small bleb at the injection site (*see* **Note 8**).
6. Wipe the foot with paper towel wet with 70% ethanol.
7. Discard the needle and syringe in a puncture resistant sharps container.
8. In control mice, inject 20 μL of PBS by the same route.
9. Return the mice to their cage.
10. Make sure that the mice are awake before leaving the room.

Intraperitonial injection of Evans blue dye (at specific days after inoculation)

1. Prepare a 1% sterile solution of Evans blue dye in PBS. If necessary, filter-sterilize the solution to remove any particulate matter that has not dissolved.
2. Aspirate 1 mL of 1% Evans blue dye solution into a syringe. Avoid all air bubbles that might have escaped into the syringe.
3. Scruff the mouse with the right hind limb immobilized and the head and body tilted downward. Hold onto the tail with the nondominant hand between the thumb and the forefinger.
4. Disinfect the right lower abdominal wall with gauze dampened with alcohol.
5. Insert the needle (small gauge, 26) at a 10–15° angle into the peritoneal cavity in the caudal right abdominal quadrant through the skin and the abdominal wall, thereby avoiding injection into the cecum or the stomach on the left side.
6. Lift the needle tip slightly and slowly inject 1 mL of 1% Evans blue dye solution.
7. Put the mouse back into its cage and observe it for 1 h.

Organ collection and extraction of Evans blue dye from the brain

1. Anesthetize the mice using Isoflurane. Keep the mice anesthetized during the whole procedure.
2. Place the mice on their backs and pin their feet on a dissection board.
3. Spray external area of abdominal and thoracic cavity with 70% ethanol. Open the abdominal and thoracic cavity using surgical scissors to expose thoracic and abdominal organs.

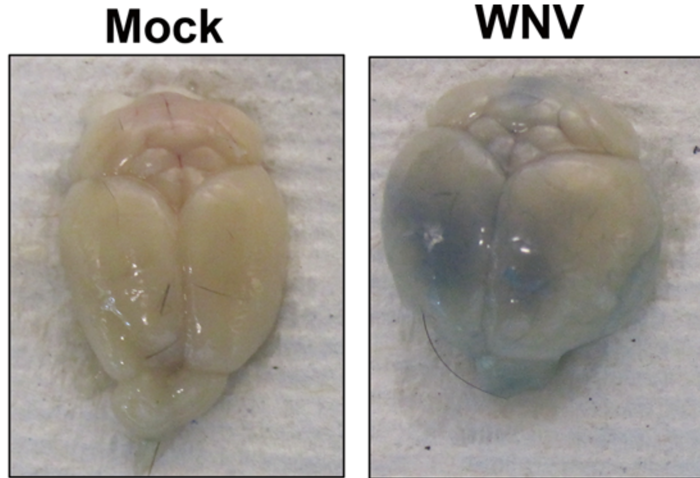

Fig. 3 In vivo BBB model. Mice were inoculated with PBS or 100 PFU of WNV via footpad and BBB permeability was measured using Evans blue dye at day 6 after infection. Mice were injected i.p. with 1 mL Evans blue dye (1 % w/v) and after 1 h were cardiac perfused with PBS. The extravasation of the dye was evident in the WNV-infected mice brain

4. Flush the heart slowly with 10–15 mL of 1× PBS using small gauge (26 G) needle (*see* **Note 9**).
5. After perfusion, harvest brains using surgical scissors and forceps.
6. Take representative pictures to show differences in Evans blue dye extravasation (Fig. 3) (*see* **Note 10**) [13]. Include all brains in the same field in order to have identical lighting conditions.
7. Collect brains in 1.5 mL tubes.
8. Weigh an empty tube and bring the balance value to zero.
9. Transfer the brain and weigh it. Repeat for all brain samples.
10. Add 500 μL formamide to each tube.
11. Transfer all tubes to a 55 °C water bath. Incubate for 24–48 h to extract Evans blue dye from the brain.
12. Centrifuge the formamide/Evans blue dye mixture to pellet any remaining tissue fragments.
13. Measure absorbance at 610 nm. Use 500 μL-formamide as blank.
14. Calculate nanograms of Evans blue dye extravasated per mg brain tissue (*see* **Note 11**).

4 Notes

1. HBCA and HBMVE cells require attachment factor to attach to the bottom of the culture flask and inserts. Coat the tissue culture flasks and inserts with 1× CSC certified attachment factor (2 mL for T-75 flasks, 100 μL for inserts) at room temperature. Wait at least 10 min before adding media or plating HBMVE and HBCA cells.
2. Evans blue dye is a dye that binds albumin. Under physiologic conditions the BBB endothelium is impermeable to albumin. Therefore, Evans blue dye-bound albumin remains restricted within the blood vessels. In WNV infection, there is increased vascular permeability and the BBB becomes permeable to small proteins such as albumin. This condition allows for extravasation of Evans blue dye in the brain tissue.
3. Always have blank inserts with no cells as controls.
4. It generally takes between 5 and 6 days to form a tight BBB model. Start checking tightness via permeability and TEER methods on day 3 after plating cells onto the insert.
5. TEER is expressed as Ω/cm^2. TEER of 450 Ω/cm^2 is considered good/tight for BBB.
6. High TEER is equivalent to higher resistance (tighter BBB).
7. Dose is based upon virus titration studies using WNV NY99 strain.
8. Use 29-G 3/8″ needle for inoculation of WNV via footpad route.
9. Perfusion of the heart with 10–15 mL of PBS is done slowly over 10–12 min.
10. In case of increased BBB permeability, brains will show significantly increased blue coloration compared to brains isolated from the mice with intact endothelium.
11. By using a standard curve for Evans blue dye, optical density measurements can be converted into milligram dye captured per milligram of tissue.

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Chapter 10

Methodology for Identifying Host Factors Involved in West Nile Virus Infection

Manoj N. Krishnan

Abstract

The West Nile virus (WNV) infection is a major medical problem for humans and some domesticated animals. WNV infection of host cells involves the interplay of the virus with several host factors. Identification of the host factors impacting on WNV infection can enhance our understanding of virus infection mechanisms, host immune defense mechanisms, and also reveal novel host targets that can be developed as antivirals. RNA interference (RNAi) is a highly efficient genetic tool to discover host genes involved in WNV infection at a genome scale. Here, we describe a protocol for conducting human genome wide RNAi screen to discover novel host factors associated with WNV infection of human cells.

Key words West Nile virus, WNV, RNA interference, RNAi, siRNA, Transfection, High-content microscopy

1 Introduction

WNV is a single plus-stranded RNA genome containing flavivirus [1]. Infection by WNV has been a recurrent human health problem in different parts of the world [2–4]. Despite the health threat posed by WNV, no effective therapeutics or vaccine is available against human infections caused by WNV [5].

Understanding the molecular mechanisms defining WNV–host cell interactions is essential for discovering novel therapeutic targets and approaches against this virus. WNV genome encodes for three structural and seven nonstructural proteins. Given their obligate intracellular life cycle with a minimal genome, it is apparent that the WNV will have to exploit a diverse compendium of host cellular molecules for completing their replication. Such host factors that support WNV infection is termed host susceptibility factors (HSFs). Contrary to this, the host cells would have evolved molecules to resist WNV infection (termed host restriction factors, HRFs). Therefore, to understand the molecular aspects of virus–host cell interactions, it is essential to discover the host factors

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involved in both supporting and restricting WNV infection. The dependence of viruses on host factors during infection also catalyzed the formation of the novel concept of host-targeted antiviral discovery, in which it is envisaged that essential infection supporting host molecules can form therapeutic targets against viruses [6].

A variety of approaches were used previously to identify the host molecules associated with WNV infection. Some of these approaches included gene expression profiling using transcript measurements, and protein–protein interaction screens using WNV proteins as baits [7–9]. Recently, a powerful forward genetics approach, RNA interference, has been increasingly exploited to dissect virus–host interactions [10, 11]. The RNAi provides unprecedented ability to discover host genes involved in supporting and restricting viral infections. Two previous genome wide RNA interference screens performed respectively in drosophila and human cells identified several host genes impacting on WNV infection [12, 13].

Here we provide a detailed account of the methodology for conducting large-scale high-throughput format RNAi screening to discover novel host genes regulating WNV infection of human cells. A schematic of the RNAi screening is given in Fig. 1.

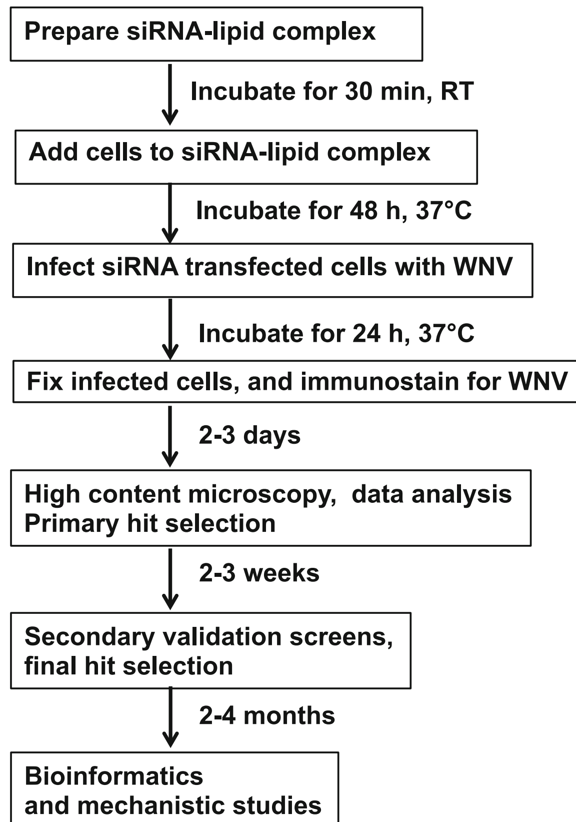

Fig. 1 Schematic of RNAi screening to identify human host factors involved in WNV infection. *h* hours, *RT* room temperature, *siRNA* small interfering RNA

2 Materials

2.1 Cell Culture

1. HeLa cells (ATCC catalogue number CCL-2).
2. Dulbeccos Modified Eagle Medium (DMEM, high glucose), containing 20% fetal bovine serum (heat inactivated) as well as serum free.
3. 0.25% trypsin-EDTA.
4. Cell culture incubator, 5% CO_2, 37 °C.
5. Pipette, cell culture type, 5, 10, and 25 ml.
6. Laboratory Pipettes, various sizes.

2.2 siRNA and Transfection Reagents

1. Positive control siRNA duplexes that blocks WNV infection, targeting human vATPase subunits ATP6V0A1 (gene ID 535) and ATP6V1A (gene ID 523). Dharmacon catalogue numbers for the siRNAs targeting ATP6V0A1 and ATP6V1A respectively are M-017618-00 and M-017590-00. Prepare a 1 μM stock of the siRNA in the resuspension buffer, and store at −20 °C.
2. Negative control siRNA that does not affect WNV infection (e.g., Dharmacon catalogue number D-001206-13-05). Prepare a 1 μM stock of the siRNA in siRNA resuspension buffer and store at −20 °C.
3. siRNA libraries. Human genome wide siRNA library containing a pool of four unique siRNAs targeting each human gene (Dharmacon catalogue number G-005000-05), arrayed at 1 μM concentration in siRNA resuspension buffer. The siRNA library can be stored at −20 °C. The human genome wide siRNA library is plated into 384 well master plates in all wells except column 1, 2, 23, and 24. The columns 1, 2, 23, and 24 contains only siRNA resuspension buffer.
4. Transfection reagent Dharmafect 1 (Dharmacon catalogue number T-2001-01).
5. siRNA resuspension buffer (Dharmacon catalogue number B-002000-UB-100).
6. Multichannel (12-channel) digital pipettes (Eppendorf ResearchPro 300 μl, and 100 μl) or automated liquid handler with 384-well head and sterile disposable tips (i-Pipette Pro, Apricot Designs).
7. Multichannel reagent reservoir compatible for 12-channel (ThermoFisher catalogue number 15075) or 384-channel (Thermo Scientific catalogue number 1064-05-7).
8. Imaging quality black wall clear bottom sterile cell culture treated 384 well plates (Corning catalogue number 3712).
9. Adhesive seal (e.g., VWR catalogue number 60941-124) for covering 384-well plates.
10. 50 ml conical polypropylene tubes.

2.3 Viral Infection

1. West Nile virus stock (10^6 pfu/ml).
2. Biosafety Level-3 laboratory.
3. 10% Bleach.

2.4 Immunostaining

1. Paraformaldehyde (4%) solution in phosphate buffered saline.
2. Anti-West Nile Virus/Kunjin Antibody detecting Envelope protein (Millipore catalogue number MAB8151).
3. Blocking buffer (phosphate buffered saline, 0.05% Tween-20).
4. Permeabilization buffer (phosphate buffered saline, 0.1% Triton X-100, 0.05% Tween-20).
5. 3 μg/ml DAPI (4′,6-diamidino-2-phenylindole, sigma catalogue number D-1388) in PBS.
6. Alexa Fluor 488 conjugated anti-mouse secondary antibody (1:2000-fold dilution of a 2 mg/ml antibody stock).

2.5 High-Content Fluorescence Microscopy

1. ImageXpress Micro high-content microscope (Molecular Devices Corporation).

2.6 Data Generation and Analysis

1. MetaXpress software.
2. Microsoft excel.

3 Methods

The methods described here explain how to perform RNAi screen in 384 well plate format to identify host factors important for WNV infection of human derived HeLa cells. This procedure employs the detection of WNV in cells through fluorescent dye Alexa Fluor 488 conjugated secondary antibody based immunostaining, and visualization by fluorescence microscopy. The siRNA transfection protocol described here involves simultaneous cell plating and siRNA transfection. In this procedure, each gene will be targeted by a pool of four unique siRNAs. The final concentration of each one of the individual siRNAs transfected into the cells is 12.25 nM (this equals to 50 nM for the combined four siRNAs in each pool). The methods given below describe how to perform the screen using both manual multichannel (12-channel) electronic pipette and automated 384-channel liquid handler. The steps below are described for performing RNAi screen in a single 384-well plate. Users can scale-up this protocol depending on the number of plates involved in specific screening experiments.

3.1 Preparation of siRNA Screen Plate

All procedures should be carried out within a biosafety cabinet.

1. Prepare a map for the 384-well plates intended for screening (termed Screen Plate), marking the location of control and test siRNA identities. Columns A3 to A22 contains siRNA library. Wells B2, C2, and D2 will receive negative control siRNA. Wells E2, F2, and G2 will receive positive control siRNA. All other wells are either left as additional control wells that would not receive siRNA, or could be used for plating additioal negative and positive control siRNAs.
2. Thaw the 1 μM siRNA master stocks at the room temperature for 30 min. Spin the plates for 2 min at 200 × *g*, to settle any liquid that may be present on the walls of the wells. Remove the plate seals.
3. Fill a reagent reservoir with 350 μl of siRNA resuspension buffer. Transfer 8 μl of siRNA resuspension buffer into all wells of the Screen Plate, using 12-channel electronic pipette. The liquid should be dropped onto the side of the well close to the bottom of the plate.
4. 2. From the 1 μM master stocks of the human genome wide siRNA library (arrayed in the 384-well plate), transfer 2 μl of siRNA into the Screen Plate, using either automated liquid handler or 12-channel electronic pipette. The siRNA should be transferred to the Screen Plate in the exact same order as in the master plate.
5. Remove 2 μl of the siRNA resuspension buffer from the wells B2-G2 (wells designated to receive negative and positive siRNA controls) using a 10 μl pipette. Then, add 2 μl of the negative controls siRNA stocks (stock of 1 μM) into the wells B2-D2, and positive control siRNAs into the wells E2-G2.
6. Gently tap the plate using hand, and let the plate sit at room temperature for 10 min, to disperse the siRNA.

3.2 Preparation of siRNA Transfection Complexes

(*Note*: go to Subheading 3.2 only after completing Subheading 3.1.)

The amount of Dharmafect1 transfection reagent needed for transfection per well is 0.05 μl. All steps should be carried out in a laminar flow cell culture hood (*See* **Notes 1–9**).

When using a manual multichannel (12-channel) electronic pipette:

1. Add 24.2 μl of Dharmafect1 into 4.84 ml of serum-free DMEM in a 50 ml conical tube, vortex at medium speed for 30–60 s. (In order to ensure efficient pipetting, an additional volume of transfection mix needed for 100 wells in excess is required. Therefore, while preparing transfection reagent for a single 384-well plate, the actual generated reagents will be for 484 wells).

2. Transfer the DMEM containing Dharmafect1 to a fresh sterile reagent reservoir using 10 ml pipette.
3. Aspirate 300 μl of the HeLa cell containing DMEM using a 300 μl manual multichannel (12-channel) electronic pipette, and drop 10 μl into each well of the Screen Plate, 15 times. Continue this process until all wells are supplied with the transfection complex.
4. Incubate the Screen Plate for 30-40 min at the room temperature for siRNA-lipid transfection complex formation.

When using automated 384-channel liquid handler Apricot I-pipettor with 125 μl capacity tips:

1. Add 57.6 μl of Dharmafect1 into 11.52 ml of serum-free DMEM in a 50 ml conical tube, vortex at medium speed for 30 s. This step prepared sufficient reagent for transfecting into three 384-well plates, although we are actually transfecting into only one plate. The 384-well block size reagent reservoir used for automatic liquid handler requires around 10–12 ml liquid to fully cover its bottom. Therefore, in order to ensure efficient pipetting, at least 10–12 ml [enough for transfcetion into three 384-well plates] of transfection complex should be added to the reagent reservoir.
2. Transfer the DMEM containing Dharmafect1 to a reagent reservoir using 10 ml pipette.
3. Aspirate 15 μl of the DMEM containing Dharmafect1 using automated 384-channel liquid handler, and drop 10 μl into the Screen Plate. Discard the remaining 5 μl of the DMEM containing Dharmafect1 from the tips.
4. Incubate the Screen Plate for 30 min at the room temperature for siRNA-lipid transfection complex formation.

3.3 Preparation of HeLa Cells for Transfection

All steps should be carried out in a laminar flow cell culture hood.

This step should be started immediately after performing Subheading 3.2, and performed in such a way that the cells should be ready when the 30 min incubation period required for siRNA-lipid transfection complex formation is completed.

1. Remove two 10 mm culture dishes containing 70–80 % confluent HeLa cells from 37 °C incubator into biosafety cabinet, aspirate the medium, rinse cells gently with room temperature PBS twice, and add 1.5 ml of 0.25 % Trypsin-EDTA.
2. Incubate the trypsin treated cells in 37 °C incubator for 3–5 min.
3. Transfer the dish containing trypsin treated cells to the biosafety cabinet, add 7 ml of 10 % serum containing DMEM to the cells, and dislodge adherent cells by passing the medium gently

through the pipette multiple times. Spin the cells in a 50 ml tube for 5 min at 200×*g*. Resuspend the cell pellet in fresh DMEM containing 20% FBS, and count the cells using hemacytometer. Prepare a diluted stock of HeLa cells at a concentration of 100,000 cells/ml in DMEM containing 20% FBS.

3.4 Transfection of siRNA into HeLa Cells

When using a manual multichannel (12-channel) electronic pipette:

1. Transfer 9.68 ml of diluted HeLa cell (100,000 cells/ml in DMEM containing 20% FBS) stock into a fresh sterile reagent reservoir (ThermoFisher catalogue number 15075).
2. Aspirate 300 μl of the HeLa cell containing DMEM using a 300 μl manual multichannel (12-channel) electronic pipette, and drop 20 μl into each well of the Screen Plate, 15 times. Continue this process until all wells are supplied with the cells.
3. Incubate the Screen Plate for 48 h in a cell culture incubator, 5% CO_2, 37 °C.

When using automated 384-channel liquid handler Apricot I-pipettor with 125 μl capacity tips:

1. Transfer 12 ml of diluted HeLa cell (100,000 cells/ml in DMEM containing 20% FBS) stock into a fresh sterile reagent reservoir (Thermo Scientific catalogue number 1064-05-7).
2. Aspirate 25 μl of the HeLa cell containing DMEM using 125 μl capacity tips, and drop 20 μl into each well of the Screen Plate, six times. Continue this process until all wells are supplied with the cells.
3. Incubate the Screen Plate for 48 h in a cell culture incubator, 5% CO_2, 37 °C.

3.5 Infection of siRNA Transfected HeLa Cells in 384-Well Plates

The virus will be added into each well in a volume of 10 μl. Dilute the stock virus in serum-free DMEM in such a way that 10 μl of the diluent medium contains enough virus particles to give an MOI of 0.3 (*See* **Note 10**).

When using a manual multichannel (12-channel) electronic pipette:

1. Transfer diluted virus stock into a fresh sterile reagent reservoir (ThermoFisher catalogue number 15075).
2. Aspirate 300 μl of the virus stock using a 300 μl capacity manual multichannel (12-channel) electronic pipette, and drop 10 μl into each well of the Screen Plate, 15 times. Continue this process until all wells are supplied with the virus.
3. Incubate the Screen Plate for 24 h in a cell culture incubator, 5% CO2, 37 °C.

When using automated 384-channel liquid handler Apricot I-pipettor with 125 μl capacity tips:

1. Transfer diluted virus stock into a fresh sterile reagent reservoir (Thermo Scientific catalogue number 1064-05-7).
2. Aspirate 15 μl of the virus stock using 125 μl capacity tips, and drop 10 μl into each well of the Screen Plate, 15 times. Continue this process until all wells are supplied with the virus.
3. Incubate the Screen Plate for 24 h in a cell culture incubator, 5% CO_2, 37 °C.

3.6 Fixing and Processing of siRNA Transfected HeLa Cells in 384-Well Plates for IFA

1. Arrange 16% PFA solution, and 10% bleach in the culture hood. Transfer the plates into culture hood.
2. Using pipette, aspirate 30 μl from each well, and discard into 10% bleach tray. This will leave each well with 20 μl of culture medium.
3. Dispense 60 μl of 16% PFA into each well, using either manual electronic pipette or automated 384-channel liquid handler Apricot I-pipettor. Spray 16% PFA to the entire plate to inactivate any medium containing virus present outside the wells.
4. Incubate in the safety cabinet for 20–30 min at the room temperature.
5. Aspirate 80 μl of the liquid from each well.
6. Dispense 80 μl PBS to each well, and incubate for 5 min. Spray PBS to the entire plate to wash any PFA present outside the wells.
7. Aspirate 80 μl of the liquid from each well.
8. Dispense 80 μl PBS to each well, and incubate for 5 min.
9. Repeat **steps** 7 and **8** four times, and remove as much of the PBS from the well as possible.
10. Add 30 μl permeabilization buffer, and incubate for 30 min.
11. Remove permeabilization buffer by aspirating 30 μl of the liquid from each well, dispense 80 μl PBS to each well, and incubate for 5 min.
12. Repeat **steps** 7–**8** four times.
13. Add 30 μl block buffer, and incubate for 30 min.
14. Remove block buffer by aspirating 30 μl of the liquid from each well.
15. Add 20 μl primary antibody (1:500 diluted in block buffer), and incubate overnight at 4 °C. Keep the plate wrapped in aluminum foil or protected from light.
16. Remove primary antibody by aspirating 20 μl of the liquid from each well, dispense 80 μl PBS to each well, and incubate for 5 min. Repeat **steps** 7–**8** four times. Keep the plate wrapped in aluminum foil or protected from light.

17. Add 20 μl anti-mouse-Alexa488 secondary antibody (1 μg/ml in PBS), and incubate for 2 h at 37 °C. Keep the plate wrapped in aluminum foil or protected from light.
18. Remove secondary antibody by aspirating 20 μl of the liquid from each well, dispense 80 μl PBS to each well, and incubate for 5 min. Repeat **steps 7–8** four times. Keep the plate wrapped in aluminum foil or protected from light.
19. Add 20 μl DAPI solution (1:2000 diluted in block buffer), and incubate for 30 min at room temperature. Keep the plate wrapped in aluminum foil or protected from light.
20. Remove DAPI solution by aspirating 20 μl of the liquid from each well, dispense 80 μl PBS to each well, and incubate for 5 min. Repeat **steps 7–8** two times. Keep the plate wrapped in aluminum foil or protected from light.
21. Seal the plate with adhesive seal. Keep the plate wrapped in aluminum foil or protected from light.

3.7 High-Content Imaging of WNV Infected HeLa Cells in 384-Well Plates

This description is based on the high-content fluorescence microscope ImageXpress Micro by Molecular Devices. Users may use different makes of high-content fluorescence microscope (*See* **Notes 11–19**).

1. Turn the microscope on.
2. Load acquisition software.
3. Set imaging parameters for imaging at 4× magnification. Green channel for virus, and blue channel for cell number.
4. Open microscope loading chamber lid, load the 384-well plate with A1 position aligning with the marked position on the plate holder, and close the lid.
5. Adjust the focus by selecting A1, A12, and A24 wells.
6. Give the command for acquisition.
7. When acquisition is completed, remove the plate from the microscope, and turn off the microscope.

3.8 Infection Quantification

1. Following the instructions provided in the MetaXpress software user manual, select the "multiwavelength cell scoring" algorithm and set the analysis parameters using the newly acquired images of WNV infected cells.
2. The selected parameters should detect both DAPI stained nucleus, Alexa-488 stained WNV.
3. The quantified outcomes from the analysis by the software should generate information on "total number of cells, total number of WNV positive cells, and percentage of WNV positive cells" in a linked excel spreadsheet.

4. Run the software to quantify WNV infection.

3.9 Data Analysis Using Mean Based Z-Score Determination

After the completion of the automated image analysis, transform the percent infection data generated in the excel spreadsheet, as given below.

1. Determine the mean percent infection of the entire plate in the excel spreadsheet using its mathematical processing options.
2. Next, calculate the standard deviation of the mean percent infection of the entire plate in the excel spreadsheet using its mathematical processing options.
3. Determine the *Z*-score using the formula $z = x - \mu / \sigma$ (x denotes the percent infection in each well, σ denotes the standard deviation of the mean percent infection of the entire population, μ denotes the plate mean percent infection).
4. Sort the phenotypes obtained for each well based on the *Z*-score corresponding to the wells, from low to high.
5. Define a *Z*-score cut off value for hit selection.

4 Notes

The following points may help to further enhance the success of the RNAi screening assays.

1. It is desirable to exclude one or two outer layers of wells to reduce the chances of edge effect occurring.
2. Some types of cells that are less adherent or nonadherent can be made adherent using specially coated plates (e.g., CellBIND plates, Corning, Cat# 3683) which received treatments that enhances the ability of the dishes to anchor the cells. It was observed that plating siRNA into these plates first (e.g., for forming the complex with lipid) often leads to a reduction of knockdown efficiency by more than 70%. While the exact reason is unknown, one possibility is that the extra treatment applied to these dishes may prevent the siRNA complex from entering the cells optimally.
3. If the liquid or cells are stuck to the walls of 384-well plate while dispensing, centrifuge the plates at around $200 \times g$ for 20–40 s.
4. To avoid air bubble created in the wells of the 384-well plate while dispensing liquids, always pipette at least 3–5 μl more liquid than is actually required.
5. To resuspend siRNA, one can also use alternative buffers than the one described here, or even RNase-free water.
6. Use of manual multichannel pipette that are not electronically controlled can introduce significant amount of variability, and can essentially compromise the quality of the assay.

7. Use of desiccated chambers to thaw siRNA plates will reduce water condensation around the outer regions of the plates during thawing.
8. For transfecting siRNA into faster growing cells, reduced initial plating cell number or growing in less serum containing medium may help to slow down cell reaching confluence.
9. The amount and type of transfection reagent useful for various cell types can vary greatly. Therefore, optimization experiments using various doses of different transfection reagents are necessary.
10. Adding virus in a very small volume (e.g., 1 or 2 μl) of inoculum into the wells can occasionally lead to localized infection (on the cells only around the area where the virus was dropped) than a uniform distribution of infected cells across a well. Infection with 10–20 μl of inoculum often yields the best result.
11. During automated scoring, the threshold intensity of immunostaining selected for scoring positive and negative signals can greatly influence the nature of the results. For example, if a 24 h infection results in 20% and 10% of the cells having very strong and weak signals for WNV respectively, then excluding the weak signal giving cells from automated scoring may enable accurate identification of infection enhancing knockdowns. This is because, under infection enhancing conditions, the weak signal giving cells will mostly also become strong signal emitting cells and hence will be readily picked by the software.
12. There are many different of statistical approaches to analyze the RNAi screen raw data and select the potential hits. Some of these are explained in several previous reports [14].
13. Many times the fluorescent tag (e.g., alexa-488) may detach from the conjugated antibody and stay in the cells. This can seriously interfere with the automated image scoring because the software may interpret the signal from fallen dye as immunostained WNV. It is always desirable to pre-test the secondary antibody to ensure that it does not cause dye detachment-based background signal.
14. Imaging of the immunostained cells can be performed at different magnifications, depending on the type of information sought by the investigator. Imaging at 5× magnification is often enough to identify the presence or absence of infection. However, in general, imaging at 5× will not enable the investigator to clearly understand the differences in localization of fluorescent signals for the WNV within the cells. Imaging at higher magnifications (e.g., 10× or 20×) may provide better information on the subcellular localizations of WNV.

15. There are no standard rules on how many replicates of the primary screen should be conducted. The primary RNAi screen can be performed in multiple ways cost effectively, such as: (a) a single round of full genome screen, followed by selection of the hit genes, and re-testing of all hits only in triplicates; (b) perform whole genome RNAi screen in duplicates; or (c) perform whole genome RNAi screen in triplicates.
16. Validation of the primary RNAi screen hits can be performed in many ways. There are two major goals for the validation of primary RNAi screen results: (a) determine whether a siRNA that gave a phenotype in the primary screen is having on-target specificity. This can be achieved by the use of multiple independent siRNAs tested against the same gene, and complementation using siRNA-resistant version of the same gene, among other possible approaches; and (b) whether the observed phenotype is due to any assay bias. This can be validated by using alternate viral load measurement assays such as plaque assay, PCR based quantification of viral RNA and detection of intracellular viral protein levels.
17. From a typical genome wide RNAi screen for virus–host interactions, assuming that all tested siRNAs are efficiently knocking down their intended target genes, three kinds of phenotypes are possible: (a) a gene knockdown that enhances viral infection. Such genes will be direct host restriction factors or genes that regulate indirect cellular processes negatively impacting on viral infections; (b) a gene knockdown that reduces viral infection. Such genes will be direct host susceptibility factors or genes that regulate indirect cellular processes positively impacting on viral infections; and (c) a gene knockdown that does not alter viral infection. Such genes do not play any direct or indirect role in viral infection.

    However, in reality, the currently available commercial siRNA libraries are not validated for their knockdown efficiency and on-target specificity. Therefore, it is very likely that one might identify several false negative and false positive hits from each RNAi screen. While false positive hits will likely be identified and eliminated through subsequent validation secondary RNAi assays, false negatives are typically never identified (unless independent RNAi screenings using additional libraries or validated siRNAs are performed).
18. Altering the time and type of measurements of viral load can provide different information. The 24 h infection described here mostly will only report changes in viral infection arising from steps preceding virion assembly and secretion.
19. A typical human whole genome RNAi screen involves targeting approximately 18,000 genes. This many genes will often require around fifty-sixty 384-well plates depending on the arraying of the siRNAs.

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Chapter 11

ELISA and Neutralization Methods to Measure Anti-West Nile Virus Antibody Responses

Brian M. Friedrich and David W.C. Beasley

Abstract

Measurements of humoral immune responses to West Nile virus (WNV) infection in mouse or other animal models are valuable components of basic laboratory investigations to assess immunogenicity of candidate vaccines or to evaluate seroconversion following challenge with WNV. Here, we outline the steps for screening or titrating of total antibodies by indirect enzyme linked immunosorbent assay (ELISA) as well as assessment of neutralizing antibody titers by immunofocus detection.

Key words West Nile virus, Humoral immunity, Antibody titration, ELISA, Neutralization, Immunofocus, FRNT

1 Introduction

Measurements of humoral immune responses to West Nile virus (WNV) infection in mouse or other animal models are valuable components of basic laboratory investigations to assess immunogenicity of candidate vaccines or to evaluate seroconversion following challenge with virulent or attenuated WNV variants/strains. This chapter provides a representative method for screening or titration of IgG or total antibodies by indirect enzyme linked immunosorbent assay (ELISA) that can be performed using in-house or commercially available recombinant protein antigens representative of envelope (E) or non-structural protein 1 (NS1), two major targets of the humoral immune response in natural WNV infections. This method is also adaptable to assessment of antibody responses in other species via the use of secondary antibodies with alternative specificity. Although several commercial immunoassays are available for testing of human serum samples for anti-WNV IgM and/or IgG antibodies, these methods can also be appropriate for experimental evaluation of field-collected human or animal serum samples.

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In addition, a method for assessment of neutralizing antibody titers is described. This method employs an immunofocus detection approach, rather than traditional detection of virus plaques, which makes it more amenable to performance in 12- or 24-well tissue culture plates, thereby reducing quantities of serum samples required for assay performance.

2 Materials

Prepare all solutions using distilled, deionized water. Prepare and store all reagents at room temperature (unless indicated otherwise). Each of the reagents described below is for example and could be readily substituted with alternative commercially available equivalents with minimal assay optimization.

2.1 Indirect ELISA Materials

1. Coating buffer: borate saline pH 9.0. Add about 900 ml of water to a glass beaker or container. Weigh 7.0 g NaCl and transfer to beaker. Weigh 3.1 g H_3BO_3 and transfer to beaker. Add 24 ml 1.0 N NaOH to beaker. Mix until all solid chemicals have gone into solution then adjust pH to 9.0. Make up to 1 l final total volume with water.
2. Phosphate buffered saline (PBS): Add one phosphate buffered saline tablet (e.g., Fisher, catalog# BP2944-100 or Sigma, catalog# P4417-100TAB) to 200 ml of water and mix to dissolve. For preparation of large quantities of PBS or washing buffer (see below), a 5× PBS stock solution (e.g., 25 tablets in 1 l of water) can be prepared and subsequently diluted 1:5 in appropriate volumes of water.
3. Blocking buffer: 3% milk or 3% bovine serum albumin (BSA) in PBS. Add about 75 ml of PBS to a glass beaker or container. Weigh 3 g dried milk (or BSA) and transfer to beaker and mix to dissolve (BSA can sometimes take several minutes to dissolve into solution). Add water to make final volume 100 ml. Mix and store at 4 °C. Sufficient quantities of blocking buffer should be prepared for a single use or for expected use over 3–5 days (*see* **Note 1**).
4. Washing buffer: PBS containing 0.5% Tween 20 (PBST). Add 0.5% v/v of Tween 20 to PBS and mix to dissolve (e.g., 5 ml Tween 20 to 995 ml PBS).
5. MaxiSorp round-bottom 96-well plates (Thermo Scientific Nunc, catalog# 449824).
6. Adhesive PCR plate foil or other adhesive plate cover.
7. 3,3′,5,5′-tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (e.g., Sigma, catalog# T0440).

Table 1
WNV antibodies

Antibody	Source/catalog number	Protein specificity	References
7H2	VRL Cat#81-002 (*see* **Note 2**)	E, domain III	[1]
5H10	VRL Cat#81-003	E, domain III	[1]
3A3	VRL Cat#81-004	E, domain III	[1]
5C5	VRL Cat#81-005	E, domain III	[1]
3D9	VRL Cat#81-006	E, domain II (fusion loop)	[1, 2]
3.1112G	Millipore Cat#MAB8152	NS1	[3]

8. Stop solution: 3 M HCl. A 3 M working solution should be prepared by appropriate dilution of a commercial hydrochloric acid stock.
9. Plate reader. (Needs to be able to read dual absorbances at 450 and 595 nm.)
10. Positive control antibodies: A range of anti-WNV E/NS1 monoclonal antibodies and polyclonal antisera are available from commercial sources or reference laboratories and can be employed as positive controls. Some examples are provided in Table 1 above.
11. HRP-labeled Secondary Antibody. Anti-Mouse IgG (whole molecule)—Peroxidase conjugated antibody produced in goat (e.g., Sigma, catalog# A4416)—or whichever species your primary antibodies are derived from (*see* **Note 3**).
12. Antigens for coating plates: Various methods have been reported in the literature for expression and purification of WNV and other flavivirus E (e.g., [4, 5]), EIII (e.g., [1, 6]), and NS1 (e.g., [4, 7]) recombinant proteins using bacterial, mammalian or insect cell expression systems. For studies in our laboratory we use an in-house prepared WNV EIII antigen; however, similar EIII antigens (e.g., SinoBiological Inc., catalog # 40345-V08Y-100) as well as 80% E (e.g., ProSpec Bio., catalog# WNV-001) and NS1 (e.g., Immune Tech, catalog# IT-006-0053p) antigens can be obtained from commercial suppliers.

2.2 Focus Reduction Neutralization Test (FRNT) Components

1. Vero growth media: MEM + 8% FBS + 1% L-glut + 1% NEAA + 1% pen/strep. To one bottle of MEM (e.g., Corning Cellgro, catalog# 10-010-CV), add 40 ml FBS (e.g., Hyclone, catalog# SH30071), 5 ml L-glutamine (e.g., Gibco, catalog# 25030), 5 ml MEM nonessential amino acid solution 100× (e.g., Sigma, catalog# M7145), and 5 ml penicillin–streptomycin (e.g., Corning Cellgro, catalog# 30-001-CI). FBS should

be heat inactivated by incubating at 56 °C for 30 min prior to use in media preparation. A bottle of FBS can be heat inactivated, aliquoted into appropriate volumes in sterile tubes and stored at −20 °C prior to use.

2. Maintenance media: MEM + 2 % FBS + 1 % L-glut + 1 % NEAA + 1 % pen/strep. To one bottle of MEM add 10 ml FBS, 5 ml L-glutamine, and 5 ml of penicillin–streptomycin.
3. 2×MEM: 2× MEM + 4 % FBS + 0.5 % phenol red + 2 % L-glut + 2 % pen/strep. To one bottle of 2×-MEM (e.g., Quality Biological 115-073-101) add 20 ml FBS, 2.5 ml phenol red Solution (e.g., Sigma, catalog# P0290), 10 ml L-glutamine, and 10 ml penicillin–streptomycin.
4. 2 % agar: Add 6 g agar (e.g., Sigma, catalog# A1296-1KG) to 300 ml water. Autoclave to dissolve and sterilize.
5. Overlay media: Make a 1:1 mixture of 2× MEM and 2 % agar (*see* **Note 4**).
6. PBS: Add 1 phosphate buffered saline tablet (e.g., Fisher, catalog# BP2944-100 or Sigma, catalog# P4417-100TAB) to 200 ml of water and mix to dissolve.
7. Vero cells (e.g., ATCC, Vero (catalog# CCL-81), Vero 76 (catalog# CRL-1587), or Vero E6 (catalog# CRL-1586)): Grow cells in culture flasks using culture media (above). A single confluent T-150 flask (e.g., Corning, catalog# 430823) generally yields sufficient cells to seed approximately five 12-well plates.
8. 12-well plates (e.g., Corning Costar, catalog# 3513, or any cell culture treated 12-well plate).
9. 96-well plate(s) (e.g., Corning Costar, catalog# 3788, or any untreated 96-well plate).
10. West Nile virus. When preparing the virus, prepare sufficient quantities of aliquots to allow repeated performances of the FRNT assay without having to reoptimize with a new virus prep each time. Please refer to Chapter 3 for methods on propagation and titration of WNV strains.
11. Serum or samples for testing (*see* **Note 5**).
12. 10 % buffered formalin (e.g., Protocol, catalog# 245-684).
13. 70 % ethanol, stored at −20 °C.
14. Primary detecting antibody (e.g., mouse anti-flavivirus MAb 4G2 [EMD Millipore, catalog# MAB10216]).
15. HRP-labeled secondary antibody. Anti-mouse IgG (whole molecule)—Peroxidase antibody produced in goat (e.g., Sigma, catalog# A4416)—or whichever species your primary antibodies are derived from (*see* **Note 3**).

16. PBS I-Block Buffer: Add 0.5% I-Block (e.g., Applied Biosystems, catalog# T2015) to PBS.
17. Precipitating substrate solution (e.g., KPL True Blue [KPL, catalog# 50-78-02] or Vector VIP [Vector Laboratories, Cat# SK-4600]).
18. Rocking platform.

3 Methods

3.1 Indirect ELISA

Carry out all procedures at room temperature unless otherwise specified. Plates should not be allowed to dry out during incubation steps—generally this should not be a problem for 60 min or shorter incubations at room temperature, but it is best to keep the plates covered while incubating.

3.1.1 Coating Plates with Antigen

1. For each ELISA plate, prepare two tubes. The first tube will have borate saline only (background control wells): measure 2700 μl borate saline into a tube. The second tube will contain antigen diluted into borate saline: EIII, 80%E or NS1 Antigen will be diluted into borate saline to a final volume of 2700 μl. For EIII, measure about 5400–6750 ng (corresponding to 100–125 ng/well) and dilute volume to 2700 μl. (*See* **Note 6** for other antigens.)
2. Pipette 50 μl per well of diluted antigen to the desired number of wells in one or more MaxiSorp ELISA plates. If appropriate, also dispense 50 μl per well of borate saline to an equal number of wells (*see* **Note 7** for plate set up).
3. Cover plate (e.g., with adhesive foil/film or a plastic lid) and incubate plate overnight in a refrigerator at approximately 4 °C.

3.1.2 Indirect ELISA

1. After incubating overnight, discard the contents of all wells, and wash once with washing buffer. Washing can be performed by hand using a squirt bottle filled with wash buffer. Fill each well with washing buffer and then invert the plate and shake over a sink to empty. Residual wash buffer can be removed from the wells by tapping the inverted plate on some paper toweling.
2. Block the ELISA plates by adding 60–75 μl/well of blocking buffer (*see* **Note 1**).
3. Cover plates with adhesive foil and incubate for 60 min to allow blocking. (Additional incubation at this step is ok.)
4. During the blocking incubation, prepare dilutions of the serum/antibody samples in wash buffer. For example, individual samples may be screened at 1:100 and 1:500 dilutions in

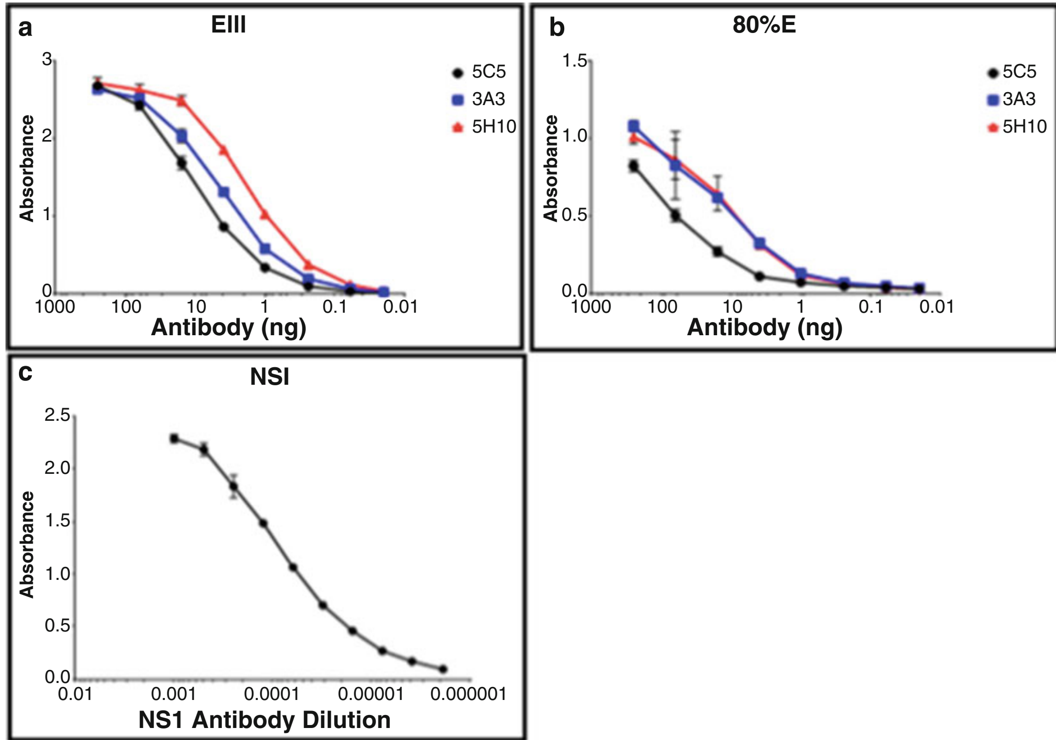

Fig. 1 Representative titrations of positive control monoclonal antibodies. EIIII-specific monoclonal antibodies (VRL) were titrated (serial fourfold dilutions) against (**a**) EIIII or (**b**) 80 % E antigens. (**c**) Titration of NS1-specific monoclonal antibody 3.112G (Millipore) (serial fourfold dilutions) against NS1 antigen ELISA. Note that the anti-NS1 antibody used was not provided by the manufacturer at a defined concentration/quantity

duplicate against antigen coated and uncoated wells. In that case, each sample will be used in four wells (duplicates for both antigen-coated and background controls), so a minimum of 200 μl (4 wells × 50 μl/well) is needed for each sample. A positive control antibody/antiserum can be included with each plate/batch, such as an anti-WNV-EIII (e.g., 7H2, 5C5, 5H10), anti-WNV-E (e.g., 3D9), or anti-WNV-NS1 (3.1112G) at a 1:1000 dilution (Fig. 1). Store diluted samples at 4 °C until ready. A negative control can be included with each plate/batch that contains "wash buffer only."

5. After blocking, wash two times with wash buffer (by completely filling wells and then discarding into the sink), then ensure any remaining wash buffer is removed from wells by tapping inverted plates on paper towels.
6. Add 50 μl/well volumes of diluted primary antibodies/sera and controls to appropriate wells. If using "borate saline only" wells/plates for background controls then dispense antibodies/sera into those as well.
7. Cover plate/s with adhesive foil and incubate for 45–60 min at room temperature.

8. Make dilution of secondary antibody: HRP-labeled anti-mouse IgG antibody at a 1:5000 dilution in PBST. (For other secondary antibodies, *see* **Note 3**.) For each plate, dilute 1 μl antibody in 5 ml PBST.
9. Wash plate/s three times with wash buffer as described above, and then ensure any remaining wash buffer is removed from wells by tapping inverted plates on paper towels.
10. Dispense 50 μl of diluted secondary HRP-labeled antibody to each well. This can be done using a multichannel pipette and trough or with a multidosing pipette.
11. Cover plate/s with adhesive foil and incubate for 45–60 min.
12. Discard secondary antibody solution from each well and wash plate(s) three times with wash buffer, and then was an additional two times with PBS alone; then remove any remaining buffer volume from wells by tapping inverted plate(s) on paper towels.
13. Add 50 μl of TMB substrate to each well using a multichannel pipette, taking care not to allow tips to touch the plate.
14. Incubate for 10 min (or until color develops sufficiently in positive control wells while remaining low in negative control wells), and then stop reaction by adding 50 μl of 3 M HCl to each well.
15. Absorbance values can then be read on a plate reader; use dual wavelength—450 and 595 nm reference values. When interpreting the data, subtract the average of the duplicate borate saline wells from the average of the duplicate antigen-coated wells of the same samples. That will give you the actual absorbance signal for the assay and subtract out any nonspecific binding or background. Cutoffs for "positive" and "negative" samples should be established for the particular types of sample(s) being tested. A good rule of thumb for a simple cutoff is a value three standard deviations above the mean absorbance of negative control wells. However, alternative cutoffs for positive and negative samples may be determined to be more appropriate depending upon the types of samples being tested or intended use of the data (positive/negative determination vs. quantitative or semi-quantitative comparison). If samples are being titrated via serial dilution, the titer typically represents the inverse of the last positive dilution (Fig. 2).

3.2 FRNT

Important: All cell culture work should be done in a clean biosafety cabinet (BSC) in BSL2 conditions. All work involving use of live WNV must be done in a BSC in a BSL3 facility. Serum samples should be heat inactivated at 56 °C for 30 min prior to use in this assay.

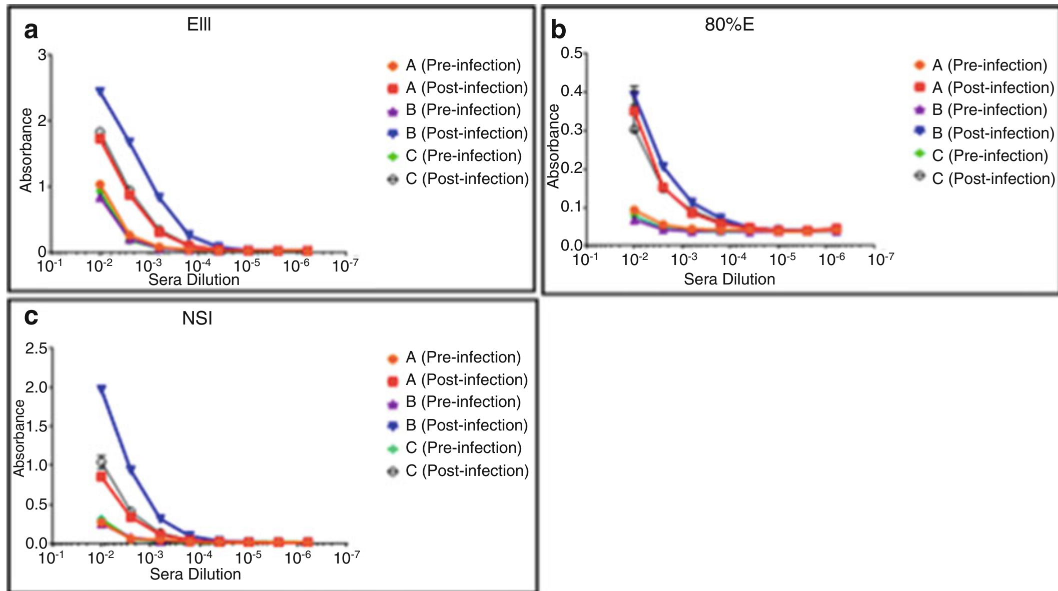

Fig. 2 Representative data from (**a**) EIII, (**b**) 80 % E, and (**c**) NS1 ELISAs to assess antibody levels in pre- and post-WNV-challenge samples from mice previously immunized with two doses of a commercial WNV veterinary vaccine. Serum samples were serially fourfold diluted from 1:100 to 1:1,638,400. The average background absorbance value (borate saline only wells) was 0.026 with a standard deviation of 0/013. Therefore the positive/negative cutoff of this particular assay would be 0.065

3.2.1 Seeding of Plates with Vero Cells

1. Seed 1 ml per well of a cell mixture containing approximately 1.2×10^5 cells/ml (Vero cells) in 12 well tissue culture plates. In the method described here, 18 wells (1.5 plates) are required per serum sample tested in duplicate, although a smaller or larger number of dilutions may be performed depending on the need to identify neutralization endpoint titers. (The recommended batch size is 15–20 total plates (10–13 samples) to minimize handling times during each of the processing steps.)
2. Plates should normally be used 1 day after seeding and should ideally be slightly less than 100 % confluent at time of infection.

3.2.2 Dilutions and Infections for FRNT

The specific range of dilutions to be tested can be varied based on available sample volumes or requirement to identify particular endpoint titer(s). In the following steps, samples are tested at doubling dilutions between 1/20 and 1/2560. Serum dilutions are prepared between 1/10 and 1/1280 in maintenance media and then mixed 1:1 with an equal volume of diluted virus to give the final dilutions in the neutralization test.

1. In a 96-well plate, pipette 108 μl of maintenance media into wells in column 1 (two rows for each sample to be tested in duplicate). (This volume would be modified for alternative starting dilutions.)

2. Pipette 60 μl of maintenance media into wells in columns 2–9.
3. Add 12 μl of serum sample(s) to wells of column 1 and mix by pipetting (this is a 1:10 dilution). Each serum sample will be diluted in two rows.
4. Using a multichannel pipette, perform serial doubling dilutions across the plate, using a transfer volume of 60 μl. Mix each dilution by pipetting and discard tips between each transfer. Discard the final transfer volume after column 8, leaving column 9 as culture medium only. The final volume in each well is 60 μl. Take particular care when using a multichannel pipette to ensure by visual inspection that all tips contain correct volumes during each transfer.
5. Prepare virus dilution. The amount of virus needed should be determined beforehand where the final assay gives approximately 40 plaques per well (*see* **Note 8**). For each serum sample tested, a minimum of 1080 μl diluted virus (9 dilutions in duplicate [18] × 60 μl/well) is required. Each virus stock tube should be thawed at room temperature and mixed by pipetting prior to use to ensure even distribution of the virus.
6. To each well of the 96-well plate(s) used for serum dilutions add 60 μl of diluted virus and mix by pipetting. A multichannel pipette can be used. Virus prep can be placed into a sterile trough, and clean tips are required for each addition. Incubate at room temperature for 60 min.
7. Following the 60 min incubation, discard supernatants from wells of 12 well plates (five or six at a time is generally ok), rinse wells with sterile PBS (~1 ml/well) and discard.
8. Pipette 100 μl of virus–serum mixture from wells of 96-well plate(s) into appropriate wells of 12-well plates (using a clean tip for each addition), rock the plate to ensure that the virus–serum mixture is distributed over the entire monolayer, then replace the lid and incubate for 30–35 min at room temperature with periodic rocking to allow virus binding.
9. Following incubation, overlay each well with 2 ml overlay media and incubate at 37 °C/5 % CO_2 for 2 days.

3.2.3 Immunostaining

1. After 2 days, fix plates by completely filling wells with buffered formalin and incubating for 60–70 min at room temperature. Ensure that formalin contacts all interior surfaces of the wells and plate lid.
2. After incubation, discard formalin from wells, remove agar plugs with a spatula and add ~1 ml of fresh buffered formalin to each well. Incubate for an additional 10–15 min at room temperature.

3. After this second incubation, plates, still containing formalin, can then be surface decontaminated with appropriate virucidal reagent (e.g., CaviCide), placed in sealed secondary containment and transferred to BSL2 for further processing.
4. Discard the buffered formalin into appropriate waste container and then wash each well two times with PBS, using approximately 2 ml per well each time (*see* **Note 9**).
5. Discard PBS and add ~0.5 ml/well ice cold 70% ethanol and incubate for 30 min at −20 °C.
6. Prepare sufficient volume of a dilution of the primary antibody (e.g., 1:2500 anti-flavivirus MAb 4G2 diluted 1:2500 in I-Block PBS buffer) to dispense 150 μl/well in the assay plates.
7. After incubation, wash plates two times with PBS as described above.
8. Discard PBS, add 150 μl/well of the primary antibody solution, replace the lid, and incubate for 80–90 min on a rocking platform at room temperature. Rocking speed should be low and sufficient to allow gentle movement of the antibody solution over the monolayer.
9. Wash cells two times with PBS (as described above).
10. Discard PBS and add 200 μl/well of the secondary antibody (e.g., 1:5000 dilution of HRP-labeled anti-mouse IgG in I-Block). Incubate 60–70 min on rocking platform.
11. Wash cells two times with PBS (as described above).
12. Stain plates with 200 μl/well precipitating substrate solution for 10–15 min on rocking platform.
13. Discard staining solution and rinse cells with dH_2O (~2 ml) when foci become clearly visible in virus-only control wells and then air dry each plate (e.g., with lid off in a fume hood).

3.2.4 Analysis and Interpretation

Count foci in each well and record on data sheets. Foci counts in all virus-only control wells for each batch are to be averaged to determine an endpoint cutoff. For example, FRNT50 titers for each sample tested can be determined by identifying the well for each containing the highest dilution of serum with a foci count <50% of the average virus control well value (Fig. 3). Titers for individual sample replicates should be within twofold. Use of alternative endpoint cutoffs (e.g., 80 or 90% neutralization) may be considered and can be calculated from the same data.

4 Notes

1. High nonspecific binding of some sera from immunized/infected mice to plates blocked with PBS containing BSA has been observed in some of our experiments, perhaps due to

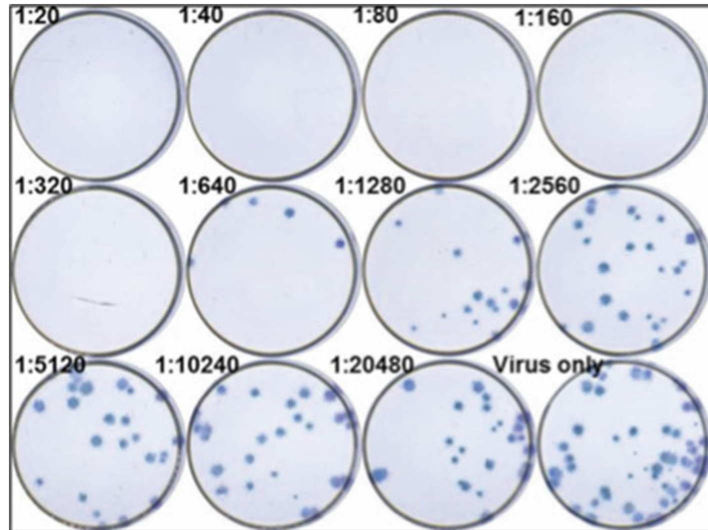

Fig. 3 Representative FRNT assay plate. The 50 % focus reduction neutralization titer (FRNT50) is the inverse of the final dilution with a foci count <50 % of the mean virus only control well counts for a batch. For this particular plate, the FRNT50 would be 1280

residual FBS in virus preparations or in some commercial WNV veterinary vaccine preparations. PBS containing 3% milk may be a preferable blocking reagent for most applications.

2. VRL was formerly known as Bioreliance.
3. Some examples of secondary antibodies for alternative species: HRP-labeled anti-human IgG antibody (e.g., Sigma, catalog# A6029-1ML), HRP-labeled anti-rabbit IgG antibody (e.g., Sigma, catalog# A6154-1ML), HRP-labeled anti-monkey IgG antibody (e.g., Rockland, catalog# 617-103-012).
4. Overlay media—2× MEM/agar. The 2% agar solution should be carefully heated in a microwave oven to return it to liquid form and then held in a 52–56 °C water bath for >20 min prior to use. 2× MEM should be removed from 4 °C storage and held in a 37 °C water bath for >20 min prior to use. After mixing equal quantities of the agar and 2× MEM to prepare overlay media, it will generally remain liquid at room temperature for at least 15 min which is sufficient time to overlay a typical batch of plates.
5. It is highly recommended to include a positive control neutralizing antibody to monitor performance from batch-to-batch. This could be a monoclonal antibody or polyclonal antiserum several of which are commercially available or can

be sourced from reference collections such as the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) or ATCC/BEI.

6. Appropriate dilutions of antigen for coating should be determined by block-titration of antigen and a known positive control antiserum/MAb, i.e., groups of wells are coated with serial dilutions of the antigen, and then detected using serial dilutions of the antiserum. Optimal dilutions of both antigen and control antiserum can be determined in this manner. In our hands, the optimal antigen coating concentrations are:
   (a) 80% E (ProSpec, catalog # wnv-001-b): 300–500 ng/well.
   (b) NS1 (Immune Technology Corp, catalog # IT-006-0053P): 200–300 ng/well.
   (c) EIII: 100–125 ng/well.
7. Plate coating and assay setup are flexible and may involve screening of sera at a single dilution, multiple dilutions, or performance of serial titrations to assess endpoint titers. Coated and uncoated wells may be on the same plate or different plates, depending on how many samples are being processed and dependent on the user. It is recommended to test sera at least in duplicate, although additional replicates may be appropriate. Also, for previously untested polyclonal sera, it is very beneficial to test against coated and uncoated (borate saline only) wells to identify any nonspecific binding. For example: if one half of the plate is coated in antigen and the other half with borate saline only, each plate can test 24 samples in duplicate at a single dilution or 12 samples in duplicate at two dilutions.
8. Virus dilutions: Prior to FRNT assay, the virus dilution must be determined that will give you approximately 40–50 foci per well in the final assay.
9. If immunostaining will occur at a later time, plates can be left with PBS in wells and stored at 4 °C for up to 2 days.

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Chapter 12

Safe Handling of West Nile Virus in the Insectary

Dexter R. McKellar and Michael J. Conway

Abstract

Working with West Nile virus (WNV) in the insectary requires specific facilities and protocols to prevent laboratory-acquired infection. Here, we review case reports of individuals infected with members of the Flaviviridae while performing biomedical research in traditional laboratories and insectaries. We highlight the most common transmission route and summarize recommendations for facilities and protocols designed to prevent laboratory-acquired infections.

Key words Biosafety, Laboratory-acquired infection, West Nile virus, Flavivirus, Mosquito, Arthropod containment level, Insectary, Biosafety level, Personal protective equipment

1 Introduction

West Nile virus (WNV) is a mosquito-borne flavivirus that naturally cycles between avian and mosquito hosts. Humans and horses are dead end hosts and infections can lead to fatal neuroinvasive disease and neurological sequelae. There is no human vaccine or specific therapy for WNV. The majority of human infections with WNV are caused by the bite of an infected mosquito; however, laboratory-acquired infections with WNV have been reported. The lack of prophylaxis and specific therapy, in addition to the possibility of severe disease, has led to the classification of WNV as a biosafety level 3 (BSL3) pathogen. This designation has led to the development of specific protocols for the safe handling of WNV in the laboratory. Protocols for safe handling of BSL3 pathogens in cell culture and mouse models have been well described. This chapter reviews the history of laboratory-acquired infections with WNV and other members of the Flaviviridae family and summarizes recommended practices and safety concerns in an arthropod containment level 3 (ACL3) facility.

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2 Laboratory-Acquired Infections

It is estimated that 500,000 workers in the USA work in research and clinical laboratories. The risk of a laboratory-acquired infection for these employees is difficult to measure as there is yet to be a federal, state, or professional society that formally tracks infections acquired in the laboratory setting. Literature that is currently available has been the basis for the safety precautions and laboratory techniques that have been implemented [1]. Our focus in this review is on laboratory-acquired infections that have taken place during biomedical research.

Viruses in the family Flaviviridae have been reported as causing many laboratory-acquired infections, although many of the transmission routes are unknown (Table 1). For example, West Nile virus (WNV) laboratory-acquired infections have been documented as early as 1954 [2]. Although the route of exposure is unknown in this early case, the laboratory staff member worked directly with infected mosquitoes and human blood samples. The patient in this case presented severe pain in his calves and thighs. He developed malaise and fatigue to an extent where bed rest was necessary. The illness affected him for more than 2 weeks [2].

Table 1
Documented laboratory-acquired infections from viruses in the family Flaviviridae

Flavivirus	Vector	Transmission route	Reference
West Nile virus	Bird brain	Percutaneous	[3]
West Nile virus	Mouse brain	Percutaneous	[3]
West Nile virus	Cell culture fluid	Percutaneous	[5]
West Nile virus	Human blood sample	Unknown	[2]
Dengue virus type 2	*Aedes aegypti*	Mosquito bite?	[6]
Yellow fever virus	Human vomit	Unknown	[7]
Yellow fever virus	*Aedes aegypti*	Mosquito bite	[7]
Yellow fever virus	Monkey blood sample	unknown	[7]
Yellow fever virus	Human blood sample	Unknown	[7]
Kyasanur forest disease virus	Cell culture fluid	Cutaneous or mucocutaneous	[9]
Tick-borne encephalitis virus	Human blood sample	Unknown	[10]
Hepatitis C virus	Human blood sample	Percutaneous	[4]
Hepatitis C virus	Human blood sample	Mucocutaneous	[4]

The most commonly reported route of transmission causing laboratory-acquired infection is the percutaneous route, often caused by accidental needlestick. Hepatitis C virus (HCV) and WNV have both led to infections via this route [3–5]. In one example, a scientist accidentally inoculated herself with a neuroinvasive stain of WNV via needlestick. Symptoms appeared 7 days after she was infected. The symptoms were biphasic and included rash, mild fever, and symptoms of meningoencephalitis. Headaches, malaise, and arthralgia were noted as second phase symptoms. Only photophobia remained after 19 days and persisted for months afterwards. An interesting aspect of this case was the scientist had been previously inoculated with the yellow fever virus (YFV) vaccine (YF-VAX, Aventis Pasteur); however, this did not prevent her from developing WNV neuroinvasive disease [5].

In 2002, the Centers for Disease Control and Prevention reported two separate cases of laboratory-acquired WNV infections [3]. In the first case, the microbiologist lacerated a thumb while using a scalpel to remove infected brain tissue from a bird. In the second case, a microbiologist punctured a finger with a contaminated needle. Both researchers were working in class II laminar flow biosafety cabinets under biosafety level 2 (BSL2) or above conditions [3]. These precautions were not sufficient to prevent the laboratory-acquired infections, suggesting that extreme care must be taken when using sharps in microbiological research or avoided entirely. Both microbiologists had mild symptoms such as malaise, fever, intermittent chills, and sweats. The microbiologist that lacerated his thumb missed 1 day of work. The other microbiologist continued to work while ill, which lasted approximately 1 week [3].

A much less common route of exposure is due to accidental bites by infected mosquitoes in the laboratory. This is likely due to the small population of researchers who work with infected mosquitoes, but is also due to stringent recommendations for proper handling of infected mosquitoes, and the short time frame where mosquitoes are transmission competent in the laboratory. Currently, there are no documented cases of laboratory-acquired infections with WNV via this route. However, YFV and much more recently dengue virus (DENV) have reported cases of laboratory-acquired infection via mosquito bite [6, 7]. Laboratory staff who work directly with YFV infected materials are urged to get vaccinated with the YFV vaccine. Vaccination has reduced the prevalence of laboratory-acquired infections with YFV. Unfortunately, vaccines are not available for the majority of viruses in the Flaviviridae family, which includes DENV. The laboratory researcher that was infected with DENV was wearing proper PPE including a gown, gloves, and eye protection. This level of protection was insufficient to protect him from being bitten by mosquito [6]. When admitted to the hospital he had a high fever marked with lethargy and

fatigue, which progressed to myalgia and severe back pain 4 days after exposure. A fine, macular, blanching rash developed 1 day later. After 3 days in the hospital all of his symptoms resolved. Interestingly, the laboratory researcher stated that the mosquito had not blood fed, suggesting that the colony may have been contaminated with DENV, the mosquito was able to transmit the virus through mechanical means, or that transmission occurred through an unknown route such as through aerosolized blood droplets or via contact with a dermal abrasion [6].

Although laboratory-acquired infections via mosquito bite are much less common than the percutaneous route, work with infected mosquitoes has increased significantly in recent years and this work includes a larger number of mosquito species and types of viruses than in previous generations. The remainder of this manuscript will summarize the recommendations for working with WNV in an arthropod containment level (ACL) and highlights safety concerns during such work, which includes procedures that increase the risk of percutaneous inoculation [8].

3 Arthropod Containment Level Facility for West Nile Virus Research

Research on arthropod vectors requires a risk assessment by the Institutional Biosafety Committee (IBC) based on the assumption that the arthropod will escape at some point in time. If the arthropod is not native to the region and may become an invasive species, or if the arthropod is infected with a human pathogen, more stringent physical barriers and protocols should be in place to prevent and neutralize escapees. Universal practices have not been prescribed due to the high diversity of arthropod vectors and diseases they transmit, in addition to the ability of the vector to colonize the region in which the research is being conducted. To guide principal investigators and IBCs, The American Society of Tropical Medicine and Hygiene/American Committee of Medical Entomology and others have developed recommendations for arthropod containment levels 1–4 (ACL 1–4) [8]. What follows is a summary of recommendations for an ACL3 facility—the facility appropriate for research with West Nile virus (WNV) in its mosquito vector. Investigators should refer to the original recommendations for complete details.

3.1 Facilities

ACL facilities are typically matched to the biosafety level (BSL) of the pathogen being investigated. For example, WNV is a BSL3 pathogen, so it is appropriate to work with WNV in an ACL3 facility that maintains *Culex* spp. Additional local considerations must be made such as the likelihood of the mosquito vector colonizing the outside environment, and the presence of alternative vectors of WNV. These possibilities may increase the ACL. The ideal facility

will eliminate any possibility of pathogen transmission within the local population.

Proper design of the ACL3 facility is critical to reducing laboratory-acquired infections or accidental release of mosquito vectors. ACL3 facilities must be placed away from main traffic corridors, and unauthorized personnel are restricted from entering by key or pushbutton locks, or key fob. Entry into the insectary occurs via two self-closing doors that includes a change room and shower. Secondary barriers such as hanging or air curtains are recommended. An autoclave may also pass through this anteroom to facilitate passage of decontaminated wastes out of the facility. Any opportunity for mosquito escape including windows or improper plumbing or ventilation should be eliminated. Exhaust air is discharged to the outside without being recirculated with appropriate barriers to prevent mosquito escape and negative pressure airflow is present in the insectary and must be monitored by personnel and audibly alert if the system fails. Notification of ongoing research with WNV infected mosquitoes should be indicated using a BSL3 biohazard sign that indicates the pathogen and vector in use, in addition to personal protective equipment (PPE) and other requirements for entry.

3.2 Equipment, Arthropod Surveillance, and Disinfection

Equipment stored within the facility should be minimized to what is required for mosquito maintenance and optimized for detection of escapees. All surfaces should be regularly cleaned and kept dry to prevent uncontrolled development of mosquitoes within the insectary. Walls and equipment should be white or clear when possible to facilitate detection of escaped mosquitoes. Uninfected mosquitoes should be physically separated from infected mosquitoes and materials. Containers and cages used to house mosquitoes should prevent escape and should not break or leak easily. Adult mosquitoes may be killed by spraying with 70% isopropanol or freezing. Larvae can be killed by incubating in 10% bleach. Only dead mosquitoes are disposed of and disposal takes place using leak-proof containers. All containers that housed arthropods should be cleaned appropriately to prevent arthropod survival and neutralize remaining virions. Metal cages should be autoclaved and plastic containers should be soaked in 10% bleach. Infected material must be autoclaved and disposed of in compliance with local requirements. Pesticide should be available for an emergency.

3.3 Training and Personal Protective Equipment

All laboratory personnel are trained in proper procedures outlined in an IBC-approved insectary safety manual and competency is evaluated on an annual basis. Training records are maintained and additional training sessions are performed as necessary. All laboratory personnel also undergo medical surveillance, including serum banking, and a medical consult, which prevents uniquely susceptible individuals from performing research on the pathogen (e.g., immunocompromised or pregnant individuals).

Laboratory personnel must protect themselves by wearing clothing that reduces skin exposure, and properly donning and doffing PPE. White laboratory coats are worn in addition to disposable solid-front gowns. Disposable gowns are disposed of before leaving the insectary. A double layer of gloves is commonly worn. The outer layer can be disinfected by spraying with 70% isopropanol prior to removal.

4 Safety Concerns Regarding West Nile Virus Work in the Insectary

The above ACL recommendations lay the foundation for a secure facility that will prevent arthropod release, and limit those who come in contact with infected mosquitoes to trained personnel. However, procedural mistakes and simple accidents can occur in the insectary, which can increase your risk of infection. Below is a list of suggestions to limit your risk of laboratory-acquired infection in the insectary.

4.1 Blood Feeding

1. Wear an appropriate respirator due to the possible generation of infectious aerosols.
2. Prepare infected blood meals in an appropriate biosafety cabinet and transport blood meals in a secondary container.
3. Prepare a "disinfection kit" with 10% bleach and 70% ethanol to clean up small blood spills, leaks, and contaminated equipment.
4. Take care when moving engorged mosquitoes from one container to another. *Culex* spp. are small and can easily get lost in wet ice and revive once the ice melts.

4.2 Microinjection

1. Wear an appropriate respirator due to the possible generation of infectious aerosols.
2. Prepare a "disinfection kit" with 10% bleach and 70% ethanol to clean up small blood spills, leaks, and contaminated equipment.
3. Identify a safe holding place for your virus loaded micropipette preferably pointing away from your body.
4. Fully anesthetize mosquitoes before microinjection. Do not rush.
5. Avoid breaking the micropipette and immediately decontaminate and remove broken glass if this does occur.
6. Keep your unused hand at a safe distance from the micropipette while microinjecting.
7. Take care when moving microinjected mosquitoes from one container to another. *Culex* spp. are small and can easily get lost in wet ice and revive once the ice melts.

4.3 Dissection

1. Fully anesthetize mosquitoes before dissection. Do not rush.
2. Clip wings to prevent flying.
3. Identify a safe position for your forceps, preferably pointing away from your body.
4. Keep your unused hand at a safe distance from the contaminated forceps when transferring tissues into sample tubes.
5. Disinfect forceps between each dissection and when the procedure is complete.
6. Take care when moving microinjected mosquitoes from one container to another. *Culex* spp. are small and can easily get lost in wet ice and revive once the ice melts.

5 Conclusion

Laboratory-acquired infections have happened in the past and they will happen in the future. The changing demands of scientific research require that our procedures adapt to new environments. The insectary is a unique research environment that requires a specific design and protocols to contain infected or transgenically modified arthropods; however, the major risk of laboratory-acquired infection likely remains the same as in traditional biomedical research laboratories—percutaneous infection. Care should be taken to both contain infected arthropods and avoid accidental exposure through infectious aerosols and sharps.

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Chapter 13

Techniques for Experimental Infection of Mosquitoes with West Nile Virus

Yang Liu and Gong Cheng

Abstract

West Nile virus is a typical mosquito-borne flavivirus, and it is transmitted between mosquitoes and birds in nature. As an incidental host, humans are susceptible to WNV infection. WNV infection in humans can result in fever, meningitis, and encephalitis. Approved human vaccines or therapies are not available for WNV infection. In this chapter, we focus on the techniques for WNV infection and detection in mosquitoes. The technical details include: (1) WNV infection in cell culture; (2) Mosquito rearing; (3) WNV infection in mosquitoes via thoracic microinjection; (4) Detection of WNV infection in mosquitoes; (5) Determination of WNV M.I.D$_{50}$ in mosquitoes; (6) WNV infection in mosquitoes via membrane blood feeding; (7) WNV infection via blood feeding on mice; (8) Immunofluorescence staining of WNV infected mosquito tissues.

Key words West Nile virus, Mosquitoes, Thoracic microinjection, Blood feeding, Viral detection

1 Introduction

West Nile virus (WNV) is a member of the *Flaviviridae* Family and is maintained in a bird-mosquito transmission cycle. Birds are the native vertebrate hosts for WNV infection. WNV rapidly replicates in birds after infection and maintains a high viremia for a prolonged period of time, which allows mosquitoes to acquire WNV through blood feeding. Humans, horses, and other non-avian vertebrates are incidental hosts [1]. Human infection can result in fever, meningitis, or encephalitis, among other symptoms [2, 3]. Approved human vaccines or therapeutics are not available for WNV, and the current preventive measures largely focus upon mosquito control [4].

WNV is a typical mosquito-borne virus. The ability of different mosquito species to transmit WNV varies widely. *Culex* spp. are the major vectors for WNV dissemination worldwide [5, 6]. *Culex pipiens*, *Culex tarsalis*, and *Culex quinquefasciatus* serve as the major vectors for WNV dissemination in different regions of the USA [7].

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In addition to *Culex* spp., WNV has also been isolated from *Aedes*, *Ochlerotatus*, and *Culiseta* mosquitoes (http://www.cdc.gov/ncidod/dvbid/westnile/mosquitoSpecies.htm). Both *Aedes aegypti* and *Culex pipiens* are major vectors for numerous flaviviruses and are ideal arthropod models for flaviviral pathogenesis and immune studies [8]. As an anthropophilic vector in and around human dwellings, both *A. aegypti* and *C. pipiens* are easily cultivated and readily susceptible to WNV in the laboratory. The virus rapidly disseminate throughout the tissues of mosquitoes following a blood meal or intrathoracic microinjection [9–13]. The genome of *A. aegypti* has been well characterized [14]. Moreover, the genome sequence of *C. pipiens* has been partially released (https://www.vectorbase.org). The advantage of genomic sequences is that they can significantly increase our understanding of WNV infection and transmission in its vectors [9, 14–16].

2 Materials

2.1 Mosquito Rearing

2.1.1 A. aegypti and C. pipiens

1. *A. aegypti* is a native vector for the transmission of dengue fever (DENV), chikungunya (CHKV), and yellow fever (YFV).
2. *C. pipiens* is one of the species of mosquitoes that spread Japanese encephalitis (JEV) and West Nile disease (WNV) in nature.

2.1.2 Mosquito Food

1. For larvae: Larvae are fed liver broth (Oxoid, Hampshire, UK) and yeast extract (Oxoid, Hampshire, UK). The liver broth and yeast extract are combined in a ratio of 3:2 (w/w) in ultrapure water (*see* **Note 1**).
2. For adult mosquitoes: Cotton balls with 10% sucrose or wet raisin are placed on the top of the mosquito cage (Bioquip, Rancho Dominguez, CA, USA).

2.1.3 Environmental Conditions

The incubator is maintained at 28 °C with 80 % humidity and a 12–12 h light–dark cycle each day. Moist towels are placed on the top of the mosquito cage to maintain the humidity.

2.2 Cell Culture

2.2.1 C6/36 Cells

1. C6/36 cells are a mosquito cell line from *Aedes albopictus* larvae.
2. Culture medium: DMEM high glucose basic medium with 10% fetal bovine serum and 1/100 (v/v%) antibiotic–antimycotic is used to culture the cells (*see* **Note 2**).
3. WNV infection medium: DMEM high glucose basic medium with 2% fetal bovine serum and 1/100 (v/v%) antibiotic–antimycotic is used for the C6/36 cells. This medium is used to maintain cells after WNV infection.
4. Culture conditions: C6/36 cells are cultured in a 30 °C and 5% CO_2 incubator (*see* **Note 3**).

2.2.2 Vero Cell

1. Vero cells are a mammalian cell line from *Cercopithecus aethiops*.
2. Culture medium: DMEM high glucose basic medium with 10% fetal bovine serum and 1/100 (v/v%) antibiotic–antimycotic is used to culture the cells.
3. WNV infection medium: VP-SFM medium (serum free medium) is used for WNV infection of Vero cells (*see* **Note 4**).
4. Culture conditions: Vero cells are cultured in a 37 °C and 5% CO_2 incubator.

2.3 Mosquito Microinjection System

1. Frozen operation platform (Portable Chill Table) (Bioquip, Rancho Dominguez, CA, USA).
2. Anatomical lens (OLYMPUS, Tokyo, Japan).
3. FemtoJet Microinjector (Eppendorf, Hamburg, Germany).
4. Dual-stage Glass Micropipette Puller (NARISHIGE, New York, USA) and capillary tube (WPI, Sarasota, FL, USA) (*see* **Note 5**).
5. Precision Model 818 High-capacity Incubator (Thermo Fisher Scientific, Waltham, MA, USA) for rearing WNV infected mosquitoes.

2.4 Quantitative-PCR (qPCR) System

1. Pestle grinder system (Thermo Fisher Scientific, Waltham, MA, USA) for homogenization of mosquitoes in RNA extraction buffer.
2. Multisource Total RNA Miniprep kit (Axygen, San Francisco, CA, USA) for total RNA isolation.
3. RNase-Free DNase Set (Qiagen, Valencia, CA, USA) for cleaning up genomic DNA contamination.
4. iScript® cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) for reverse transcription of total RNAs.
5. Taqman® qPCR system includes primers, FAM-labeled Taqman® probes and EasyTaq® DNA polymerase (Transgene Biotech, Beijing, China).

2.5 Membrane Blood Feeding System

The Hemotek® membrane blood feeding system (Hemotek, Lancaster, UK) includes electronic heating equipment, reservoirs, and a semipermeable membrane.

2.6 Immunostaining

1. Silane-Prep slides (Sigma-Aldrich, St. Louis, USA): the mosquito tissues can be firmly attached onto the slide after drying.
2. Liquid-repellent Slide Marker Pen (Daido Sangyo, Tokyo, Japan): write a circle with the pen on the slide and place the tissues in the circle.
3. 4% paraformaldehyde (PFA) to fix the tissues.

4. BD Perm/Wash® buffer (BD Biosciences, San Jose, CA, USA).
5. ProLong Gold® anti-fade reagent (Life Technologies, Carlsbad, CA, USA): mount the stained tissues.
6. Primary antibody: Mouse monoclonal antibody D1-4G2-4-15 (EMD Millipore, Billerica, MA, USA) is used for WNV staining (1:500 dilution).
7. Secondary antibody: Goat anti-mouse IgG, Alex Fluor® 546 (Invitrogen, Waltham, USA) (1:1000 dilution).

3 Methods

West Nile virus is a bio-safety level 3 (BSL-3) agent. All procedures involving WNV infection should be restricted to a bio-safety hood in an authorized BSL-3 lab.

3.1 WNV Infection in Cell Culture

WNV is grown in mosquito C6/36 and mammalian Vero cells (*see* **Note 6**).

3.1.1 WNV Infection in C6/36 Cells

1. Before WNV inoculation, the C6/36 cells should reach at least 90% confluence (*see* **Note 7**).
2. Inoculate approximately 1×10^4 pfu (plaque forming unit) of WNV into C6/36 cells in a 75 cm^2 cell culture flask. Culture the cells at 30 °C and 5% CO_2 for 4–8 h.
3. After the incubation, wash the cells twice with PBS and add fresh DMEM medium supplemented with 2% FBS (WNV infection medium).
4. The infected C6/36 cells are maintained at 30 °C and 5% CO_2 for 3–4 days. Check the cells every day until 50% cytopathic effect (CPE) (*see* **Note 8**) of the cells is observed (*see* **Note 9**).
5. Collect the supernatant and centrifuge it at $1000 \times g$ and 4 °C for 10 min to remove the suspended cells and cell debris.
6. Aliquot the WNV supernatant into 1.5 ml centrifuge tubes on ice, and store it in a −80 °C freezer or liquid nitrogen (*see* **Note 10**).

3.1.2 WNV Infection in Vero Cells

1. Before WNV inoculation, the Vero cells should reach at least 90% confluence.
2. Inoculate approximately 1×10^4 pfu of WNV into Vero cells. Further culture the cells at 37 °C and 5% CO_2 for 4 h.
3. Wash the cells twice with PBS and replace the supernatant with fresh VP-SFM medium.
4. Maintain the infected Vero cells at 37 °C and 5% CO_2 for 2–3 days. Check the cells every day until 50% cell death is observed (*see* **Note 11**).

5. Collect the supernatant and centrifuge it at 1000× at 4 °C for 10 min to remove the suspended cells and cell debris.
6. Aliquot the WNV supernatant into 1.5 ml centrifuge tubes on ice, and store it in a −80 °C freezer or liquid nitrogen.

3.2 Mosquito Rearing

C. pipiens, a member of the *Culicinae* subfamily, is a natural vector for WNV transmission [5, 6]. WNV has also been isolated in native *A. aegypti* or other *Aedes* species (http://www.cdc.gov/westnile/transmission/). Because *A. aegypti* and *C. pipiens* are easy to cultivate and their genomes have been well characterized, they are ideal insect models for viral pathogenesis and immune studies [14–16]. Both of these mosquitoes are susceptible to WNV infection by thoracic microinjection and blood feeding.

3.2.1 *A. aegypti* Eggs

1. Adult mosquitoes emerging 2 weeks later are used to lay eggs (*see* **Note 12**).
2. A container with clean water and a piece of filter paper is placed into the mosquito cage for *A. aegypti* to lay eggs. After a blood meal (*see* **Note 13**), the female mosquitoes will lay eggs on the surface of the wet filter paper within 4 days (*see* **Note 14**).
3. Harvest the filter papers with mosquito eggs and dry the papers on clean cotton in a petri dish (15 cm in diameter) at room temperature (RT) (*see* **Note 15**).
4. Stock the eggs at least 1 week at RT before the next hatching.

3.2.2 *C. pipiens* Eggs

1. Adult mosquitoes emerging 2 weeks later are used to lay eggs.
2. A container with clean water is placed into the mosquito cage for *C. pipiens* to lay eggs. After a blood meal, the female mosquitoes will lay eggs in the water within 4 days.
3. Transfer the mosquito eggs to another container with clean water. The eggs will hatch in the new container (*see* **Note 16**).

3.2.3 Mosquito Hatching

1. Place the mosquito eggs in a flat tray (larvae tray) with clean water at 28 °C. After 1–2 days of incubation, the eggs will begin to hatch (*see* **Note 17**).
2. Feed the larvae with 1–3 drops of larvae food (Ref. to Subheading **2.1.2**, **step 1**). The amount of food should be increased gradually in relation to larvae growth (*see* **Note 18**).
3. After approximately 15–20 days, collect the pupae into a container with clean water. DO NOT continue to feed the pupae.
4. Move the container with the pupae into mosquito cages. The pupae will emerge to adult mosquitoes approximately 3 days later (*see* **Note 19**).
5. The adult mosquitoes are maintained with cotton balls soaked in 10% sucrose or wet raisins. Change the food every day to avoid microbial contamination.

6. The female mosquitoes emerging in 5–7 days (*see* **Note 20**) are used for WNV infection via thoracic microinjection, and the mosquitoes emerging in 7–15 days are used for the infection through blood feeding.

3.3 Thoracic Microinjection

1. The female mosquitoes emerging in 5–7 days are collected using an aspirator and anesthetized on ice (*see* **Note 21**).
2. The microinjection needles are made using a needle puller.
3. Precool the plastic microscope slides on a frozen operation platform. Transfer the mosquitoes onto the cold slides after thoroughly anesthetizing the mosquitoes (*see* **Note 22**), and then, place the slides with the mosquitoes onto the frozen platform prepared for microinjection (*see* **Note 23**).
4. Dilute the virus with sterile PBS and subsequently microinject the virus into the thorax of the mosquitoes (Fig. 1) (*see* **Note 24**). For each mosquito used for functional investigation, 10 M.I.D.$_{50}$ of WNV is inoculated, while 1000 M.I.D.$_{50}$ of WNV is inoculated in the mosquitoes used for studying gene expression.
5. Drop the injected mosquitoes into sealed paper cups (16 oz), and maintain the infected mosquitoes in a Precision® Model 818 incubator at 28 °C and 80% humidity.
6. The mosquitoes are maintained with cotton balls soaked in 10% sucrose, which is placed on the top of the culture cups. The sucrose cotton balls are replaced every day to avoid microbe contamination.
7. After 6 days rearing, the infected mosquitoes will be sacrificed in a −80 °C freezer for further analysis.

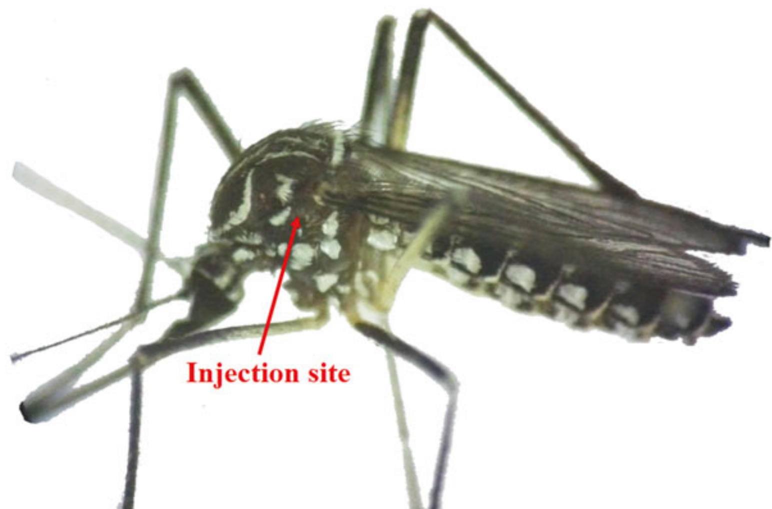

Fig. 1 The injection site for thoracic microinjection of *Aedes aegypti*. The mosquito is anesthetized on ice and placed on the side for microinjection. The injection site locates in the middle of lateral thorax

3.4 Detection of WNV Infection in Mosquitoes

1. The infected mosquitoes are sacrificed in a −80 °C freezer (*see* **Note 25**), and the whole mosquito is homogenized in RI buffer (Multisource Total RNA Miniprep kit) with a Pestle grinder system (*see* **Note 26**).
2. Isolate total RNA from these mosquitoes and synthesize cDNA using an iScript® cDNA synthesis kit (*see* **Note 27**).
3. The number of genomic copies of WNV is detected by Taqman® qPCR (*see* **Note 28**).

3.5 Determination of WNV M.I.D.$_{50}$ in Mosquitoes

The WNV for the in vivo experiments is titrated in mosquitoes infected by thoracic microinjection prior to the study. The WNV titration in the mosquitoes is measured using the Mosquito Infective Dose 50% (M.I.D.$_{50}$).

1. The WNV is diluted by tenfold serial dilutions (i.e., 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) in PBS.
2. The mosquitoes (12 in each group) are inoculated in the thorax by microinjection with 300 nl of diluted virus.
3. The inoculated mosquitoes are maintained in a Precision Model 818 incubator at 28 °C and 80% humidity for 6 days.
4. The infected mosquitoes are sacrificed, and total RNA is isolated. The viral burden is determined by Taqman® qPCR
5. The M.I.D.$_{50}$ is estimated using the Reed–Muench method (*see* **Note 29**).

3.6 WNV Infection in Mosquitoes via Membrane Blood Feeding

3.6.1 Blood Preparation

1. Human or murine blood is collected using heparin anticoagulant tubes (*see* **Note 30**). Store the blood at 4 °C for no more than 12 h.
2. Centrifuge the whole blood at 1000 × *g* and 4 °C for 10 min to separate the plasma from the blood cells.
3. Remove the plasma in the upper layer and transfer it to a clean centrifuge tube.
4. The plasma is incubated at 55 °C for 1 h to inactivate the complement proteins (*see* **Note 31**).
5. Meanwhile, wash the blood cells with PBS 3–5 times, and centrifuge it at 1000 × *g* and 4 °C for 10 min during each wash (*see* **Note 32**).
6. Mix the inactivated plasma and pre-washed blood cells together. The treated bloods are stored at 4 °C for mosquito blood feeding within 1 week.

3.6.2 Membrane Blood Feeding

Both *C. pipiens* and *A. aegypti* can acquire WNV infection via membrane blood feeding.

1. Mix treated blood and WNV supernatant at a ratio of 1:1 (v/v) (*see* **Note 33**).

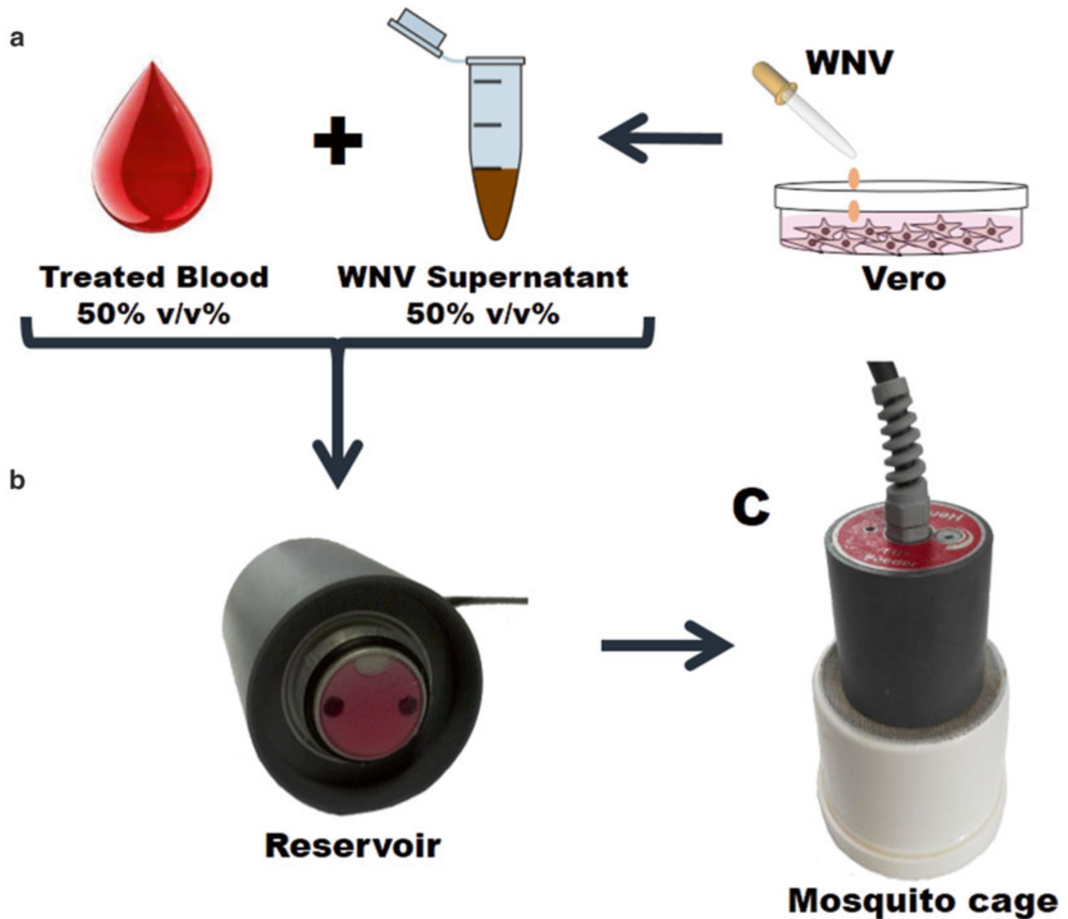

Fig. 2 The schematic diagram of mosquito membrane blood feeding. (**A**) The treated blood and WNV supernatant from Vero cells are mixed at a ratio of 1:1 (v/v). (**B**) The mixture is added into the reservoir of the Hemotek® feeding system. (**C**) The installed reservoir is placed on the top of the mosquito cage

2. Add the mixture into the reservoir (*see* **Note 34**) of the Hemotek® feeding system. The feeding system keeps the reservoir at 37 °C for mosquito blood meal.
3. Place the installed reservoir on the top of the mosquito cages (Fig. 2). Keep the reservoir there for approximately 30 min until most of the mosquitoes are engorged in blood (*see* **Note 35**).
4. After feeding, the mosquitoes are anesthetized on ice (*see* **Note 36**). The fed mosquitoes are picked out and transferred into a new sealed container for rearing.
5. The fed mosquitoes are maintained in a Precision® Model 818 incubator at 28 °C and 80% humidity for 8–10 days for further analysis.

3.7 WNV Infection via Blood Feeding on Mice

C. pipiens, but not *A. aegypti*, can efficiently acquire the infection through blood feeding on WNV infected mice.

1. Inoculate approximately 1×10^8 pfu of WNV into a C57BL/6J mouse through intra peritoneal (I.P.) inoculation.
2. The infected mice are anesthetized with Nembutal (100 μl/13 g) (*see* **Note 37**) and placed on the top of the mosquito cages. Allow the *C. pipiens* mosquitoes to feed on the blood meal for 30 min.
3. After blood feeding, the mosquitoes are anesthetized on ice. The engorged mosquitoes are picked out and transferred into a new container for rearing.
4. The fed mosquitoes are maintained in a Precision® Model 818 incubator at 28 °C and 80 % humidity for 8–10 days for further analysis.

3.8 Immunofluorescence Staining of WNV Infected Mosquito Tissues

1. Infect female mosquitoes by thoracic microinjection using 1000 $M.I.D._{50}$ of WNV or membrane blood feeding.
2. Dissect various mosquito tissues, including the salivary glands, hemocytes, midgut, ovaries, and Malpighian tubule (*see* **Note 38**).
3. Place the tissues on Silane-Prep slides (*see* **Note 39**), and fix them using 4 % PFA at RT for 30 min or at 4 °C overnight (O/N).
4. Wash the tissues five times with PBS.
5. The tissues are permeabilized with a PBS buffer containing 0.1 % Triton X-100 and 0.1 % BSA at RT for 30 min.
6. Wash the tissues three times with PBS.
7. Block the tissues with Perm/Wash buffer at RT for 30 min.
8. Wash the tissues three times with PBST (PBS containing 0.05 % Tween 20).
9. The primary antibody is diluted in a Perm/Wash buffer at a 1:500 (v/v%) ratio and incubated with the tissues at RT for 2 h.
10. Wash the tissues three times with PBST.
11. The secondary antibody is diluted in the Perm/Wash buffer at a 1:1000 ratio (v/v%) and incubated with the tissues at RT for 1 h.
12. Wash the tissues five times with PBST.
13. Mount the slides with 50 μl of ProLong® Gold antifade reagent (*see* **Note 40**).
14. Now, the slides are ready for microscope examination and can be stored at 4 °C in darkness for over 1 year.

4 Notes

1. Larvae food can be stored at 4 °C for no more than 1 month.
2. The cells grow better without antibiotics; however, the addition of antibiotics does not influence WNV amplification in the cells.
3. C6/36 cells are semi-adherent cells. The cells attach to the bottom of the culture flask but can be detached by powerful blowing using an automatic pipette. Digesting C6/36 cells with trypsin during subculture is not recommended.
4. VP-SFM is specific for virus maintenance in mammalian cells and increases viral viability in the culture medium. Also, this serum free medium can eliminate the interference caused by FBS in subsequent experiments.
5. The tip of the capillary tube needle should be cut before microinjection. Also label the capillary tube using a proper scale (i.e., 1 mm per interval).
6. WNV derived from C6/36 cells is used for microinjection, while WNV derived from Vero cells is used for membrane blood feeding.
7. More cells improve virus yield but excess cell growth is not recommended. Too old cells are not susceptible to viral infection.
8. The Cytopathic Effect (CPE) phenomenon: for C6/36 cells, the cells are inflated, and the cell surface is rough. Sometimes, cell fusion will also be observed in C6/36 cells. For Vero cells, the cells are apoptotic and detach from the bottom of the culture container [17].
9. To determine the best time to harvest WNV, the culture medium should be collected every half day to extract viral genomic RNA for RT-QPCR determination. The infected supernatant will be collected when the virus genomic RNA reaches the peak during the time course detection.
10. The aliquoted volume of WNV is variable for different experiment. Specifically, 50 μl per tube is suitable for WNV microinjection, and 1 ml per tube is best for WNV membrane blood feeding.
11. The infection of WNV shows more virulent in Vero cells than in C6/36 cells.
12. The mated female mosquitoes are more efficient in blood feeding than the fresh mosquitoes.
13. The mosquitoes feed on an anesthetized mouse placed on the top of mosquito cage.
14. Female mosquitoes can be reused for laying eggs.

15. The eggs of *A. aegypti* can be stored at RT for several years.
16. The eggs of *C. pipiens* are laid into water and cannot be stored on filter paper. Therefore, *C. pipiens* mosquitoes should be maintained generation by generation.
17. A low density of larvae in each container is good for the health (activity and body size etc.) of mosquitoes.
18. The water needs to be refreshed when it becomes turbid, about every 7 days for the first to the third instar larva, and every 4 days for the fourth instar larva. Excess food is not recommended.
19. Usually, the pupae emerge to adults within 4 days.
20. The age of mosquitoes is speculated from the time that pupae emerge to adults.
21. *A. aegypti* is easily anesthetized on ice within 3–5 min, however *C. pipiens* shows more tolerance for low temperatures and should be anesthetized for up to 30 min.
22. Please make sure the mosquitoes are thoroughly anesthetized during microinjection. Keeping the environmental temperature lower than 20 °C, especially for the *C. pipiens.*
23. Do not leave the mosquitoes on the frozen operation platform more than 10 min. The mosquitoes may be freezed and dead.
24. The mosquitoes should be placed on their side for microinjection. The injection site locates in the middle of lateral thorax.
25. For mosquito sacrifice, the mosquitoes should be kept at −80 °C for more than 10 min.
26. After the mosquitoes are homogenized in RI buffer, the material can be stored at −80 °C for up to 1 month before RNA extraction.
27. An one-column DNase treatment is used to remove the genomic DNA contamination.
28. The gene of WNV envelope (E) protein is used for RT-QPCR detection of WNV genomic copies.
29. The Reed–Muench method is a common statistic approach for determining 50% endpoints [18].
30. EDTA can also be used for anticoagulation during blood preparation. However, this chemical may influence mosquito metabolism, and therefore is not recommended.
31. Shake the plasma several times during the heating incubation to inactive the complement proteins completely. The activation of complement proteins can dramatically impair WNV infection in mosquitoes.
32. The purpose of washing blood cells is to get rid of the heparin (anticoagulation) on the surface of blood cells, which may influence WNV infection in the mosquitoes.

33. The operation should be gentle to avoid the damage of erythrocytes.
34. For the membrane feeding by Hemotek® system, the volume of 1 ml mixture of blood and virus is enough to feed 50 mosquitoes approximately.
35. Mosquitoes should be fed blood in darkness.
36. Anesthetize *A. aegypti* for 5 min; the time should be prolonged more than 15 min for *C. pipiens.*
37. Nembutal does not influence WNV infection in mosquitoes.
38. Refer to the video of *A. aegypti* tissue dissection [19].
39. Make a circle by liquid-repellent slide marker pens, and then place the tissues in the circles. A droplet of diluted antibody can be added into the circle for staining.
40. Place coverslips on the stained sample gently to avoid generating bubbles.

Acknowledgement

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Chapter 14

Field Surveillance Methods for West Nile Virus

Samuel B. Jameson and Dawn M. Wesson

Abstract

Surveillance of West Nile virus (WNV) in populations of mosquitoes or sentinel animals is of primary importance when assessing the risks to human health posed by naturally circulating WNV. In this chapter we focus on methods for detection of both WNV and its enzootic transmission. Methods for virus detection include CDC mini light trap, CDC gravid trap, and dead bird surveillance. Methods for transmission detection include passive box traps, chicken-baited traps, and sentinel chickens.

Key words West Nile virus, Light trap, Sentinel chickens, Passive box trap, Gravid trap, Dead bird surveillance

1 Introduction

The primary question that should be asked prior to any attempt to collect field data on West Nile virus (WNV) is, "What is being measured?" This chapter outlines the most popular and promising methods for detection of West Nile Virus and its transmission in nature. The methods are separated based on whether each measures the presence or transmission of WNV. The distinction between presence and transmission is not a trivial matter. Detection of WNV nucleic acid in mosquito pools does not necessarily indicate enzootic cycling of the virus. Non-infectious viral particles, non-disseminated virus, and non-competent vectors may all confound the results of commonly used quantitative rtPCR assays. To detect enzootic transmission of WNV, sentinel animals or passive box traps must be used. The sero-conversion of sentinel animals definitively confirms enzootic transmission of WNV in the area of the animal while passive box traps are highly suggestive of enzootic transmission [1, 2].

Because human West Nile Virus (WNV) disease is the result of an aberrant zoonotic infection, prediction of human disease burden relies heavily on monitoring the enzootic cycle of WNV in birds and mosquitoes. In the USA, sentinel chickens are the most

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frequently used birds to detect enzootic transmission as the capture and testing of live wild birds is strictly regulated, expensive, labor intensive and requires specialized training. In other regions, wild birds and non-avian sentinel animals, like horses, are used to detect enzootic transmission [3–6]. Appropriate sentinel animals demonstrate a profound immunological response to WNV infection but do not exhibit high viremias. This allows for detection of seroconversion without contributing to further transmission of the virus. Testing mosquito vectors for WNV has become widely favored by many US mosquito control districts over sentinel chickens in recent years given its relative cost-effectiveness and ability to detect circulating virus well before human cases are detected. In the USA, sentinel chickens provide valuable information about enzootic transmission of WNV, but they do not provide a reliable method for detecting WNV prior to human cases of WNV disease [7]. However, this may not be the case for other regions. The biology and behaviors of local vectors as well as the region's history with WNV inform the most appropriate sampling method for a given region. The most comprehensive assessment models for human WNV infection in the USA are informed by sentinel chicken, dead bird, mosquito, and climatic data [8, 9]. Other models have shown that reasonable predications of human WNV disease in the USA can be made using only climatic data, historic health data, and publically available bird census data [10]. Limited funding for WNV surveillance is the reality in most locations. In such instances it is advisable to focus efforts on a single method to obtain robust, quality data sets.

2 Materials

2.1 Virus Detection

2.1.1 Carbon Dioxide-Baited, CDC Light Trap (See Note 1)

1. Six volt, twelve amp hour, sealed, lead acid battery.
2. CDC miniature light trap.
3. Mesh collection bag.
4. Light duty rope or cord for hanging trap.
5. Dry ice.
6. Ice.
7. Cooler.
8. Label tape.
9. Permanent marker.

2.1.2 CDC Gravid Trap

1. Six volt, twelve amp hour, sealed, lead acid battery.
2. CDC gravid trap.
3. Mesh collection bag.
4. Gravid trap infusion (*see* Table 1 for recipes).

Table 1 Recipes for gravid trap infusions

Ingredients	Equipment	Preparation	Citation
• Straw • Milk or milk albumin • Water	• Large mesh bag • 65-gallon plastic trashcan	1. Stuff mesh bag with hay 2. Fill trashcan with water 3. Add hay bag and small amount of milk or milk albumin to trashcan 4. Leave in a sunlit area for at least 1 week	[16]
• 0.45 kilograms of timothy hay • 20 L of aged tap water	• 20 L plastic container	1. Place hay in plastic container 2. Fill container with aged tap water	[17]
• 0.45 kilograms of timothy hay • Four cups of rabbit chow • 20 L of aged tap water • One packet of brewers yeast	• 20 L plastic container	1. Place all ingredients except for water in plastic container 2. Fill container with aged tap water 3. Allow infusion to age outdoors for 3 days	[17]
• Softball-size amount of fresh horse or cow manure • Commercial algaecide • Straw or hay • Tap water	• Large plastic trashcan or other convenient container	1. Fill trashcan with tap water to desired volume 2. Add algaecide according to manufacturer's directions 3. Add manure 4. Add one handful of straw or hay for each gallon of water used 5. Allow to age outdoors for 4–5 days	[18]
• Fish oil emulsion • Hay • Water	• Large plastic trashcan or other convenient container	1. Fill trashcan with hay 2. Fill with tap water 3. Allow to age outdoors for 3–4 days 4. Add one fluid ounce of fish oil emulsion per gallon of water used 5. Allow to age outdoors for an additional 3–4 days	[19]

5. Infusion pan.
6. Dry ice or ice.
7. Cooler.
8. Label tape.
9. Permanent marker.

2.1.3 Dead Bird Surveillance (Focus on Family Corvidae, Crows and Jays)

1. Sterile cotton swabs.
2. VecTest West Nile virus antigen assay or similar commercial test.

2.2 Transmission Detection

2.2.1 Passive Box Traps

1. Passive Box Trap.
2. Nucleic acid preservation cards.
3. Honey.
4. Compressed carbon dioxide cylinder with regulator.
5. Dry ice or ice.
6. Cooler.
7. Label tape.
8. Permanent marker.
9. Fipronil (optional) (*see* **Note 2**).
10. Octenol bait (optional) (*see* **Note 3**).

2.2.2 Chicken-Baited Traps

1. Lard can trap adapted to hold chicken (Figs. 1 and 2).
2. One chicken per trap.
3. Insect-free holding facility.
4. Sterile, 23 G needles.
5. Sterile, 5 mL syringes.
6. Red-top Vacutainer tubes.

2.2.3 Sentinel Chickens

1. Between seven and ten seronegative chickens per sentinel flock (*see* **Note 4**).
2. Predator-proof chicken coop located near suspected vector habitat.
3. Sterile, 23 G needles.
4. Sterile, 5 mL syringes.

3 Methods

3.1 Virus Detection

3.1.1 Carbon Dioxide-Baited, CDC Light Trap

1. Fill the insulated canister with dry ice and securely attach the lid.
2. Attach the cover of the trap to the bottom of the dry ice container hang the apparatus from a branch or other fixed object.

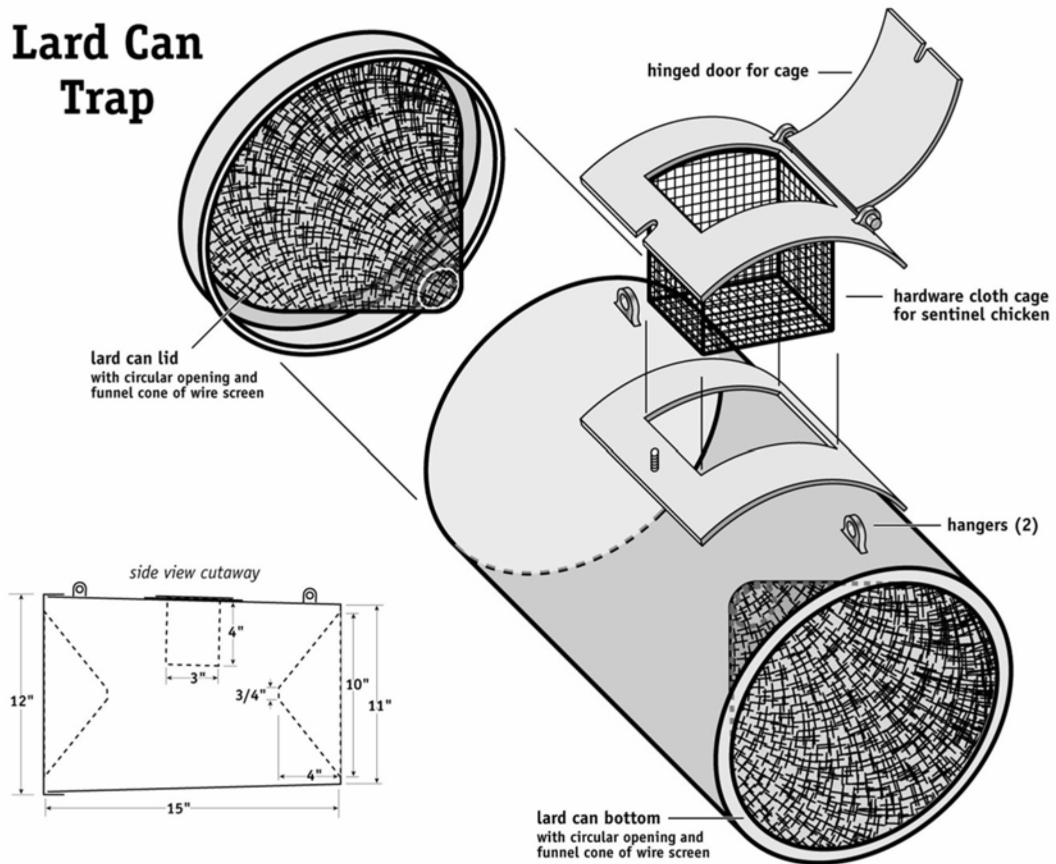

Fig. 1 Diagram of a lard can trap adapted for use with sentinel chickens. This trap will collect all the mosquitoes attracted to the sentinel chicken as well as provide transmission data by checking for seroconversion 2 weeks post-exposure. Photo credit: Jim Newman, University of Florida—Florida Medical Entomology Laboratory

3. Place the traps such that the fan assembly is roughly 1 and 1.5 m from the ground in an area relatively free of surrounding obstructions. If multiple traps are to be used at a single area, space them greater than 25 m apart (*see* **Note 5**).
4. Attach a clean collection bag to the bottom of the fan assembly and connect the wires securely to the battery terminals (*see* **Note 6**).
5. Place traps in the field shortly before dusk and collect the following morning shortly after sunrise (*see* **Note 7**).
6. Carefully remove the collection bag while the trap is still running.
7. Label the collection bag with label tape and a permanent marker.

Fig. 2 Photograph of an unmodified lard can trap (foreground) that is properly deployed. This collection is baited with dry ice contained in the blue cooler (background). Carbon dioxide is released from a simple tube connection between the cooler and the interior of the trap. Photo credit: Jim Newman, University of Florida—Florida Medical Entomology Laboratory

8. Place the collection bag in a cooler containing ice or dry ice.
9. Keep mosquitoes cold or frozen until testing.

3.1.2 CDC Gravid Trap

1. Place infusion pan in on flat ground at least 1 m away from buildings, trees, or large shrubs.
2. Assemble CDC gravid trap by placing a clean, double-ringed collection bag over the exhaust end of the trap.
3. Place the arms of the trap on the long sides of the infusion pan and add infusion to the pan until it is two and one-half or three centimeters below the intake side of the trap.
4. Connect the trap's wires firmly to the terminals of the 6 V battery (*see* **Note 6**).
5. Place traps in the field shortly before dusk and collect the following morning shortly after sunrise (*see* **Note 7**).
6. Carefully remove the collection bag while the trap is still running.
7. Label the collection bag with label tape and a permanent marker.
8. Place the collection bag in a cooler containing ice or dry ice.
9. Keep mosquitoes cold or frozen until testing.

3.1.3 Dead Corvid Surveillance (See Note 8)

1. Corvids determined to be dead for fewer than 48 h should be refrigerated and sampled the same day or frozen at −20 °C and sampled in fewer than 4 days.
2. Obtain samples by swabbing the oral cavity and proximal esophagus of the bird with a sterile cotton swab.
3. Place the swab directly into 1 mL of VecTest grinding buffer (included in test kit).
4. Perform test following manufacturer's directions.

3.2 Transmission Detection

3.2.1 Passive Box Trap

1. Soak nucleic acid preservation cards overnight in honey. Optionally, fipronil may be added to the honey at a final concentration of six hundredths of one percent.
2. Suspend passive box trap from a tree or other fixed object such that the bottom of the trap is approximately 1 and 1.5 m from the ground (*see* **Note 9**).
3. Attach the regulator of the cylinder of compressed carbon dioxide to the trap using the supplied hoses (multiple traps can be run from a single cylinder, though traps should be separated by no fewer than 20 m).
4. Supply each trap with 250 mL of carbon dioxide per minute.
5. If desired, place an octenol bait outside of the opening to the trap after it is set up.
6. Remove nucleic acid preservation cards for testing.
7. Label passive box trap with label tape and permanent marker and place in cooler.
8. Keep mosquitoes cool or frozen for species identification or further testing.

3.2.2 Chicken-Baited Traps

1. Suspend each lard can trap horizontally from a tree branch or other stationary object such that the bottom of the trap is approximately 1 and 1.5 m from the ground (*see* **Note 9**).
2. Place the chicken in the protected, bait section of the trap.
3. Expose each chicken for only one night.
4. House chicken in insect-free facility for at least 2 weeks to allow for seroconversion.
5. Take a 2–3 mL sample of blood from the brachial vein of each chicken using a 23 G needle and 5 mL syringe (*see* **Note 10**).
6. Allow blood to clot for greater than 30 min in a sterile, additive-free Vacutainer tube.
7. Centrifuge at 3000 times gravity for 10 min.
8. Serum can be used immediately or frozen at −20 °C until testing with a commercial antibody detection kit.

3.2.3 Sentinel Chickens

1. Keep flocks of between seven and ten hens in secure, predator-proof coops situated in environments favorable to the suspected vector species.
2. Take a 1–2 mL sample of venous blood from the brachial vein and prepare serum as previously described in **steps 6** and 7 of Subheading 3.2.2.
3. Serum can be used immediately or frozen at −20 °C until testing with a commercial antibody detection kit.

4 Notes

1. The most common trap for host-seeking WNV vectors is the CDC light trap. This trap can be configured in many ways to best attract the targeted species. Many mosquito control districts have found that running the trap with a source of carbon dioxide and the light turned off provides the best results. Carbon dioxide may be supplied by either dry ice contained in a large, insulated beverage cooler with small holes drilled in it or from a small cylinder of the compressed gas. The amount of carbon dioxide released is directly proportional to the number of mosquitoes collected by these traps [11]. It is most common to use 1 kg of dry ice per trap per night. The flow rate of gas can be roughly controlled by adjusting the size of the holes in the insulated container.
2. The use of fipronil in Passive Box Traps is reserved for instances where dead mosquitoes are desirable or a large catch number is suspected. The fipronil does not reduce feeding on the honey or effect downstream arbovirus detection, but kills the mosquito shortly after feeding. This may be desirable with large catches as it reduces competition for the honey-soaked card. To reduce desiccation of dead samples, a moist sponge may be included in the trap [12].
3. Octenol lures will additionally attract mosquito species like *Aedes aegypti* and *Aedes albopictus* that prefer to feed on humans [13].
4. Although the most often cited number of chickens in a sentinel flock is ten, some research has indicated that a flock size of 6–7 chickens produces the same quality data as a ten-bird flock at a reduced cost [14].
5. The precise height of the trap is not what is of concern here. Rather, all traps in a given collection scheme should be hung at the same height so as to reduce variability amongst collections. It is important that these traps are hung away from the ground and on an object free of ants. CDC light traps should be hung away from competing sources of light (if collecting at night)

and out of direct sunlight (if collecting during the day) to improve trap performance.

6. Ensure the battery terminals are free from corrosion and wires are not damaged or frayed. Always check the airflow of trap after connecting the battery. Most mosquito traps will readily run in reverse if the wires are connected to the opposite battery terminal. In wet areas, small waterproof plastic boxes may be useful in protecting the battery components of a trap.
7. The relative timings of dawn and dusk are preferable to fixed times of day (except in extreme latitudes). Vector species of WNV are most likely to be active in the span just before sunset until just after sunrise.
8. Although many different kinds of birds may be tested for the presence of WNV, oral swab tests work best on corvids (like magpies, jays, and crows). To reliably test other types of birds, necropsies must be performed and tissue samples tested. If necropsies are used, heart tissue is a reliable indicator of WNV infection in a wide range of birds [15].
9. The precise height of the trap is not what is of concern here. Rather, all traps in a given collection scheme should be hung at the same height so as to reduce variability amongst collections. It is important that these traps are hung away from the ground and on an object free of ants.
10. The size of the syringe used for blood collections is a matter of preference. Five-milliliter syringes are stouter than 1- or 3-mL syringes and easier to collect blood using one hand. Only 23-G needles should be used. Needles larger than this are likely to rupture the brachial vein. Needles smaller than this are likely to hemolyze the sample. Hemolysis can be minimized by drawing the plunger of the syringe with the least required force.

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Chapter 15

Detection Protocols for West Nile Virus in Mosquitoes, Birds, and Nonhuman Mammals

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Abstract

West Nile virus is the most widespread mosquito-borne virus in the world, and the most common cause of encephalitis in the USA. Surveillance for this medially important mosquito-borne pathogen is an important part of public health practice. Here we present protocols for testing environmental samples such as mosquitoes, nonvertebrate mammals, and birds for this virus, including RT-PCR, virus isolation in cell culture, and antigenic assays, as well as serologic assays for antibody detection.

Key words West Nile, Flavivirus, Surveillance, RT-PCR, ELISA, PRNT

1 Introduction

1.1 Biology and Epidemiology

West Nile virus (*Flavivirus*, *Flaviviridae*) is a spherical enveloped virion, ~50 nm in diameter, with a capsid surrounded by a host-derived lipid membrane containing two glycoproteins, the envelope (E) and the membrane protein (M). Multiple copies of the capsid protein surround single-stranded positive sense RNA, approximately 11 kb in length, which contains a single open reading frame that is translated cotranslationally and posttranslationally by cellular and viral proteases into three structural proteins, capsid (C), premembrane (prM)/membrane (M), and envelope (E) at the 5′ end of the genome and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) at the 3′ end.

WNV is currently the most widely distributed arbovirus in the world, occurring on all continents except Antarctica. The virus was first isolated from the blood of a febrile woman in the West Nile district of Uganda in 1937 [1]. Prior to 1996, occasional epidemics occurred in Africa, Eurasia, Australia, and the Middle East, but few cases of West Nile neuroinvasive disease (WNND) were observed [2]. Virus activity increased noticeably from 1996 to 1999 in the Mediterranean basin, southern Romania, and the

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Volga delta in southern Russia; then in 1999, WNV was introduced into the Western Hemisphere, where it spread rapidly in the USA, Canada, and Central and South America over the next four years. WNV is now the leading cause of encephalitis in the USA and Canada.

Worldwide, WNV is maintained in nature in an enzootic cycle between ornithophilic mosquitoes and susceptible avian hosts. Epizootics/epidemics occur in horses and humans, but these incidental hosts are considered "dead-end" because the level of viremia is below the threshold required to infect mosquitoes. Approximately 65 different mosquito species and 326 bird species have been found infected in the USA, although not all are competent hosts [3]. *Culex* species mosquitoes are the major vectors globally, with the particular species dependent on geographic location. In the northeastern USA, it has been estimated that *Culex pipiens* L. is likely responsible for 80% of human WNV infections [4]. In the western USA, *Cx tarsalis* is the predominant vector to humans, and in the southern USA, *Cx quinquefasciatus*. Birds belonging to the order *Passeriformes* are the major amplification hosts.

Phylogenetic analysis reveals two major lineages of WNV, I and II [5], and several possible lineages that are newly recognized, III [6], IV [7], and V [8]. Lineage 1 includes three sublineages: 1a, which is distributed in Africa, the Middle East, Europe, and the Americas; 1b, found in Australia, also known as KUN; and lineage 1c, which includes strains isolated in India. Lineage II is generally confined to sub-Saharan Africa and Madagascar. Migratory birds play a major role in the spread of WNV throughout the world. Migratory routes from Africa to Europe and Asia are well understood, and WNV virus and antibodies have been isolated from migrating species [9, 10]. Successful spread of the virus by birds is dependent on avian viremia that is sufficiently high and long lasting to survive during migratory travel and remain infectious to mosquitoes.

Infection with the virus leads to a wide range of disease symptoms from mildly febrile to severely neurologic, but asymptomatic infections occur most frequently. While the disease appears to be more severe in older individuals, all ages may become severely ill. Until 2008, severe WNND in humans almost exclusively had been associated with lineage 1 strains, but since 2004, lineage 2 strains have been found circulating in Central, Southern and Eastern Europe, and have been responsible for major outbreaks in Hungary, Austria, Greece, Romania, Italy, and the Volgograd region of Russia [11–13]. There have been approximately 42,000 cases of WN disease in the USA between 1999 when the virus was introduced into the New York City area and December 2014 (Arbonet, Arboviral Diseases Branch, CDC).

1.2 Clinical

Laboratory diagnosis of WNV infection is predominantly serological [14], although caution is advised because of the high degree of cross-reactivity among flaviviruses [15, 16]. Paired acute and convalescent sera are recommended for confident determination of etiology of disease, where a fourfold rise between the two is necessary for confirmation [17], or a fourfold difference between related flaviviruses. Diagnosis by serological methods is particularly difficult in areas where Japanese encephalitis virus (JEV) and/or dengue virus (DENV) are co-circulating, e.g., India and tropical Americas, respectively, and more recently, Zika virus (*ZIKV*; *Flavivirus, Flaviviridae*). Secondary infections present additional problems in interpretation. Serologic assays include the standard IgM and IgG enzyme-linked immunosorbent assay (ELISA), immunofluorescent antibody assay (IFA), microsphere immunoassay (MIA), and confirmatory plaque reduction neutralization test (PRNT). Many horses are now vaccinated against WNV and therefore the diagnostician must be certain to distinguish naturally occurring antibody from that resulting from vaccination. Laboratory protocols for the standard PRNT assay are described below (Subheading 3.11). Procedures for RT-PCR and virus isolation assays (Subheadings 3.6–3.8) are seldom used for clinical samples because of transient and low viremia.

1.3 Surveillance

Surveillance for medically important mosquito-borne pathogens is an important part of public health practice. It is meant to provide an early warning of increased levels of viral activity allowing control measures to be undertaken. Field specimens, especially mosquitoes and dead birds, collected as part of surveillance programs, are tested for the presence of viral nucleic acid by standard or real-time RT-PCR (Subheadings 3.6 and 3.7, respectively), viral antigen by IFA (Subheading 3.9) and antigen-capture dipstick assay (Subheading 3.10), or infectious virus (Subheading 3.8). Rapid test protocols have been developed in response to the expansion of WNV in the USA [18–20]. In some locations, sentinel birds are tested for antibody by indirect ELISA on serum (Subheading 3.13), using protocols similar to those used in equine and human diagnostics. A flowchart for testing field specimens is presented in Fig. 1. In addition, serological surveys using indirect ELISA (Subheadings 3.13) and PRNT (Subheading 3.11) of various wildlife populations such as small mammals, deer, and wild birds have yielded important information about distribution of WNV; however, the problem is one cannot be certain where or when the infection occurred. Horses and other equids that die following neurologic symptoms should also be tested, first for rabies, then for WNV, by cell culture virus isolation or RT-PCR.

1.4 Safety

It is important to note that WNV is classified as a BSL-3 agent by CDC [21]. Once WNV is identified in a diagnostic specimen, sub-

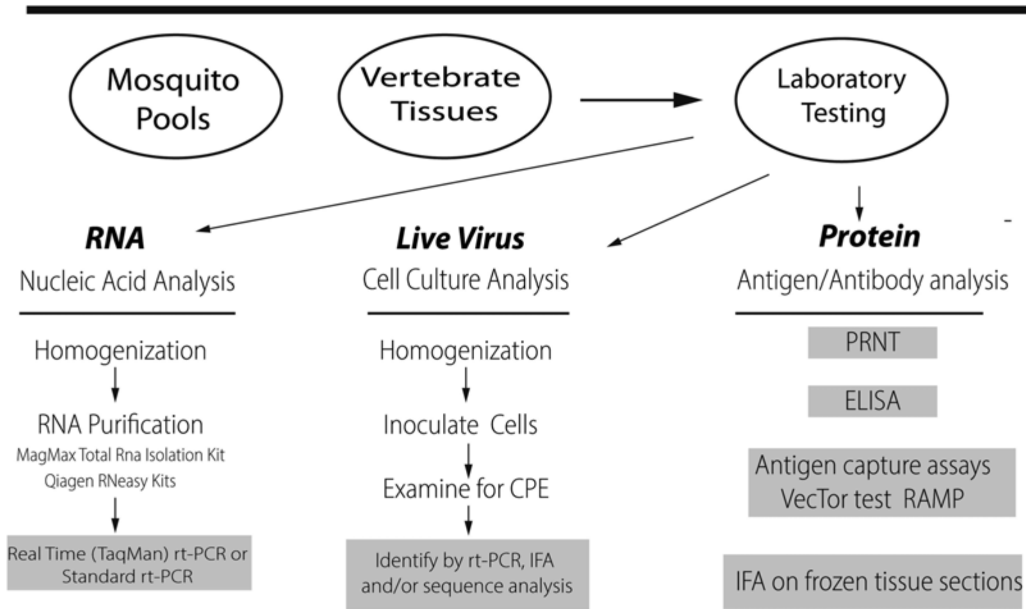

Fig. 1 Typical workflow management of field collected samples

sequent handling of infectious virus from that sample should be conducted in a Biosafety Level-3 laboratory using a Class II biological safety cabinet (BSC) by laboratory staff who are trained to work at this level of containment. Staff must wear appropriate personal protective gear including lab coats, goggles, and gloves to protect themselves from infection. It is best practice for all submitted specimens to be handled as infectious material.

2 Materials

2.1 Preparation of Mosquito Test Samples

1. Class II biological safety cabinet (BSC).
2. Retsch Mixer Mill, MM 301 (Retsch, Inc., Newtown, PA) (*see* **Note 1**).
3. TissueLyser adapter set (holds 2×24 microfuge tubes) for Mixer Mill 301 (Qiagen Inc., Valencia, CA).
4. Qiagen benchtop centrifuge, Model 4-15C, with 2×96 plate rotor and deep well buckets (*see* **Note 2**).
5. Daisy 4.5 mm steel airgun shot (BBs), zinc plated.
6. Mosquito diluent: PBS supplemented with 20% heat-inactivated fetal bovine serum (FBS), 100 units/mL of penicillin,

100 μg/mL of streptomycin, 10 μg/mL gentamicin, 1 μg/mL Fungizone (Amphotericin B).

7. BA-1 Diluent: M-199 medium with Hanks' salts, 1% bovine serum albumin, 350 mg/L of sodium bicarbonate, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 10 μg/mL gentamicin, 1 μg/mL of Fungizone in 0.05 M Tris, pH 7.4. Store at 2–8 °C, expiration 3 weeks.
8. RLT Lysis buffer for RNA purification using RNeasy Mini Kit protocol (*see* **item 4** in Subheading 2.4).
9. Lysis/Binding Solution for RNA purification by MagMAX technology (*see* **item 12** in Subheading 2.5).

2.2 Preparation of Nonhuman Mammal Test Samples

1. Materials listed for mosquitoes (*see* Subheading 2.1).
2. Scalpels, sterile disposable.

2.3 Preparation of Avian Test Samples

1. Materials listed for mosquitoes (*see* Subheading 2.1).
2. Scalpels, sterile disposable.
3. Cotton swabs with plastic shafts or swabs made of polyester (polyester fiber tipped applicator, Thermo Fisher Scientific) (*see* **Note 3**).

2.4 RNA Purification by Qiagen RNeasy (See Note 4)

1. 95–100% ethanol, ACS grade or higher quality.
2. β-mercaptoethanol.
3. RNeasy Mini Kit (Qiagen, Inc.). Each kit contains spin columns, 1.5 mL collection tubes, 2 mL collection tubes (additional tubes may be purchased separately), RNase-free water, and buffers RLT, RW1, and RPE (*see* **items 4–6** in this section for buffer preparation and storage and *see* **Note 5** for safety precautions).
4. Buffer RLT (Lysis Buffer): check buffer for precipitate and if present incubate at 80 °C until dissolved. Add β-ME (10 μL/mL RLT), store at RT and use within 1 month (*see* **Note 6**).
5. Buffer RW1 (Wash 1 Buffer): ready to use without additions, Store at RT.
6. Buffer RPE (Wash 2 and 3 Buffer): add four volumes of 96–100% ethanol to prepare a working solution. Store at RT.
7. Microcentrifuge.

2.5 RNA Purification by MagMAX Technology

1. Polystyrene U bottom 96-well plates and lids.
2. 100% ethanol, ACS grade or higher quality.
3. 100% isopropanol, ACS grade or higher quality.
4. β-mercaptoethanol.
5. Orbital shaker for 96-well plates.

6. Magnetic stand for 96-well plates: Ambion 96-well magnetic stands are recommended.
7. MagMAX-96 Total RNA Isolation Kit (Ambion/Thermo Fisher Scientific). Each kit contains Lysis/Binding Concentrate, Wash Solution 1 Concentrate, Wash Solution 2 Concentrate, RNA Rebinding Concentrate, Elution Buffer, MagMAX Turbo DNase Buffer, RNA Binding Beads, Lysis/Binding Enhancer, and TURBO DNase (*see* **items 8–11** in this section for buffer preparation and storage).
8. Wash Solution 1: Add 6 mL 100% isopropanol to bottle labeled Wash Solution 1 Concentrate. Store at RT.
9. Wash Solution 2: Add 44 mL 100% ethanol to bottle labeled Wash Solution 2 Concentrate. Store at RT.
10. RNA Rebinding Solution: Add 6 mL 100% isopropanol to the bottle labeled RNA Rebinding Concentrate and mix well. Store at RT.
11. Bead Mixture (prepare on day of use). Vortex the RNA Binding beads at moderate speed to form a uniform suspension before pipetting. Combine equal volumes of RNA Binding Beads and Lysis/Binding Enhancer. The final volumes required are 20 μL per reaction or 2.2 mL per plate (includes 10% overage, which is recommended to account for pipetting error). Place the prepared bead mix on ice and use within 1 day.
12. Lysis/Binding Solution (prepare on day of use). For each reaction add 63 μL of β-mercaptoethanol to 77 μL of Lysis/Binding Solution Concentrate or add 9 mL of β-mercaptoethanol to 11 mL of Lysis/Binding Solution Concentrate (entire bottle). Mix thoroughly, store at RT and use within 1 day (*see* **Note 6**).
13. Diluted TURBO DNase (prepare during Wash **step 2**, see item 7 in Subheading 3.5). For one reaction add 1 μL of DNase to 49 μL of MagMAX TURBO DNase Buffer. For 96 reactions, add 5.4 mL of TURBO DNase to 5.4 mL of MagMAX TURBO DNase Buffer.

2.6 Standard RT-PCR

1. Thermocycler, Applied Biosystems, Model 2720.
2. PCR Enclosure, Labconco Purifier.
3. Qiagen One-step RT-PCR kit (cat. No. 210212).
4. Primers (Table 1, Sets 4–5). Prepare 25 μM stocks in RNase/DNase-free water, and store in aliquots at −20 °C in a manual defrost freezer.
5. Owl EasyCast horizontal B2 mini gel system (Owl Separating Systems Inc. Portsmouth NH).
6. Gel electrophoresis combs (variety of sizes).
7. Ultrapure agarose (Invitrogen/Thermo Fisher Scientific).

Table 1
Primers and probes for detection of WNV by standard or real-time RT-PCR

Set	Target	Description	Sequence (5′–3′)	Size (bp)	Reference
1	WNV env	1160 Forward 1229 Reverse 1186 Probe	TCA-GCG-ATC-TCT-CCA-CCA-AAG GGG-TCA-GCA-CGT-TTG-TCA-TTG 6FAM-TGC-CCG-ACC-ATG-GGA-GAA-GCT-C-TAMRA	70	[19]
2	WNV NS5	NS5 Forward NS5 Reverse NS5 Probe	GCT-CCG-CTG-TCC-CTG-TGA CAC-TCT-CCT-CCT-GCA-TGG-ATG 6FAM-TGG-GTC-CCT-ACC-GGA-AGA-ACC-ACG-T-TAMRA	168	[40]
3	WNV NS1	3111 Forward 3239 Reverse 3136 Probe	GGC-AGT-TCT-GGG-TGA-AGT-CAA CTC-CGA-TTG-TGA-TTG-CTT-CGT 6FAM-TGT-ACG-TGG-CTG-AGA-CGC-ATA-CCT-TGT-TAMRA	149	[18]
4	WNV capsid PrM	212 Forward 619 Reverse	TTG-TGT-TGG-CTC-TCT-TGG-CGT-TCT-T CAG-CCG-ACA-GCA-CTG-GAC-ATT-CAT-A	432	[19]
5	WNV NS5	WN 9483 WN 9794	CAC-CTA-CGC-CCT-AAA-CAC-TTT-CAC-C GGA-ACC-TGC-TGC-CAA-TCA-TAC-CAT-C	326	[19]

8. GelRed nucleic acid gel stain, 10,000× in water (Biotium, Inc.) (*see* **Note 7**).
9. Tris–acetate–EDTA (TAE) Buffer, 50× (Thermo Fisher Scientific). Dilute the 50× Buffer to a working concentration of 1× before use. For each electrophoresis fresh 1× buffer should be used. The 1× composition is 40 mM Tris, 20 mM Acetate and 1 mM EDTA with a pH around 8.6.
10. 10× BlueJuice gel loading buffer (Invitrogen/Thermo Fisher Scientific) (*see* **Note 8**).
11. 1 kb ladder (1 μg/mL) (Invitrogen/Thermo Fisher Scientific). Working Solution: 10 μL 1 kb ladder, 10 μL 10× BlueJuice, and 80 μL H_2O.
12. UV transilluminator.
13. Digital camera such as Canon Power Shot A590 IS with UV-Light filter and appropriate hood or a Polaroid camera with UV-light filter (use with Polaroid film 667).

2.7 Real-Time (Quantitative) RT-PCR

1. Thermocyler: ABI Prism 7500 Real-time PCR System (Applied Biosystems/Thermo Fisher Scientific).
2. Eppendorf centrifuge, model 5810R with A-2-DWP rotor, or similar centrifuge that will handle 96-well plates.

3. TaqMan RNA-to-C_T 1-Step Kit (Applied Biosystems cat.no. 4392653). The kit has two components. Vial 1: DNA polymerase mix (2×) contains AmpliTaq Gold DNA Polymerase, dNTPs with dUTP, Passive Reference 1, and optimized buffer components. Vial 2: RT enzyme mix (40×) contains ArrayScript Reverse Transcriptase and RNase Inhibitor.
4. 96-well optical reaction plates (Applied Biosystems).
5. Optical adhesive covers (Applied Biosystems).
6. Primers and probes (Table 1, Sets 1–3). Prepare stocks of primers (100 μM) and probes (25 μM) in RNase/DNase-free water, and store in aliquots at −20 °C in a manual defrost freezer.
7. WNV Standards: Purify RNA from a WNV viral stock with known titer, dilute to 1×10^6 PFU/mL, and prepare serial 10-fold dilutions equivalent to 1000, 100, 10, 1, and 0.1 PFU/10 μL. Store in 100 μL aliquots at −70 °C.
8. Negative RNA extraction controls: prepare by including extraction buffer without sample in each RNA extraction procedure (RNeasy or MagMAX).
9. Positive extraction control: homogenize tissue from a known WNV-positive sample in RLT lysis buffer (*see* **item 4** in Subheading 2.4), and store at −70 °C in 100 μL aliquots. For each RNA extraction dilute an aliquot 1:10 with lysis buffer and include in the RNA purification and RT-PCR procedures (*see* **Note 9**).

2.8 Immunofluorescence Assay for Viral Antigen

1. BSC.
2. Fluorescence microscope equipped with epi-illumination and excitation and emission filter sets designed for the fluorophore used.
3. Incubator (37 °C, 5 % CO_2).
4. Staining trays designed to hold slides in a horizontal position.
5. Moist chamber (Plexiglas or plastic container with lid that will hold staining trays above moist paper towels or similar substrate).
6. Staining jars or dishes.
7. Warming Tray (e.g., Cole-Parmer slide warmer Model 771).
8. 10-well microscope slides (Diagnostic slides, Thermo Scientific).
9. Cell scrapers.
10. 5 mL Pipettes.
11. Cover slips, 22 × 50 mm.
12. Acetone (prechilled to −20 °C).
13. Blotting paper.
14. PBS, pH 7.6 (enough to fill staining dishes/Coplin jars twice).

15. Primary antibodies—antisera to viral antigen(s) for which assay is performed.
16. Secondary antibodies (conjugate)—Anti-IgG antibodies conjugated with fluorescent marker (usually FITC). This antibody must recognize the species from which the primary antibody was derived. FITC has excitation and emission spectrum peak wavelengths of approximately 495 and 519 nm, respectively. Like most fluorochromes, it is prone to photobleaching.
17. Evans Blue counterstain, 1.0% stock in sterile Millipore water, store in aliquots at −20 °C. Use at a 1:1000 dilution in PBS (0.001%). This azo dye has a high affinity for serum albumin and will stain cell cytoplasm. It fluoresces with excitation peaks at 470 and 540 nm and an emission peak at 680 nm.
18. Mounting medium: VECTASHIELD hardset antifade mounting medium (Vector Laboratories).

2.9 Isolation of Live Virus in Cell Culture

1. BSC.
2. Incubator, 37 °C, 5% CO_2.
3. Inverted light microscope with phase contrast optics.
4. Confluent cell monolayers: Cell lines commonly used for infection of arboviruses are listed in Table 2. Cells are diluted to 2×105 c/mL, and seeded into 6-well plates at 3 mL/well or T25 or T75 flasks at 5 or 15 mL/flask, respectively. The monolayer should be ready for infection 3–5 days later. Before infecting, examine cell monolayers for density and vitality under the microscope. Monolayers should be at least 90% confluent, but not overgrown. Maintenance medium (cell line-specific growth medium with FBS reduced to 2%) is used after the cells reach confluency and during virus infection.
5. Test samples: homogenized mosquito or vertebrate tissue (Subheading 3.1), infected cell culture samples, oral swabs, serum, or CSF.
6. PBS-2%: calcium/magnesium-free PBS supplemented with 2% FBS.

Table 2
Cell lines commonly used for arbovirus isolation

Cell line	ATCC	Derivation	Culture medium
Vero	CCL-81	African green monkey kidney	Eagle's MEM+10% FBS
C6/36	CRL-1660	*Aedes albopictus* (Asian tiger mosquito)	Eagle's MEM+10% FBS
BHK	CRL-10314	Baby hamster kidney (Syrian golden hamster)	DMEM+10% FBS

7. BA-1 Diluent (*see* **item 7**, in Subheading 2.1).
8. Mosquito Diluent (*see* **item 6** in Subheading 2.1).

2.10 VectorTest WNV Antigen Assay

1. VectorTest WNV Antigen Assay Kit (VecTOR Test Systems, Inc., Thousand Oaks, CA, cat. no. WNV-K050) containing Antigen Assay dipsticks, Grinding solution, Copper-coated BBs, tubes and racks.
2. Vortex.
3. Centrifuge.
4. Swabs (polyester fiber tipped applicators, Thermo Fisher Scientific).

2.11 Plaque Reduction Neutralization Test (PRNT)

1. CO_2 incubator (5% CO_2, 37 °C).
2. Light box for visualization of viral plaques.
3. BSC.
4. Confluent Vero cell monolayers in 6-well plates (*see* **item 4** in Subheading 2.9).
5. BA-1 Diluent (*see* **item 7**, in Subheading 2.1).
6. Growth Medium (2× MEM + 10% FBS): 2× Minimal Essential Medium with 10% Fetal Calf Serum, 200 units/mL penicillin, and 200 μg/mL streptomycin. Store at 4 °C, expiration 3 weeks.
7. 1.2% Oxoid Agar: add 1.2 g of Oxoid agar to 100 mL of H_2O in a 250 mL Wheaton bottle. Autoclave on liquid cycle, store at RT, and microwave to liquefy before use.
8. Neutral Red Solution, 3.3 mg/mL.
9. 96-well cell culture U-bottom plates.
10. Test tubes, snap-cap.
11. Virus stocks, with known Vero cell titer in PFU (plaque forming units)/mL. Choice of virus stocks used in each assay is based on the patient's history, including travel.
12. PRNT-confirmed positive and negative serum specific for each virus employed in the assay. Positive control must neutralize virus at 1:10 dilution or greater. Negative control must show no neutralizing ability at lowest dilution (1:10).
13. Nutrient agar overlay (prepare this overlay during infection of cells and use within 1 h). Combine equal parts of 45 °C 2× MEM + 10% NCS and 1.2% agarose that has been melted by microwave and cooled to 45 °C; hold the mixture at 45 °C until use.

2.12 Preparation of Crude WNV Antigen for Indirect ELISA

1. BSC.
2. Sonicator, Branson Digital Cell Disrupter, Model S 250D, equipped with large capacity cup horn.

3. Vero cell monolayers in T75 flasks (*see* **item 4** in Subheading 2.9).
4. WNV stock virus with a known Vero cell titer.
5. 0.2 M glycine, pH 9.5.
6. 0.5 % Triton X-100 in PBS.

2.13 Indirect ELISA for Detection of WNV Antibody in Avian Serum

1. PBS with 0.05 % Tween 20 (PBS-T). Store at RT, prepare fresh weekly.
2. Coating Buffer: 0.3975 g Na_2CO_3, 0.7325 g NaH_2CO_3, and 250 mL of ddH_2O. Stir until dissolved, adjust pH to 9.6, store at RT, prepare fresh weekly.
3. PBS with 0.05 % Tween 20 and 0.5 % bovine albumin (PBS-T + BA). Stir until dissolved, store at 4 ° C, prepare fresh weekly.
4. Blocking Buffer (BB): PBS with 0.05 % Tween 20 and 5.0 % skim milk powder. Add 50 g Difco skim milk powder to 1 L of PBS-T. Stir until dissolved, store at 4 °C, and make fresh weekly. Warm if necessary to completely dissolve the powder.
5. HCL 1:20: 300 mL dH_2O, 15 mL hydrochloric acid (*see* **Note 10**). Mix, store at RT. No expiration.
6. Immulon 1B flat-bottom 96-well plates.
7. ELISA AutoPlate Washer, ELx 405.
8. ELISA Ultra Microplate Reader, ELx 808, Bio-Tek Instruments, Inc.
9. Humidity chamber (e.g., plastic container with moist paper towels).
10. WNV-positive control avian serum.
11. WNV-negative control avian serum.
12. Goat anti-bird IgG-heavy and light chain antibody, conjugated with HRP (Bethyl Labs, Montgomery, TX).
13. TMB 2-Component Microwell Peroxidase Substrate Kit (KPL, Inc., Gaithersburg, MD). Bring to RT before mixing components for use.

3 Methods

3.1 Preparation of Mosquito Test Samples

1. Perform all procedures in a BSC in the BSL-3 laboratory.
2. Mosquitoes, after collection in the field, should be sorted by species, and pools of 10–50 placed in 2.0 mL safe-lock microfuge tubes, each containing a single BB. Store samples at –70 °C until homogenized.
3. Determine how each mosquito pool should be tested. To prepare samples for both live virus isolation and RT-PCR, follow

steps 4 and **5** in this section. For samples to be tested by RT-PCR only, follow **step 6** in this section (*see* **Notes 11** and **12**).

4. Remove mosquito sample tubes from the freezer and place on ice. Add 0.75–1.0 mL of Mosquito Diluent to each tube of mosquitoes.
5. Place the tubes in the 24-well Mixer Mill adapter racks that have been prechilled at 4 °C, and then secure in the Mixer Mill. Homogenize for 30 s at 24 cycles/s, and then place on ice for 5 min. It is important to keep the samples as cold as possible to minimize the activity of RNases released from the tissue during homogenization. Microfuge at 4 °C for 5 min at 8000 × *g*. Transfer the supernatant to a 1.5 mL microfuge tube and store at −80 °C. This preparation is ready for live virus isolation (Subheading 3.8) and purification of RNA by RNeasy or MagMAX (*see* Subheading 3.4 or 3.5, respectively).
6. For mosquito samples that will not be used for live virus isolation, homogenize directly in RNA lysis buffer (RLT buffer for the RNeasy procedure and Lysis/Binding Solution for MagMAX). Follow the same procedure described for Mosquito Diluent (*see* **item 5** in this section).

3.2 Preparation Nonhuman Mammal Test Samples

1. Place vertebrate tissue obtained from necropsies performed off-site in specimen jars, send to the laboratory, and store at −80 °C. For detection of WNV brain tissue is tested most often. For safety during handling, all mammalian samples should be confirmed as negative for rabies virus before shipment to the lab.
2. Place the frozen tissue in its jar on a bed of ice in a BSC. Thaw the tissue slightly and cut three sections of less than 1 mm^3 from different areas, since the virus may not be evenly distributed throughout the tissue. A 3 mm cube of tissue weighs approximately 30 mg. Place the sections together in a 2.0 mL safe-lock tube containing 1 BB and 1 mL of buffer: RLT lysis buffer for RNeasy purification, Lysis/Binding Solution for MagMAX RNA purification, or BA-1 diluent for live virus isolation.
3. Place the tubes in the Mixer Mill 24-well adapter racks. Homogenize for 4 min at 24 Hz, and then place on ice for 5 min. To achieve uniform homogenization, stop the mixer mill after 2 min and rotate the tubes in the sample adapters.
4. Clarify by microcentrifugation at 4 °C for 5 min at 8000 × *g*, and transfer the supernatant to a new microfuge tube.
5. Use aliquots of this preparation for RNA purification and RT-PCR or live virus isolation in cell culture, depending on the buffer used for homogenization. Or store at −80 °C for later use.

3.3 Preparation of Avian Test Samples

1. Dead bird carcasses collected for WNV testing are deemed acceptable if collected within 48 h of death and appear to be in relatively good condition (intact, no physical decay or appearance of insect larvae) then frozen at −20 °C [22]. Three categories of samples may be collected: (1) Brain, heart, kidney, and spleen, (2) Feathers: vascular and non-vascular, and (3) Oral and cloacal swabs.
2. Brain, heart, kidney, and spleen: Perform necropsies aseptically; store harvested tissues at −70 °C. For testing thaw the tissues at 4 °C and place on ice. Using a sterile disposable scalpel excise a 50 mg portions (3 × 3 × 6 mm) from each tissue and place in a microfuge tube containing 1 mL of buffer appropriate for the downstream application. Homogenize and clarify as described in Subheading 3.2.
3. Feathers: At necropsy examine each bird for wing flight feathers (remiges) and tail flight feathers (retrices) that contain vascular pulp (*see* Fig. 2). Pull feathers containing vascular pulp from the feather follicle and aseptically cut at the distal end of the umbilicus. Place the umbilicus containing vascular pulp in a microfuge tube containing 1 mL of mosquito diluent, and freeze at −70 °C. For testing, thaw sample and with forceps remove vascular pulp from the umbilicus and create a 10%

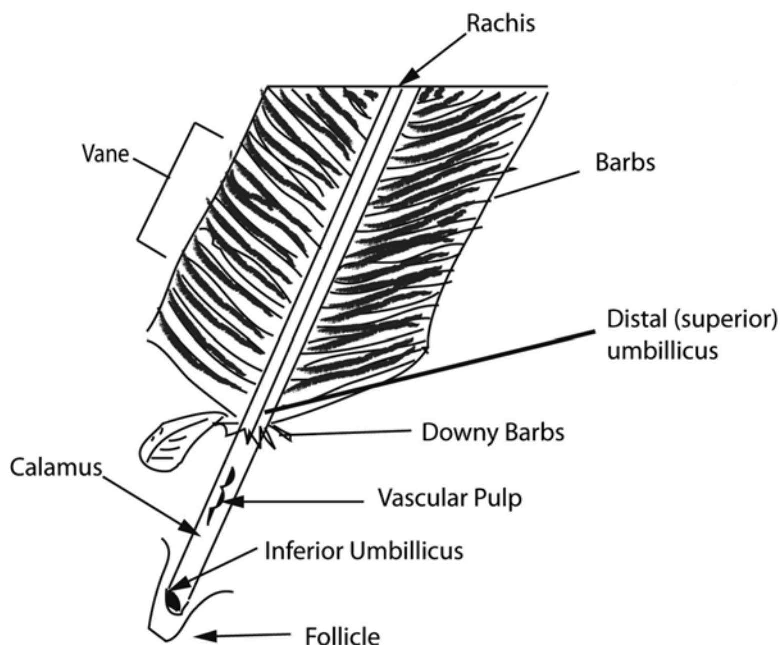

Fig. 2 The schematic representation of a typical bird feather and the location of the vascular pulp. The feathers are pulled from the feather follicle and aseptically cut at the distal end of the umbilicus. The umbilicus contains the vascular pulp

(wt/volume) suspension in 1 mL buffer appropriate for the downstream application. Vortex the sample until it appears homogeneous and clarify by microcentrifugation at 4 °C for 5 min at 8000 × *g*. Proceed with isolation of infectious virus or RNA purification and RT-PCR (*see* **Note 13**). For VectorTest samples, pull feathers containing vascular pulp from wing or tail and then express the pulp onto a swab. Mix the swab in VectorTest buffer (*see* Subheading 3.10).

4. Oral and cloacal swabs. Swab birds in a BSC, if possible. Place entire carcass in a plastic bag with the head protruding. Open the bird's beak and place a swab into the oral cavity, then move it vigorously around the oropharyngeal cavity and the proximal esophagus. Place each swab into an appropriate buffer (depending on downstream application) and swirl it for at least 20 s. Press swab on side of tube to ensure release of material into buffer. Vortex or swirl sample until it appears homogeneous, then clarify by microcentrifugation for 5 min at 8000 × *g*. Take multiple swabs from each bird. Store samples at 4 °C for further processing, or −70 °C for future processing. Downstream processing consists of RNA isolation by RNeasy or MagMAX followed by RT-PCR, isolation of live virus, or VectorTest for detection of protein (*see* **Note 14**).

3.4 RNA Purification by RNeasy Mini Kit

1. All RNeasy procedures should be carried out at room temperature (15–25 °C) *See* **Note 4**.
2. Prepare test material, as described in Subheadings 3.1–3.3.
3. For samples that were homogenized in RLT buffer, place 350 μL of clarified homogenate in a microfuge tube, add 350 μL of 70 % ethanol, and mix well by pipetting up and down. For samples that were homogenized in Mosquito Diluent (mosquitoes), add 100 μL of clarified homogenate to 350 μL of RLT, and then add 250 μL of 96–100 % ethanol and mix well. Do not centrifuge after the addition of ethanol (*see* **Note 15**).
4. Bind RNA to the silica-based membrane by transferring the 700 μL sample with ethanol to an RNeasy mini spin column positioned in a 2 mL collection tube. Microfuge for 15 s at 8000 × *g*. Carefully transfer the spin column to a clean 2 mL collection tube.
5. Wash the membrane with bound RNA three times. For the first wash, add 700 μL of RW1 Buffer to the column, microfuge for 15–30 s at 8000 × *g*, and then carefully transfer the spin column to a clean 2 mL collection tube.
6. For the second wash, add 500 μL of RPE Buffer to the column, microfuge for 15–30 s at 8000 × *g*, and then carefully transfer the spin column to a clean 2 mL collection tube.

7. For the third wash, add 500 μL of RPE Buffer to the column, microfuge for 2 min at 8000 × *g*, and then carefully remove the column and place it in a 1.5 mL collection microfuge tube (*see* **Note 16**).
8. To elute RNA from the column add 30–50 μL of RNase-free water onto the center of the membrane without touching, and allow it to adsorb for 1–10 min. Centrifuge for 1 min at 8000 × *g*. If the expected yield is greater than 30 μg, repeat the elution with another 30–50 μL of water, reusing the collection tube.
9. The purified RNA is ready for use in standard or real-time RT-PCR assays. Store at −20 to −70 °C.

3.5 RNA Purification by MagMAX Technology

1. The MagMax-96 total RNA lysis kit is designed for high throughput purification of total RNA in a 96-well plate format. It can be used with either multichannel pipettes or with robotic liquid handlers (*See* **Note 4**).
2. Sample preparation. Disrupt and homogenize samples in freshly prepared Lysis/Binding Solution or Mosquito Diluent using standard homogenization procedures (*see* Subheadings 3.1–3.3). For samples homogenized in mosquito diluent, add 50 μL of each clarified homogenate to the well of a polystyrene U bottom plate, and then add 50 μL Lysis/Binding Solution. For samples homogenized in Lysis/Binding Solution add 100 μL of each clarified homogenate to the 96-well plate. For all samples add 60 μL of 100% isopropanol, place lid on plate, and shake for 1 min.
3. Add bead mixture: Gently vortex the bead mixture to resuspend the magnetic beads. Add 20 μL of bead mixture to each sample. Cover the plate and shake for 5 min on an orbital shaker at maximum speed to bind the RNA to the RNA Binding Beads.
4. Magnetically capture the RNA binding Beads. Move the processing plate to the magnetic stand to capture the RNA Binding beads. Leave the plate on the magnetic stand until the beads have completely pelleted; when the mixture becomes transparent the capture should be complete (*see* **Note 17**).
5. Carefully aspirate and discard the supernatant without disturbing the beads, and remove the processing plate from the magnetic stand.
6. Wash 1. Add 150 μL of Wash Solution 1 to each sample and shake for 1 min. Recapture the beads on the magnetic stand beads. Carefully aspirate and discard all supernatant without disturbing the beads, and remove plate from the magnetic stand. It is critical to remove the plate from the magnetic stand before the subsequent wash step.

7. Wash 2. Add 150 μL of Wash Solution 2 (ethanol added) to each sample and shake at maximum speed for 1 min. Recapture the beads on the magnetic stand, aspirate and discard the supernatant, and remove the plate from the stand. Be sure to remove all of Wash Solution 2 before the DNase treatment step. Prepare Diluted TURBO DNase during the Wash 2 step.
8. Turbo DNase Treatment. Add 50 μL of Diluted TURBO DNase to each sample and shake the plate for 10–15 min at maximum speed (*see* **Note 18**).
9. Final RNA cleanup. Add 100 μL of RNA Rebinding Solution to each sample and shake for 3 min at maximum speed. In this step, the RNA is bound to the RNA Binding Beads again. Magnetically capture the RNA Binding Beads and discard the supernatant. Wash twice with 150 μL Wash Solution 2. Dry beads by shaking for 2 min at maximum speed (*see* **Note 19**).
10. Elute the RNA by adding 50 μL of Elution Buffer to each sample and shaking vigorously for 3 min at max speed. Capture the RNA Binding Beads on the magnetic stand. Transfer the supernatant, containing purified RNA, to a nuclease-free container appropriate for your application, such as a 96-well plate.

3.6 Standard RT-PCR

Real-time RT-PCR assays are specific and high throughput. For bird and mosquito surveillance, specimens are confirmed as WNV when positive results are obtained with two different primer/probe sets. If results are equivocal, RNA often is reextracted from the tissue homogenate by RNeasy and a standard RT-PCR assay is performed.

1. Prepare samples by isolating RNA from test samples by RNeasy or MagMAX technology (*see* Subheadings 3.4 and 3.5).
2. Prepare Master Mix (MM) in a PCR hood that is located in a room separate from the thermocycler and gel electrophoresis procedures. Keep all reagents on ice or use cold blocks. Use the reagents supplied in the Qiagen One-step RT-PCR kit. The WNV primer sets used for standard RT-PCR are sets 4 and 5 listed in Table 1 [19]. Each reaction requires 40 μL of MM and 10 μL of sample. Determine total volume of MM needed for the entire set of reactions and prepare enough for an extra 2 reactions (Table 3).
3. Set up the reaction tubes by pipetting 40 μL MM into each RT-PCR tube, and then add 10 μL of the RNA sample. Secure caps on the tubes and place in the thermocycler.
4. Thermocycler conditions for both WNV primer sets (Table 1, sets 4 and 5) consist of 50 °C for 30 min to synthesize the first-strand cDNA; 95 °C for 15 min to inactivate the reverse transcriptase and to activate DNA Taq polymerase; 35 cycles of

Table 3
Master mix for standard RT-PCR (Qiagen OneStep RT-PCR kit)

Component	μL per reaction
Water, DNase/RNase-Free	14
OneStep RT-PCR Buffer (5×)	10
dNTP mix, 10 mM each	2
Forward Primer (25 μM)	1
Reverse Primer (25 μM)	1
Q-Solution	10
RT-PCR Enzyme Mix[a]	2
Total	40

[a]Contains Omniscript reverse transcriptase, Sensiscript reverse transcriptase, and HotStarTaq DNA polymerase

94 °C for 45 s, 56 °C for 45 s, and 72 °C for 1 min for PCR amplification; a final elongation at 72 °C for 10 min, then hold at 4 °C until ready for gel electrophoresis.

5. Assemble Owl EasyCast Horizontal electrophoresis box in the casting position.
6. Prepare 1.5% agarose gel: weigh 1.5 g ultrapure agarose and dissolve in 100 mL 1× TAE buffer in an appropriate vessel (1-L Erlenmeyer flask). Microwave until dissolved (1–2 min) and allow to cool to 60 °C. Add 10 μL Gel Red per 100 mL of cooled agarose.
7. Pour warm gel into the casting stand and immediately place the appropriate comb into the gel (10- or 20-well). Allow approximately 20 min for the gel to solidify.
8. To load the gel, fill the gel box with approximately 750 mL of 1× TAE buffer, up to fill line on gel box. Mix each sample with BlueJuice (four parts sample one part BlueJuice). Load samples into the wells (up to 20 μL per 12-well comb; up to 15 μL per 20-well comb). Load at least one additional well with a 1 kb ladder sample.
9. Run the gel: connect the gel apparatus to a power supply and run at 150 V Constant (VWR model 300 power supply). Typical run time is 30 min, when DNA bands are adequately separated.
10. Visualize the gel: remove the gel and place it on a transilluminator. With safety shield in place turn on the UV-light to visualize bands. Photograph the gel for a permanent record.

3.7 Real-Time (Quantitative) RT-PCR

1. Prepare MM in a PCR hood that is located in a room separate from the thermocycler and any downstream processing. Keep all reagents on ice or use cold blocks. Use the reagents supplied in the ABI TaqMan One-Step RT-PCR Kit. The WNV primer sets used for standard RT-PCR are sets 1–3 listed in Table 1 [18, 19, 23]. Each reaction consists of 40 μL of MM and 10 μL of sample. Determine total volume of MM needed for the entire set of reactions and prepare enough for an extra 2 reactions (Table 4).
2. TaqMan plate setup. Place a 96-well optical reaction plate in a cold block or on ice. Add 40 μL of MM to each well (may be done with a multichannel pipette). Add 10 μL of RNA template, serial dilutions of WNV standards or DNase/RNase-free water (No Template Control) to each well. Cover the reaction plate with an optical adhesive cover, seal well. Centrifuge the reaction plate so that all liquid is in the bottom of the wells (1000 × *g* for 3 min).
3. Place the plate in the ABI 7500 instrument and turn it on. Open the software and choose File > New. Refer to the ABI Absolute Quantification Getting Started Guide (pp. 40–45) to set up the run [24]. Enter sample information and concentrations of the standards.
4. Set the thermocycler conditions and start the run. The thermocycler conditions for the WNV primers listed in Table 1 are: 30 min at 48 °C and 10 min at 95 °C (rep 1); 15 s at 95 °C and 1 min at 60 °C (rep 40). Save the file as a *.sds file. You can also save as a *.sdt (template) file for future use.
5. Download data. When the run is complete, go to Results/Amplification Plot; select the entire plate (upper left corner) and click Analyze. Using the Results Tab you can view the results, change parameters, omit samples, and manually set

Table 4
Master mix for real-time RT-PCR (TaqMan RNA-to-C_T 1-Step Kit)

Component	μL per reaction
Water, DNase/RNase-Free	12.25
2× Universal PCR Master Mix	25.00
40× MultiScribe + RNase Inhibitor	1.25
Forward Primer (100 μM)	0.5
Reverse Primer (100 μM)	0.5
25 μM TaqMan Probe	0.5
Total	40.00

baseline and threshold. If any parameters are changed the data must be reanalyzed. The default baseline is automatically set from 6 to 15 cycles. Reset the cycles if the amplification begins before a CT of 15. The threshold is automatically set by the software, and should be located in the geometric phase of the amplification curve. For the WNV primer sets the threshold is usually maintained at 0.2. View the Standard Curve, which displays the values for the samples designated as standards.

6. Analyze the data. The CT value is the amplification cycle at which fluorescence increases above threshold. The Rn value is relative change in fluorescence at the end of the amplification. These two values are used to analyze the data. Export these values for each test and standard sample into an Excel file. Express results as CT values or Relative PFU calculated by linear regression from the standard curve. A sample is determined to be positive if the CT value is equal to or less than the threshold CT value and the Rn value is two or more times the average of eight negative wells.

3.8 Isolation of Live Virus in Cell Culture

Changes in cell morphology caused by infectious virus, called cytopathic effect (CPE), are an important tool for surveillance. Cell monolayers prepared from cell lines that are permissive for the virus are infected with the test material, incubated for a period of time and observed for morphological changes. For surveillance purposes, the presence of CPE is indicative of the presence of virus that must be further identified or confirmed with additional testing, usually by PCR or immunoassay.

1. To infect the cells, decant media from a 6-well plate (or T-25 flask) containing a confluent monolayer of cells. Vero cells are used most often (*see* **item 4** in Subheading 2.9 and **Note 20**). Inoculate each well with 100 μL (200 μL for T-25 flask) of test material such as mosquito or vertebrate homogenate, swabs, or feather pulp (*see* Subheadings 3.1–3.3).
2. Mock-infect at least one well or flask for each assay, by inoculating with 100 μL (or 200 μL) of BA-1 diluent. This is an important control which is used to evaluate CPE by comparing uninfected and infected monolayers.
3. Incubate at 37 °C, 5% CO_2, for 60 min, rocking plates gently every 15–20 min to allow absorption of virus to the cells.
4. At the end of the infection period, add 3 mL (5 mL for T-25 flask) of cell maintenance medium to each well. Do not remove the inoculum. Incubate at 37 °C, 5% CO_2, for up to 7 days.
5. Examine plates each day post infection under the phase contrast microscope, using the uninfected control for comparison. CPE may consist of cell rounding, fusion, swelling or shrinking, death, or detachment from the surface, and is rated on a

scale of 1+, which means less than 25 % of the cells are affected, to 4+ where all of the cells in the monolayer are involved (*see* **Note 21**).

6. When no or questionable CPE is observed, the samples may be passed for two additional rounds as follows. Decant media from a fresh uninfected cell monolayer (6-well plate or T-25 flask). Transfer medium from the first passage to the new cells (0.5 mL for 6-well plates and 1.5 mL for T-25 flask). Include another uninfected control. Adsorb, overlay, incubate, and examine for CPE as described for the initial infection. If no CPE is observed after 1 week, repeat the procedure for a third round.
7. CPE-positive samples may be identified further by IFA of infected cells (*see* Subheading 3.9) and/or RT-PCR. For analysis of the viral culture by PCR, purify RNA from 100 μL of culture fluid by Qiagen RNeasy kit and perform standard RT-PCR (*see* Subheading 3.6) or real-time RT-PCR (*see* Subheading 3.7).
8. When 50 % of the monolayer is exhibiting CPE, virus stocks may be harvested. Add 800 mL of culture supernatant to a cryotube containing 200 μL of FBS. Mix well, a using micropipette, and transfer 500 μL to a second tube to give two 500 μL aliquots. Store at −80 °C.

3.9 Immunofluorescence Assay for Viral Antigen

1. Prepare slides by applying infected cells exhibiting 2–3+ CPE to microscope slides (*see* Subheading 3.8 for infection procedure). Using a pencil, label slides with sample information. Gently but thoroughly scrape infected cell monolayer into the media. Break down clumps of cells by gentle trituration, using a 1000 μL micropipette. Transfer material to a 15 mL centrifuge tube and vortex at high speed to further disrupt clumps of cells. Dispense 20 μL to each well of a 10-well slide. Allow slides to air-dry in the BSC for 2 h at RT or ½ h on warming tray. Fix slides by immersing in −20 °C acetone for 30 min. Remove from acetone and allow to air-dry. Use immediately for IFA or store in slide boxes at −80 °C. Prepare uninfected and positive control slides in addition to test samples.
2. Prepare appropriate dilutions of primary and secondary antibodies in PBS. Secondary antibody may be diluted in PBS containing Evans Blue counterstain (0.001 %).
3. Prepare a moist chamber by placing soaked paper towels in the bottom of closable chamber.
4. Prepare enough PBS, pH 7.6, to fill the staining dishes twice and add enough PBS to staining dishes to completely immerse slides.

5. Stain slides with primary antibody as follows. Place slides to be stained on a tray in moist chamber. Add 18 μL primary antibody to each well. Incubate in the moist chamber at 37 °C for 1 h.
6. To rinse slides, carefully remove contents of each well by aspiration with micropipette or vacuum, avoiding cross contamination between wells. Dip each slide in PBS, then immerse slides in fresh PBS for 15 min. Gently shake-off excess PBS and allow to air-dry for 20 min at RT or 5 min on a warming tray.
7. Stain slides with secondary antibody. Add 18 μL of diluted secondary antibody to appropriate wells. Incubate in the moist chamber at 37 °C for 30 min. Briefly rinse each slide by dipping in PBS, then immerse in fresh PBS bath for 15 min.
8. To mount coverslips, remove slides from PBS bath and shake gently to remove excess liquid. Blot bottom and sides of slides and dry as much of the space between the wells as possible without disturbing the cells in the wells. Add a drop of mounting medium into each well, then carefully lower a coverslip over the wells, trying to avoid air bubbles. Allow to set for 15 min.
9. Read slides on a fluorescent microscope using a 20× objective. Concentrate readings on areas where cells are well separated (not clumped). Positive cells will fluoresce bright green while negative cells will appear dull background green or red if Evans Blue was used. The percentage of positive cells in a positive culture will vary between 30% and 80% (*see* **Note 22**).

3.10 VectorTest WNV Antigen Assay

The VectorTest is a dipstick format, qualitative, immunochromatographic test that uses type-specific monoclonal antibodies to detect WNV antigen [25].

1. Mosquito Specimens: Place 1–50 mosquitoes into the provided plastic culture tube provided in the VectorTest kit.
2. Mix the Grinding Solution by inverting the bottle five times. Dispense 2.5 mL onto mosquitoes and add 4 BBs to tubes.
3. Vortex the capped tubes for 1 min at high speed until the mosquito pool is homogenized into a slurry. A centrifugation step may be performed to remove excess mosquito debris before running the test
4. Dispense 250 μL of mosquito homogenate into the conical tube provided and insert a test strip with arrows pointing down.
5. To interpret the test, remove the test strip from the solution and compare it to the pictorial sample provided in the test kit (*see* Fig. 3). The assay results should be read within 30 min of performing the test.

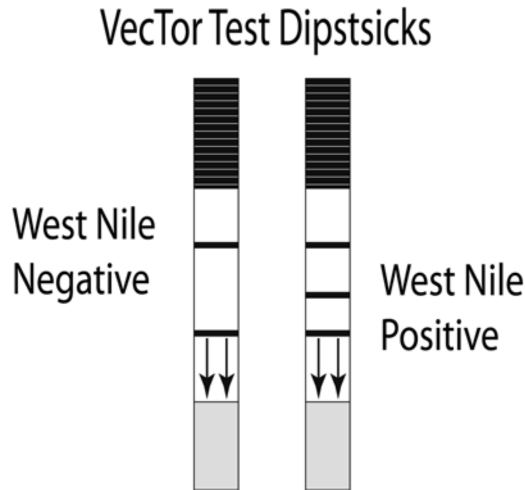

Fig. 3 Diagram of the VectorTest Assay. The reagent zone of the test strip is coated with WNV monoclonal antibody (mAb) conjugated to colloidal gold. These antibodies will migrate up the test strip with the test material. The detection zone contains immobilized unlabeled WNV mAb that will capture WNV antigen as it migrates up the strip. The control zone contains immobilized reagents that will capture any gold-conjugated monoclonal antibodies that do not bind to the detection zone to demonstrate that the sample migrated through the test zone. The bottom of the dipstick, indicated by *arrows*, is placed in a tube containing the test material, which will be wicked onto the strip and migrate through the three zones. After 15 min the strip is removed and read. A positive sample will develop a reddish-purple color in both the control and the detection zones. Negative samples develop color only in the control zone. If no color develops, the test is invalid

6. Test oral, cloacal, or feather pulp swabs (*see* **steps 3** and **4** in Subheading 3.3) by swirling each swab for at least 20 s in 1 mL of Grinding Solution in the tube provided in the kit. Press the swab on the side of the tube to ensure release of material into solution. Vortex the sample until it appears homogeneous, and clarify by microcentrifugation for 5 min at 8000×*g*. Proceed with the VectorTest (**steps 4** and **5** in this section). Vertebrate tissue also may be tested by taking swabs directly from the parenchyma of the tissue, and processing as for oral swabs.

3.11 Plaque Reduction Neutralization Test

The plaque-reduction neutralization test (PRNT) is considered the gold standard procedure for the identification of arbovirus isolations [26–28]. Adapted for the "clinical" setting, it may be used to assist in the diagnosis of arboviral infections. Since the test is costly, time-consuming, and requires a BSL-3 facility, it usually is not used as a clinical screening tool. Other useful applications of this procedure include epidemiological studies of antibody seroprevalence in human, animal, or wild bird populations [29–31] and evaluation

of current or new vaccine efficacy [32]. A common screening method with a large number of samples for serosurvey is to do ELISA and confirm with PRNT [31, 33]. The protocol described here is for clinical situations where two serum samples from the same individual are compared to detect a recent infection.

1. Preparation of 96-well plates with diluted serum. Dilute test and control serum 1:2.5 in BA-1 Diluent, and heat-inactivate at 56 °C for 30 min to remove nonspecific neutralizing substances (*see* **Note 23**). Aliquot 100 μL of BA-1 diluent in columns 2–12 of a 96-well plate, and then add 100 μL of diluted and heat-inactivated serum samples to columns 1 and 2, using one row for each test or positive control serum (*see* **Note 24**). Make twofold serial dilutions of test sera as follows. Mix contents of column 2 with a multichannel micropipette, and transfer 100 μL to column 3. Repeat the dilution procedure in columns 3–12, and discard the last 100 μL. Prepare an identical plate for each virus used in the assay, including positive and negative controls.
2. Preparation of virus dilutions. The viruses chosen for neutralization in each PRNT assay are determined by patient history, with information on travel to areas endemic for arboviruses such as DEN, JEV, ZIKV, and YFV of high importance. Thaw each virus stock and dilute to 200 PFU/100 μL in fresh BA-1, preparing a sufficient amount to apply 100 μL for each serum dilution plus extra for back titration to verify its concentration. For back titration, take an aliquot of the 200 PFU/100 μL dilution and prepare 100, 10 and 1 PFU/100 μL dilutions in BA-1.
3. Neutralization. Add 100 μL of the 200 PFU/100 μL virus stock to each dilution of test serum and control serum. Each well now will contain 100 PFU/100 μL of virus, and the starting dilution of each test serum sample (in column A) usually will be 1:10. Add the virus control back-titration dilutions to three vacant wells of the same 96-well plate. Incubate the plate at 4 °C overnight or 37 °C for 1 h.
4. Infection of cells. At the end of the neutralization incubation, infect the Vero cell monolayers with the neutralized virus. Remove the medium from 6-well plates containing confluent cell monolayers (*see* **item 4** in Subheading 2.9). Inoculate 100 μL of each virus/serum mixture into a separate well. For back-titration of each virus, inoculate duplicate wells with 100 μL of each dilution. Inoculate one plate with BA-1 only to serve as an infection control. Rock the plates gently to evenly distribute the inoculum over the monolayer, and incubate at 37 °C for 1 h to allow adsorption of virus to the cells. During the infection period, prepare nutrient agar overlay. At

the end of the 1 h infection period, add 3 mL of agar overlay to each well, allow it to solidify at room temperature and incubate at 37 °C, 5 % CO_2 until plaques develop (approximately 2 days for EEEV, 3 days for WNV, 5 days for SLEV, and 4–5 days for JEV).

5. Count plaques and determine neutralization titer. When plaques start to appear stain the cells by adding to each well a second agar overlay, similar to the first but with the addition of neutral red (2 mL stock solution per 100 mL overlay, final concentration 66 μg/mL). After an overnight incubation the plaques should be visible and ready to count. Using a light box, count plaques and record numbers daily until no further significant increase in plaque number is observed (*see* **Note 25**). Neutralization titers are determined as the highest dilution of test serum or positive control serum that inhibits formation of at least 90 % of the plaques as compared with the virus control back titration. The back titration of the 100 PFU/100 μL dilution should exhibit 30–100 plaques per well. If more than 20 or less than 100 plaques develop, the assay should be repeated.
6. Interpretation of results. A fourfold difference in PRNT titers between related flaviviruses as well as a fourfold rise in titer between paired acute and convalescent sera are required for confident determination of etiology of disease. If paired acute and convalescent sera are not included in the PRNT, it is not possible to determine if neutralizing antibody detected by the assay is due to a recent or past infection. When secondary infections with a different flavivirus occur, results can be confusing and easily misinterpreted.

3.12 Preparation of Crude WNV Antigen for Indirect ELISA

1. Virus infection. Seed two T-75 flasks with Vero cells and allow to grow to 90–95 % confluency (*see* **item 4** in Subheading 2.9). Infect one flask with WNV at a multiplicity of infection of 0.1 PFU/cell in 1 mL of culture medium. Mock-infect the other flask with 1 mL of culture medium. After adsorption of the virus at 37 °C for 1 h, add 14 mL of medium and incubate at 37 °C until CPE reaches 3+ (most cells rounded, usually at 48 hpi). Freeze the flasks with their contents at −70 °C overnight. The mock-infected flask will be processed as negative crude antigen.
2. Harvest virus. Thaw the cultures and scrape the cells off the flask surface into the culture medium with a cell scraper. Always handle the uninfected flask first and take great care not to cross-contaminate the samples. Centrifuge media and cell debris for 15 min at 3000 × *g*, and discard the supernatant medium. Resuspend the cellular fraction in 1.5 mL PBS per

T-75 flask (represents a 10× concentration). Freeze the preparations at −70 °C overnight.

3. Preparation antigen. Thaw and add 1.5 mL of 0.2 M glycine, pH 9.5, to the contents of each flask, and then transfer to 50 mL centrifuge tubes. Sonicate indirectly in an ice water bath at 100 mV for three bursts of 20 s. Allow 1 min of cooling between bursts. Place tubes in a 37 °C water bath for 4.5 h, vortexing every 45–60 min.
4. Inactivate antigen: Add an equal volume of 0.5% Triton X-100 in PBS (3 mL/t-75 flask) and leave at 4 °C for 2 h with occasional vortexing. Centrifuge at 10,000 × *g* for 10 min at 4 °C. Store the supernatant in aliquots at −70 °C.
5. To confirm inactivation of virus in the antigen preparation, inoculate Vero cell monolayers and monitor for CPE (*see* Subheading 3.8). Triton X-100 will produce cell toxicity; therefore, pass at least one time to eliminate the possibility that apparent toxicity is not the result of viral infection.
6. Titrate antigen at a dilution range of 1:50–1:1000 to determine the optimal concentration for use in ELISA (*see* **steps 1** and **2** in Subheading 3.13 and **Note 27**).

3.13 Indirect ELISA for Detection of WNV Antibody in Avian Serum (see Note 26)

1. Determine optimal dilutions for each preparation of negative and positive antigen, negative and positive serum controls, serum test samples, and anti-species conjugate by checkerboard titration (*see* **Note 27**).
2. Crude antigen: Optimal concentrations of crude antigen are defined as those yielding the highest mean P/N value for known positive samples and P/N values closest to unity (one) for known negative samples. Generally a 1:100 or 1:200 dilution is optimal. Consider Positive and Negative crude antigen as a paired set and use identical dilutions for each batch (*see* **Note 28**).
3. Negative and positive serum controls: Determine optimal dilutions by checkerboard titration
4. Serum test samples usually are tested at concentrations of 1:50–1:200. Initial screening assays often use only one dilution, usually determined by the nature and amount of test material available. If an end point ELISA titer is required, samples should be serially diluted across the plate.
5. Secondary antibody (anti-species conjugate) optimal dilutions are suggested by the manufacturer. However, it is advisable to test this sample by checkerboard, also (*see* **Note 29**).
6. Coat 96-well ELISA plates with antigen. Dilute negative and positive crude antigen in coating buffer and apply 50 μL per well, placing negative antigen in columns 1, 4, 7, and 10

and positive antigen in columns 2, 3, 5, 6, 8, 9, 11, and 12. Incubate in a moist chamber at 4 °C overnight (or 37 ° C for 1 h).

7. Wash excess antigen from the plate. Using the plate washer, aspirate the antigen and wash three times with PBS-T. Blot plates by inverting over paper towels and tapping gently.
8. Block plates by adding 100 μL of Blocking buffer (BB) to each well. Incubate plates in the humidity chamber at 37 °C for 1 h. Aspirate BB with the plate washer, but do not rinse.
9. Apply test serum. Dilute each test serum sample in PBS-T + BA while the ELISA plate is incubating with BB. After removing the BB add 50 μL of each serum sample to three wells (one coated with negative antigen and two with positive antigen). Include blank (PBS-T + BA) and appropriately diluted positive and negative control serum samples in each assay. Incubate the plates in the humidity chamber at 37 °C for 1 h. Wash with PBS-T using the plate washer, and blot gently.
10. Apply HRP conjugated secondary antibody. Dilute HRP conjugated goat anti-bird antibody in PBS-T + B, add 50 μL to each well, and incubate in the humidity chamber at 37 °C for 1 h. Wash with PBS-T using the plate washer and blot by inverting and gentle tapping.
11. Apply HRP TMB substrate. Prepare the substrate by mixing equal volumes of room temperature TMB Peroxidase Substrate and Peroxidase Substrate Solution B in a clean polypropylene or glass container immediately before use. Add 50 μL to each well, cover the plates, and incubate in the dark at RT for 8 min. A deep blue color will develop. Stop the reaction by adding 50 μL of 1:20 HCL to each well. The color will turn yellow (*see* **Note 30**).
12. Measure optical density. Wipe the bottom of the plate and read in the ELISA microplate reader within 30 min at a wavelength of 450 nm.
13. Compute the positive/negative (P/N) value of each sample by dividing the mean OD of positive antigen-containing wells by the OD of the negative antigen-containing wells. Samples with a P/N values less than 2 are considered positive and often are tested further by PRNT for confirmation [28].

4 Notes

1. If a ball mixer is not available, a hand operated homogenizer system (Thomas Scientific cat. no/1191H97) may be substituted, using disposable pestles (Kimble-Kontes cat. no. Z359947).

2. Since this centrifuge does not have aerosol tight containers, the entire centrifuge has been placed in a biological safety cabinet to comply with safety rules for the BSL-3 laboratory.
3. Cotton swabs with wooden shafts may contain materials which inhibit PCR. Plastic or metal shafts are preferable.
4. RNase precautions: before working with RNA, clean the lab bench and pipettes with an RNase decontamination solution such as Ambion RNaseZap, RNaseKiller or any other commercial RNASE decontaminating solution. Wear laboratory gloves for all RNA procedures to protect you from the reagents and protect the RNA from nucleases present on the skin. Use RNase-free pipet tips to handle the reagents.
5. Buffer RLT contains guanidine thiocyanate, Buffer RLC contains guanidine hydrochloride, and Buffer RW1 contains a small amount of guanidine thiocyanate. Guanidine salts can form highly reactive compounds when combined with bleach. If liquid containing these buffers is split, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with bleach.
6. Dispense β-ME into Buffer in a fume hood and wear appropriate protective equipment.
7. GelRed is a fluorescent nucleic acid stain designed to replace highly toxic ethidium bromide (EB) for staining DNA and RNA in agarose and polyacrylamides gels. It is more sensitive than EB and has the desirable features of long term stability, optical properties identical with EB, and it can be discarded directly down the drain or in regular trash because it is environmentally safe.
8. BlueJuice 10× loading buffer contains 65% (w/v) sucrose, 10 mM Tris–HCl (pH 7.5), 10 mM EDTA, and 0.3% (w/v) Bromophenol Blue. When mixed with the DNA sample, the sucrose makes the sample heavier than the running buffer and prevents it from diffusing away from the well, and the blue dye allows tracking of the sample when the gel is run.
9. The WNV positive control used in our laboratory is prepared from a crow that is highly positive by real-time RT-PCR, having a CT value of less than 16. Thus, the homogenized sample may be diluted before RNA purification.
10. Safety Note: make sure to add the acid to the water.
11. WNV-positive mosquito pools of up to 50 individuals may contain only one infected mosquito, and thus, the pool cannot be split to process for live virus isolation and RT-PCR separately. If both assays are to be performed, homogenize the pool initially in mosquito diluent to prevent degradation of the

virus by lysis buffers (*see* **Note 12**). Then clarify the homogenate by centrifugation and take aliquots for isolation of infectious virus in cell culture and/or RNA purification by RNeasy or MagMAX procedures.

12. Lysis buffers (RLT from the RNeasy Kit and Lysis/Binding Buffer from the MagMAX procedure) contain guanidine salts which are chaotropic agents that destroy the three-dimensional structure of proteins, thus inactivating RNases that are released during homogenization of the tissues. The RNA from mosquitoes homogenized in these lysis buffers is well-preserved, but the integrity of virus is compromised and the samples cannot be used for live virus isolation. When mosquito samples are homogenized initially in mosquito diluent, the quantity and quality of RNA obtained from mosquito samples will be somewhat compromised due to the release of RNases. It is recommended that the samples be kept chilled during all steps of the procedure to minimize RNA degradation.
13. One study [34] comparing WNV present in avian tissues, oral and cloacal swabs, and feather pulp found that viral titers in the vascular pulp samples were up to four times higher than tissue samples and swabs. Another study [35] using the Vector antigen test found that feather pulp was an excellent source of WNV antigen in certain infected bird species.
14. Oral and cloacal swabs have been used for detection of WNV in other vertebrate species [36–38].
15. Ethanol promotes selective binding of RNA to the silica-based membrane in the column
16. It is very important that all ethanol be removed from the sample to prevent degradation of RNA; use care in removing the column after the last spin so that the column does not contact the flow-through. If carryover is observed or suspected, transfer the spin column to a clean 2 mL collection tube and repeat the centrifugation.
17. The capture time depends on the magnetic stand used; with the Ambion 96-well Magnetic-Ring Stand, the capture time is 2–3 min.
18. When the Diluted TURBO DNase is added to the sample, nucleic acids are released from the RNA Binding Beads and genomic DNA is degraded.
19. Drying the beads removes residual ethanol which otherwise could interfere with downstream applications.
20. Although WNV will amplify in C636 cells, CPE usually is not observed, and plaques do not form. Therefore this cell line is not suitable for detection of virus by the presence of CPE, or for immunofluorescence assays.

21. Early examination of inoculated cells may reveal damage caused by toxicity from a component of the inoculum. The distinction between CPE and cytotoxicity often can be resolved during subsequent daily examinations, since CPE due to viral infection is progressive, spreading through the monolayer with time.
22. It is important to compare "positive cells" to the positive and negative controls. Some antibodies create substantial background "fluorescence" which can be misinterpreted. Persistent background fluorescence may be reduced by adding 0.2 % BSA to the PBS used for antiserum dilutions.
23. Storage of serum samples and mild hemolysis has not been observed to affect cell growth or reproducibility of results.
24. If an insufficient volume of test serum is available, a higher starting dilution, usually 1:10, may be used [28]. Diluted positive and negative serum controls may be used for at least 3 months provided they remain contaminant free (not cloudy, no pH change) and do not lose reactivity.
25. Stained monolayers that appear to be dried out with uncountable plaques may be caused by allowing the monolayers to dry during infection or incubation. Take care to keep the time that the plate covers are removed to a minimum. Drying also could be caused by adding the agar overlay when its temperature is too hot. Light staining of the cells may be due to precipitation of neutral red out of solution, which may be avoided by adding the neutral red to 56° agar before combining the agar with 2× medium. Light staining may also be due to unhealthy cells, which will not take up the stain optimally.
26. This screening assay has been used to study the prevalence of WNV infection within wild bird populations [28, 39]. A similar assay may be used for other wildlife populations, as well as for detection of IgG in clinical samples.
27. A checkerboard titration (CTB) is an ELISA designed to determine the optimal concentration of one or two ELISA assay components The CBT assay is accomplished by serial dilution of one reagent across the plate and serial dilution of a second reagent down the plate. This design permits you to analyze different concentrations of two reagents in each well to determine the optimal combination of both reagents. The maximum number of reagents that can be titrated on one plate is two. However, if you have a reasonable estimate of the optimal concentration, it is possible and efficient to test fewer dilutions, and thus include more than one CBT on a plate.
28. It is important that enough antigen is available on the surface of wells for antibody binding. Excess antigen is wasteful. It is critical that the immobilized antigen molecule be coated to the

surface as a monolayer to ensure the greatest precision. An ideal monolayer coverage has no void areas on the surface (indicating the concentration is too low) or unstable multilayer formation from protein-protein interactions (indicating the concentration is too high). Excessive concentrations of coating protein may actually lead to less coating.

29. Common dilutions are 1:1000–1:2000. Avoid using it too concentrated (wasteful and may cause background) or too dilute (may not detect weak positives).

30. TMB (3,3′,5,5′-tetramethylbenzidine) is a chromogen that yields a blue color when oxidized by HRP. The blue color change can be read on a spectrophotometer at a wavelength of 650 nm, and the blue color continues to develop over time enabling collection of sequential OD readings for a kinetic or non-stopped ELISA. When the reaction is stopped by addition of acid (HCL) a stable yellow color results that may be read at 450 nm.

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Chapter 16

Field Methods and Sample Collection Techniques for the Surveillance of West Nile Virus in Avian Hosts

Sarah S. Wheeler, Walter M. Boyce, and William K. Reisen

Abstract

Avian hosts play an important role in the spread, maintenance, and amplification of West Nile virus (WNV). Avian susceptibility to WNV varies from species to species thus surveillance efforts can focus both on birds that survive infection and those that succumb. Here we describe methods for the collection and sampling of live birds for WNV antibodies or viremia, and methods for the sampling of dead birds. Target species and study design considerations are discussed.

Key words West Nile virus, WNV, Avian, Surveillance, Sample collection

1 Introduction

The spread, maintenance and amplification of West Nile virus (WNV; *Flaviviridae*: *Flavivirus*) is enabled by vector mosquito blood meal selection among a wide variety of avian hosts. Numerous avian host competence studies [1, 2] have demonstrated the varying viremia response of different bird species to infection and therefore the differing roles that they play in the transmission of WNV [3, 4]. Some species such as columbiforms (doves and pigeons) and galliforms (chickens and quail) produce very low viremias and generally survive WNV infection to produce robust antibody responses. Other species such as corvids (crows, jays, and magpies), American robins (*Turdus migratorius*), and house finches (*Haemorhous mexicanus*) can produce elevated viremias, sufficient to infect mosquitoes, and may or may not succumb to infection. Because of their importance in the maintenance and amplification of WNV, surveillance efforts can be enhanced by including avian reservoirs that survive and succumb to infection.

Determining the best surveillance strategy for detection of WNV in wild bird populations is contingent upon the research question and species of interest. Generally, there are four possible WNV states observed for individuals within avian populations:

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(1) susceptible [never infected]—virus and antibody negative for WNV, (2) immune [survived infection]—virus negative but antibody positive for WNV, (3) acutely infected at capture—infectious virus particles detectable in blood (viremic) but usually antibody negative, and (4) dead—succumbed to infection and WNV positive. A sero-surveillance program, where wild birds are captured alive and blood samples collected can identify susceptible, immune, and acutely infected birds. However, sample collection and testing methods vary depending on intent to detect antibodies, viral RNA or infectious virus. In addition, it is important to consider what a positive test result means in terms of the viral transmission cycle.

1.1 Considerations

Birds found antibody positive for WNV have survived one or more WNV infections or have been infected with a cross-reacting flavivirus such as St. Louis encephalitis virus (SLEV). Generally, birds produce robust and long-lived antibody responses to WNV [5]. Antibodies can be detected in serum or plasma using traditional antibody detection assays such as plaque reduction neutralization test (PRNT) or enzyme immune-assay (EIA). A bird that demonstrates a specific antibody response to WNV has presumably survived infection and is now immune for life; however, antibody titers may decay with time post-infection. Subsequent infections result in the boosting or reemergence of circulating antibody titers [6, 7]. The timing of WNV infection cannot be directly inferred from a positive antibody result, but may be delimited, in part, by the age of the bird. For instance, a nestling or hatch year bird must have been infected sometime between hatch and capture dates. Recaptured banded birds that previously were antibody negative then found positive were infected between encounter events.

Surveillance for WNV can target either infectious virus by plaque assay or viral RNA by RT-PCR. Although acute viremias have been reported in free-ranging birds [8, 9], this is not a common approach for general WNV surveillance, because the viremic period is generally <1 week, limiting the probability of detection. Acute infection surveillance is generally performed on very young birds that have not had sufficient time to develop an antibody response or when detection of acute viremia is important to the research question.

Because susceptibility to WNV varies at the species and individual level, this variation must be considered when interpreting surveillance data. For instance species that are refractory to WNV infection, such as pigeons [1], doves [10], and quail [11], are commonly found antibody positive for WNV and are generally not found WNV positive in dead bird surveillance programs. Western scrub-jay (*Aphelocoma californica*) are moderately susceptible to WNV; e.g., in an experimental infection study 3 out of 5 WNV-infected jays succumbed to infection [12]. These moderately susceptible birds are often found both positive for WNV antibody

[13] as well as dead and positive for WNV RNA [14]. American crows (*Corvus brachyrhynchos*) are highly susceptible and almost always succumb to WNV infection [15], are one of the most commonly encountered species in dead bird surveillance programs [14], but rarely are found antibody positive for WNV [16].

1.2 Data Collection

Dead bird surveillance programs are useful for identifying active WNV transmission activity, but the success of these programs relies on the public encountering and reporting carcasses and on laboratory tests confirming that WNV was present at the time of death. If relying on the public to report dead birds, repeated outreach programs are critical for optimizing dead bird detection and reporting. This passive surveillance is biased by human population density necessary to detect carcasses, but is useful for locating urban and periurban transmission foci not anticipated by public health agencies.

Live bird surveillance includes both sentinel and wild bird sero-surveillance programs. Spatial coverage is, by design, biased by surveillance site selection. Sentinel programs take repeated samples from confined birds placed at locations where transmission is anticipated. Mosquito attractive and WNV incompetent birds such as chickens or pigeons typically are used to prevent amplification of virus near the location of sentinel deployment. Wild bird sampling programs are by necessity biased by the method of collection and usually focus on limited subsets of species; for example, small passerines collected by mist nets. Baited traps can catch large numbers of birds but data may be biased towards specific species based on baits used, or within species, for certain "trap happy" individuals. If wild bird programs can target epidemiologically important species, then sero-prevalence levels can measure "herd immunity" and be useful in forecasting WNV outbreak risk [17].

2 Materials

2.1 Permits and Protocols

1. Refuge and local permits and land-owner permission.
2. State collection permits.
3. Federal and international banding and take permits.
4. Institutional animal care and use committee (IACUC) protocols.

2.2 Wild Bird Sero-surveillance

1. Approved permits and protocols.
2. Mist net pole system and tethers. Mist nets, mesh size depends on target species, 38 mm is appropriate for most passerines (*see* **Note 1**).
3. Baited traps: traps can vary in size; smaller traps generally have ground level cone entrances (sparrow trap) and are improved by adding catch boxes to aid in removal of trapped birds.

Larger walk-in traps have a funneled channel-type opening (crow trap). Cone size and channel width can be adjusted to suit target species. Bait choice is also an important consideration and is species dependent (*see* **Note 2**).

4. Bird holding bags.
5. Bird bands: in the USA bird bands are supplied and regulated by the US Geological Survey (USGS) Bird Banding Laboratory located at Patuxent Wildlife Research Center. Band sizes are specific for each species and contain unique band numbers. All banding data must be reported to the Bird Banding Laboratory.
6. Reference guides for identification, aging and sexing birds such as the *Identification Guide to North American Birds Part I and II* [18, 19] (*see* **Note 3**).
7. Wing rule—for taking wing chord measurements.
8. Calipers—for taking various measurements used to age, sex, or speciate.
9. Scale: spring or digital.
10. Optivisor binocular headband magnifier: useful hands-free magnifier for improved visualization of skull ossification, molt patterns, or sample collection.
11. Data sheets.

2.3 Blood Sample Collection

1. Gloves, suitably sized for maximum dexterity, thicker gloves are more resistant to tearing but can reduce dexterity.
2. 70% isopropanol or ethanol to clean venipuncture site and wet feathers.
3. 27 G × 1 in. hypodermic needles for pricking ulnar veins.
4. 28 G × 1 or ½ in. hypodermic needles on 0.5 or 1.0 mL syringes for drawing 100 μL of blood from most bird species, 27 G needles can be used on larger (crow-sized) birds.
5. Heparinized capillary tubes.
6. Capillary tube clay sealant.
7. Capillary tube bulb for expressing blood.
8. 2.0 mL screw-top, leak-proof microcentrifuge tubes.
9. Biohazardous Sharps containers.
10. Labels for sample tubes containing unique sample numbers.
11. Phosphate buffered saline (PBS).
12. Virus transfer medium (VTM): Dulbecco's modified eagle medium, 500 U/mL penicillin, 0.5 mg/mL streptomycin, 250 μL/mL amphotericin B, and 20% fetal bovine serum.
13. Centrifuge.
14. Pipettes.

15. Pipette tips.
16. 18 G × 1½ in. blunt tipped needles on 1 mL syringes, for removing plasma from capillary tubes.

2.4 Collection of Dead Birds

1. State and federal permits.
2. Sealable bags, sized to fit birds small (sparrows), medium (jays and crows) and large (raptors) sized birds.
3. Gloves.
4. Cooler.
5. Ice or ice pack.

2.5 Sample Collection from Dead Birds

1. Gloves, *see* Subheading 2.3, **item 1**.
2. Biosafety cabinet.
3. Sharps container.
4. Dissection scissors and forceps for necropsy.
5. Sterile polyester tipped applicator for collection of oral swabs.
6. Microcentrifuge tubes 2.0 mL or compatible with downstream applications.
7. 3.0 mL syringes.
8. 16 G × 1½ in. hypodermic needles for collection of brain aspirates.
9. Sample storage solution.
   - Lysis buffer (compatible with downstream RNA protocols).
   - VTM (from Subheading 2.3, **item 12**).
   - RNASound cards (Fortis Bio) or other lysis impregnated filter paper.

3 Methods

3.1 Permits and Protocols

US law states that "A banding or marking permit is required before any person may capture migratory birds for banding or marking purposes or use official bands issued by the Service [USGS Bird Banding Laboratory] for banding or marking of any migratory bird." (50 CFR 21.22). Collection of blood and "take" of dead birds require additional permitting. In addition to collection permits, animal care and use protocols may also be required by your institution or funding agency. Permits should be established well before the start of any field work. Capture, handling, and sample collection of wild birds requires a special skill set and should be conducted by well-trained personnel.

3.2 Wild Bird Surveillance

Trapping methods depend on target species. McClure [20] provides detailed descriptions of trap designs. Baited traps and mist nets are most commonly used for WNV surveillance.

1. *Capture method*. To achieve maximal performance from baited traps, begin baiting prior to the first trapping day, and check traps to ensure target species are visiting the trap prior to attempting capture. Mist nets are most effective within the first hours of dawn when bird activity is generally highest and nets are difficult to see in early morning shadows. According to guidelines provided by the North American Banding Council (NABC) [21], traps or mist nets should not be operated when temperatures are above or below 0–35 °C, or when raining or excessively windy. Traps/nets should not be placed in direct sun, water should be constantly available inside baited traps, nets should be checked every 30 min and traps should be checked every 60 min, checks should be more frequent during the breeding season or if suspected predators are in the area.
2. *Capture capacity and effort*. It is important to anticipate your catch size and plan staffing accordingly. If banding at a new location or during migration periods, it is better to start the day opening a conservative number of nets/traps to prevent being overwhelmed by a heavy catch. If numbers become larger than what can be processed in 1 h, close traps/nets and work through the backlog before reopening. Depending on surveillance purpose, interpretation of data may need to be adjusted for effort. If effort will be considered, develop a consistent trapping routine and record net and/or trap hours per outing.
3. *Prior to processing*. Once birds are captured, place each bird individually into an appropriately sized bird bag. The bird should have enough space to move freely in the bag, but the bag should not be too big that removal unduly stresses or allows the bird to escape. Bird bags should be secured with a drawstring or tie wrapped around the neck of the bag and back through one of the loops of the tie. Bags should be made out of a "breathable" fabric and be free from loose thread that can entangle bird toes. Hang bird bags so they are shaded, fully suspended and out of the way to prevent injury of captive birds.
4. *Determination of species, age, and sex*, birds should be identified to species and aged and sexed where possible. Skull ossification, plumage and molt patterns can be used to identify ages. Use of an Optivisor wearable magnifier may improve visualization of skull ossification patterns. Plumage, measurements, and presence of brood patch or cloacal protuberance can be used to identify sex. Useful guides for familiarization of these fea-

tures are the *NABC North American Bander's Study Guide* [21] and the *Guide to the identification of North American Birds Part I and II* [18, 19] (*see* **Note 4**).

5. *Banding*. Marking each bird captured with a leg band is useful if samples are taken repeatedly in the same area and there is a need to identify recaptures or individuals. Bird bands are provided by the USGS Bird Banding Lab, some studies also require the use of color bands that are used to identify individuals without recapture. Use of color bands (auxiliary markers) must be approved on banding permits. All banding data must be reported to the USGS banding lab.
6. *Taking measurements*. There are many measurements that banders collect depending on the question at hand, but two basic measurements are wing chord and weight. Wing chord can be measured with a wing rule, available from banding supply houses. Weight can be taken with a spring or digital scale. If using a spring scale, weigh the bird in the bird bag, then subtract the weight of the bag. If using a digital scale, an efficient way to weigh a passerine is to slip it head first into a cup, the bird should just fit in the cup, thus preventing it from flapping its wings or flipping around to escaping. Tare the cup before putting the cup and bird onto the scale.
7. *Record keeping*. Field records should be kept in an orderly fashion for each bird and, if banding, include information required by the USGS. Critical data include, but are not limited to: capture date, capture location, species, band number, age, sex, how age and sex determinations were made, weight, wing chord, samples collected, and sample numbers.
8. *Securing nets and traps*. When bird capture activities are completed for the day, make certain to take down or completely secure mist nets and to leave traps in non-capture-mode so that birds can freely move in and out of traps. Mist nets and traps either unintentionally left open or accidentally changed into capture-mode can inadvertently catch birds and lead to unacceptable levels of mortality.

3.3 Sample Collection

The most common sample collected from living birds for WNV surveillance is blood. Blood samples can be used to test either for WNV antibodies (previously exposed) or for WNV viremia (acutely infected) (*see* **Note 5**). Handling of possibly infectious birds should be done using proper personal protective equipment (PPE) as high quantities of virus can be present in all samples, including feces. Gloves should be worn at all times during blood collection, and all sharps biohazardous waste deposited in and disposed of in appropriate hard walled containers. The amount of blood collected and method used for collection are important considerations.

Guidelines published by the Ornithological Council [22] state that no more than 1% of body weight can be collected per sampling and no more than 2% can be collected in a 2-week period. Therefore, 100 μL of whole blood can be collected from a 10 g bird. Treatment and handling of blood samples in the field is determined by downstream assays.

1. *Bird restraint*. Blood collection should be done only after all other processing of the bird is complete, because newly clotted veins are susceptible to reopening during handling. Regardless of the method of blood collection, develop a safe restraint hold that prevents the bird from moving and exposes the vein of interest. Most holds are modified versions of the bander's grip where the head of the bird is held between the first and middle finger and the thumb, ring, and little finger wrap loosely around the bird and are used for manipulating the bird. Always handle bird wings with caution, as small passerines can be especially susceptible to wing strain; therefore, handle wings only when necessary and do not manipulate wings out of normal planes of movement. Feathers can be dampened with 70% ethanol or isopropyl to enhance vein visualization, but do not drench the bird in alcohol, especially in cool weather. The best application method is to keep a small cup of alcohol available, dip finger in the alcohol, then touch it to the venipuncture site. If using the prick and capillary tube method (described below), alcohol should be allowed to evaporate before pricking the vein, because alcohol contamination can interfere with some downstream assays.
2. *Blood collection*. There are several effective blood collection methods for birds (*see* **Note 6**). Blood can be collected from the right jugular, cutaneous ulnar, or metatarsal veins. The jugular vein is the best choice for syringe venipuncture due to its size and clotting capacity, but there are some cases where alternate sampling locations are indicated. The ulnar or brachial veins are optimal for densely feathered birds, such as columbiforms, where the jugular vein is not readily visualized, or where the size of the bird makes restraint for jugular venipuncture awkward. However these veins, especially the ulnar, are more prone to hematoma and bleeding post-release. For most birds 28 G needles on 1.0 or 0.5 mL syringes are ideal for syringe venipuncture. Blood can also be collected from the ulnar vein by pricking the vein with a 27 G needle and collecting the blood with a heparinized capillary tube.
3. *Post-collection*. After blood collection it is important to stanch the flow of blood. For syringe venipuncture, before removing the needle, place the thumb gently over the venipuncture site, remove the needle, and then apply more pressure. The amount of pressure applied should be sufficient to stop the flow of

blood, but still allow blood to flow through the vessel. Keep the bird in the blood draw grip and apply pressure for a minimum of 10–20 s before checking for clotting, and then observe the venipuncture site for 10 s to ensure bleeding has ceased. If bleeding is noticed, apply pressure for a further 30 s to 1 min; if bleeding appears to have stopped observe the site for a further 10 s to ensure bleeding has stopped before releasing the bird. Leaving the bird in the bleeding grip will allow you to easily apply pressure to and observe the venipuncture site. If blood has flowed onto the skin, the blood can stick to a gloved finger and cause the vein to reopen when the finger is lifted. The corner of an alcohol prep pad can be applied to the venipuncture site and a thumb placed over the pad to apply pressure to prevent sticking. Be sure only a small corner of the pad is in contact with the bird, as full contact can drench the bird's feathers. If there was an irregularity in any part of the blood draw, and a hematoma is suspected, apply pressure for a longer period of time before checking the site. For the prick and capillary method, after blood collection apply pressure as described above; hold for at least 30 s before checking for clotting and observe the venipuncture site for at least 10 s before releasing the bird.

4. *Sample handling*. For a typical surveillance program, it is effective to draw 100 μL from all birds over 10 g, thus streamlining the sample handling process and keeping dilutions uniform. Serum or plasma is required for most antibody assays. If collecting serum for antibody assays, performing a field dilution in PBS (phosphate buffered saline) is recommended. Microcentrifuge tubes (2.0 mL) with leak-proof screw top lids can be prefilled with 450 μL PBS and labeled in advance. In the field 100 μL of whole blood can be added to the PBS for a 1:10 serum dilution (*see* **Note 7**), presuming approximately a 50% hematocrit or packed red blood cell volume. Heparinized tubes can be used if whole plasma is required, but avoid potassium EDTA as it can cause hemolysis in some bird species. Mix all tubes after adding blood by rocking back and forth. Filled capillary tubes should be immediately dispensed into a 1:10 field dilution using a capillary tube bulb or stopped with clay sealant. In the field, samples for serology should be stored in a cooler on blue ice. Serum samples should be allowed to clot for at least 30 min prior to centrifugation. Samples prepared for serology also can be tested by RT-PCR for WNV RNA. If virus isolation is planned, samples should be diluted 1:10 in VTM and frozen on dry ice in the field.

5. *Sample Processing*. Consult with your testing lab to determine optimal tubes, centrifugation, and storage conditions for your samples. To clarify samples, plasma should be centrifuged at

2000–5000 × *g* (gravity) for 5 min and serum should be centrifuged at 6000–11,000 × *g* for 7 min. Confirm that selected tubes are appropriately sized for your centrifuge, if planning to spin capillary tubes, a special rotor or centrifuge may be necessary. Serum should be pipetted from the red blood cell pellet and transferred to a new microcentrifuge tube. Plasma is removed from capillary tubes using a syringe and 18 G blunt tipped needle. Store antibody samples at −20 or −80 °C. Be sure to note the field dilution on the sample tube or datasheet, and use a sample numbering system that will easily track back to field data and bird band numbers. Samples that will be tested for WNV RNA or used for virus isolation should be stored at −80 °C.

3.4 Dead Bird Surveillance

Dead bird reporting programs can be organized so that members of the public report dead birds that are then collected and tested for WNV. The California Department of Public Health, for example, operates a hotline where members of the public can call or utilize online forms to report dead birds. Operators attempt to identify the species and collect data on collection location, date, and condition of the reported birds, and then transfer the reports to local agencies such as vector control districts or public health agencies that retrieve the birds and, if appropriate, submit specimens for testing. Robust dead bird surveillance programs rely on effective public information campaigns. The spatially aggregated reports can indicate major avian die-offs, and laboratory testing can determine if these deaths were attributable to WNV.

1. *Target species.* Many species have been reported dead and found to be positive for WNV. In California, the five species with greatest WNV prevalence of infection at death were: Yellow-billed magpie (*Pica nutalli*), Western scrub-jay, American crow, Red-tailed hawk (*Buteo jamaicensis*), and house finch; however, 169 species have been found dead and tested WNV positive from 2004 to 2012 [14]. Many species are found WNV positive, but not all contribute equally to transmission or are common encounters. Some species, such as mourning doves (*Zenaida macroura*) and rock pigeons (*Columba livia*) may be excluded from surveillance, as these birds are known to usually survive infection, and therefore are not good indicators of WNV activity. Surveillance can be further narrowed by only testing the most susceptible species, such as corvids or only American crows. Data analysis should consider the regional distribution of target avian species as well as human population density (to detect dead birds) and the distribution of vector mosquito species.
2. *Carcass handling.* Dead birds should always be handled with gloves, “double bagged” in sealable bags, and transported in a cooler on blue ice. Birds can be stored at 4 °C prior to sampling.

3.5 Dead Bird Sampling

Prior to sampling, confirm the bird species identification and note visible trauma. The type of sample taken depends on the species of bird and the level of virus present at death. Crows can be reliably sampled by oral swab, whereas other species do not produce as elevated viremias and relying on an oral swab can lead to false negatives [23]; collection of kidney or brain tissue is the preferred sampling method for these species. When present, maggots can be used for WNV surveillance in all bird species, with preference for younger instars containing visible (often red colored) tissue material within the gut.

1. *Safety.* Sampling of dead birds should take place in a biosafety cabinet, by trained personnel. Dead birds infected with WNV can harbor high loads of infectious virus that may be aerosolized during sampling. Gloves and proper PPE should be used and care taken to avoid punctures from sharp instruments as well as beaks and claws.
2. *Sample handling.* Oral swabs and tissue samples can either be placed directly into a lysis buffer that is compatible with downstream RNA extraction protocols, or if virus isolation will be attempted, placed into VTM and stored at −80 °C. Alternatively oral swabs can be rubbed onto RNASound cards where virus will be inactivated and RNA protected for up to 7 days of room temperature storage (ideal for a compact, room temperature shipment). RNA stability is increased when cards are stored −80 °C. Punches can be removed from the card and used for subsequent RNA extraction and detection by RT-PCR.
3. *Oral Swabs.* American crows produce very high tittered infections, frequently exhibit oropharyngeal bleeding at death, and therefore an oral swab is a reliable sample to test for WNV infection [23]. To take an oral swab, carefully open the crow's beak (*see* **Note 8**), and swab the proximal part of the mouth along the tongue, twist the swab to collect a dense sample, place the swab into the selected diluent and snap off the stem of the swab. Even completely desiccated crows can be sampled by oral swab by first moistening the swab in collection diluent and then swabbing as described above.
4. *Kidney snips.* Kidney collection requires dissection of the lower abdomen, as the kidneys lay along the dorsal rib cage, on either side of the vertebral column, proximal to the cloacal opening. Dissections should be performed with the bird lying on its back and can be done with dissecting scissors and forceps.
5. *Brain aspirates.* Collection of brain aspirates is less invasive than collection of kidney snips and can be used for all bird species. To collect the aspirates use a 16 G needle on a 3.0 mL syringe and carefully puncture the side of the cranium or the ear opening for entry into the braincase. Draw back on the

plunger to create a vacuum inside the skull, and brain tissue will be pulled into the syringe. Once collected, pipette up and down in the collection diluent to ensure all brain tissues are washed from the syringe and needle.

6. *Collection of maggots.* Often birds are collected in an advanced state of decay. If maggots are present, they can be collected and tested for WNV. Select early instar maggots with tissue material visible within the midgut of the maggot. Collect 5–10 maggots from each carcass and place in lysis buffer for RNA detection.

4 Notes

1. Mist nets are available in a range of mesh sizes and configurations, but permits must be in place before mist nets can be purchased from supply houses. Mist nets are an effective tool for the capture of wild birds but required training before they can be used safely and effectively. Birds often become very entangled in net material and require great skill to remove safely. Checking nets frequently is critical to limit the degree of entanglement.
2. Traps should be configured and baited based on the species of interest. Examples of targeted bait include: wild bird seed (millet and sunflower) to catch sparrows, finches, quail, doves, and jays; peanuts to catch jays; cafeteria scraps to catch crows. Bait choice can limit unintended captures, however, most bait types will attract rats and squirrels that can be very disruptive to a trapping program and even kill captured birds. Traps must be monitored frequently to prevent injury of trapped animals.
3. Aging and sexing birds can rely on subtle characters such as skull ossification and feather molt patterns that are contingent upon the time of year and species of bird. Specialized knowledge and experience is essential to collect accurate data, and field personnel should be thoroughly trained in safe and accurate bird handling techniques.
4. There are times of the year when there will be ambiguities in age and sex, if one is unsure of the age, sex, or species of a bird, field data should be left as unknown. How a bird was aged or sexed should always be noted on the datasheet so that others can infer the validity of aging and sexing criteria. The USGS Bird Banding Laboratory provides codes that can be used to describe how birds were aged and sexed [24].
5. Enzyme immunoassays are generally inexpensive, rapid, broadly reactive, and relatively sensitive. They are useful for rapidly detecting positives, but require confirmation.

Because *Flaviviruses* native to North America such as SLEV readily cross react with WNV, it is necessary to specifically identify antibody positive sera using a quantitative plaque reduction neutralization assay (PRNT). For definitive diagnosis, the identified virus should have an end point titer ≥4× the competing virus. Therefore, the collector is frequently torn between minimizing sample size to preserve bird health and maximizing sample size to provide sufficient material for screening and confirmatory analysis. The recommended 1:10 field dilution generally provides 450 μL of diluted serum and is usually sufficient for testing.

6. Researchers new to avian blood collection techniques should be mentored by experienced personnel such as an avian researcher or veterinarian. When performed properly, blood collection should not endanger the bird. However, recognize that when collecting blood from small passerines hematomas can be fatal. Blood collection should only be performed by well-trained and experienced individuals.
7. When whole blood samples are collected, post-centrifugation serum often "gels" and is impossible to pipette. When only 100 μL of blood is collected, this can severely impact sample yield. To reduce "gelling," samples can be diluted in the field. Thorough mixing of the diluted blood generally reduces, but does not completely eliminate this problem. When diluting samples it is important to consider what kind of dilution you are making. If downstream assays rely on serum dilution for interpretation then field dilutions should be made accordingly. Whole blood is approximately 50% serum, thus a 100 μL sample of whole blood contains 50 μL of serum, to make a 1:10 serum dilution, add 100 μL of whole blood to 450 μL PBS.
8. When opening the beak of a crow it can be difficult to pry the mandibles apart. The tip of the beak can be sharp and has the potential to puncture the skin of the individual sampling the bird. If the bird's mandibles will not open easily, reverse action pliers can be used to carefully pry them apart.

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Chapter 17

Statistical Tools for the Interpretation of Enzootic West Nile virus Transmission Dynamics

Kevin A. Caillouët and Suzanne Robertson

Abstract

Interpretation of enzootic *West Nile virus* (WNV) surveillance indicators requires little advanced mathematical skill, but greatly enhances the ability of public health officials to prescribe effective WNV management tactics. Stepwise procedures for the calculation of mosquito infection rates (IR) and vector index (VI) are presented alongside statistical tools that require additional computation. A brief review of advantages and important considerations for each statistic's use is provided.

Key words *West Nile virus*, Mosquito surveillance, Surveillance indicators, Mosquito infection rate, Vector index

1 Introduction

Public health officials must continuously monitor various *West Nile virus* (WNV) surveillance indicators to make informed decisions to attempt to minimize human infection risk. Ideally, effective surveillance indicators provide geographically sensitive and specific advanced warnings of imminent increases in human WNV risk. Since human infection risk is preceded by pathogen amplification within animal reservoirs, environmental WNV surveillance attempts to measure pathogen presence or intensity of the enzootic (animal–mosquito–animal) transmission cycle. Mosquito-transmitted pathogen surveillance should be sufficiently spatially and temporally resolved to provide disease management officials the necessary information to direct control efforts to specific areas prior to the elevation in human infection risk. At a minimum surveillance should indicate areas that are experiencing increased human risk to potentially prevent additional human infections. The sensitivity and specificity of surveillance systems must be balanced with fixed financial, labor, and equipment resources. Many of these indicators have been more extensively reviewed in Chaps. 14–16 of this book and elsewhere [1]. Table 1 lists surveillance indicators for WNV

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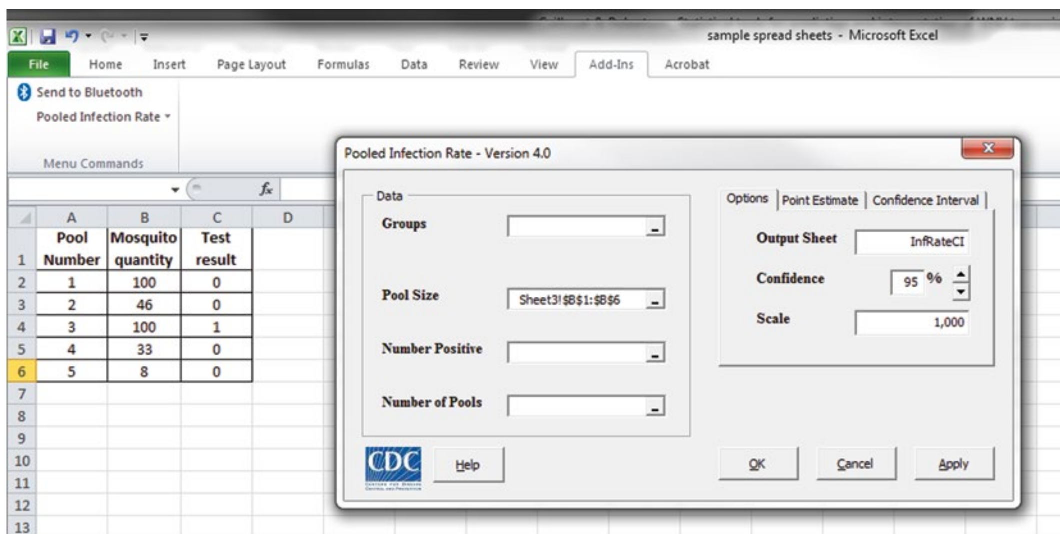
Table 1
Enzootic *West Nile virus* surveillance indicators

Category	Surveillance indicator	Advanced warning of human infections	Usefulness	Cost	Primary disadvantages
Sentinel animals	Antibody seroconversion	++	++	$$$	Requires phlebotomy and antibody testing, logistically difficult
Reservoir hosts	Dead bird presence/ abundance	++	+	$$	Requires pathogen testing, population dependent reporting
	Antibody or pathogen prevalence	+/−	+	$$$$	Requires phlebotomy and antibody testing, logistically difficult; low sample size
	Host species presence	+/−	+	$$	Requires species identification skills; logistically difficult across space
	Host community index	++	+++	$$	Requires species identification skills and computation; logistically difficult across space
Vectors	Species presence	+	++	$	Requires species identification skills
	Species abundance	+	+++	$	Requires species identification skills
	Pathogen presence	+++	++++	$$$	Requires species identification skills and pathogen testing capability
	Mosquito infection rate (MIR)	+++	+++++	$$$	Requires species identification skills, pathogen testing capability, and computation
	Vector index (VI)	+++	++++++	$$$	Requires species identification skills, pathogen testing capability, and computation

and includes information on their relative cost and usefulness as advanced warning of elevated human WNV risk. Though monitoring elements of both hosts and vectors can be useful in environmental WNV surveillance, most widely used indicators focus on vector-specific factors for three reasons: (1) they are relatively inexpensive and can be scaled easily to achieve moderate spatial resolution across wide areas; (2) they often provide advanced warning (1–3 weeks) of pending human infections [2]; and (3) due to the lack of an effective human vaccine, management of human exposure to vector populations is currently the only option for WNV risk reduction [1]. Mosquito-centric WNV surveillance indicators include monitoring vector presence, vector abundance, pathogen presence in vectors, pathogen prevalence (infection rate) in vectors, and abundance of infected vectors. Traditional entomologic measures of describing transmission dynamics for pathogens such as malaria including the entomologic inoculation rate, vectorial capacity, and the basic reproductive rate are not commonly used in WNV surveillance. New or novel applications of existing calculations such as the WNV host community index and force of infection give insight into vector–host interactions that are difficult to observe, but particularly important in the transmission of zoonotic arboviruses. Each of these indicators require increasing levels of skill, cost, and computation, but the additional information gained usually is accompanied by increasing level of correlation with human infection risk.

Relatively few statistical tools have been developed to aid in the estimation of human WNV infection risk. Some of these tools, such as the Centers for Disease Control and Prevention's Pooled Infection Rate calculator (Fig. 1) [3], are available at no



	A	B	C
1	Pool Number	Mosquito quantity	Test result
2	1	100	0
3	2	46	0
4	3	100	1
5	4	33	0
6	5	8	0

Fig. 1 Designating fields in the Pooled Infection Rate calculator Microsoft Excel Add-in

cost, in user-friendly software formats. All WNV surveillance indicators, regardless of the complexity of computation, require basic knowledge of how they are generated and how they may be biased in order to appropriately apply their estimation to the practice of WNV risk management. This chapter reviews the statistical tools and processes currently available for the estimation and interpretation of WNV infection risk to humans using enzootic transmission indicators.

2 Materials

2.1 Surveillance Data Storage

Chapters 14 and 15 outlined tools and mosquito collection devices for WNV surveillance. Once mosquitoes are collected using any type of collection device, at a minimum they should be enumerated and recorded with corresponding information regarding how they were collected (i.e., collection method), where they were collected (i.e., site identification), and when they were collected (i.e., date). If possible, mosquitoes should be identified to species using morphological taxonomic keys (such as [4]) or molecular methods. Species information should also be stored along with the collection information (*see* Tables 2 and 3). It is also a good practice to enumerate male mosquito specimens and to note the abdominal status of individual female mosquitoes (e.g., whether the female mosquito's abdomen is filled with eggs (gravid), blood, or empty). The storage and retrieval of mosquito surveillance data is greatly aided by the use of a digital database or spreadsheet software. Digital storage of surveillance data in a database file can be easily integrated into mapping software to create a geographic information system (GIS) to enable easier visualization and communication of the data.

2.2 Software to Calculate Mosquito Infection Rates

The Centers for Disease Control and Prevention has developed freely available software for the calculation of mosquito infection rates for pooled specimens. The infection rate calculator, Pooled Infection Rate 4.0 [3], is available for download at http://www.cdc.gov/westnile/resourcepages/mosqsurvsoft.html. The software operates as an "add-in" in Microsoft Excel. Follow the accompanying documentation for installation of the software. *See* also Fig. 2.

3 Methods

3.1 Vector Species Presence and Abundance

Though the monitoring of vector species abundance is a critical element of WNV control, it is not always directly correlated with WNV intensity or even pathogen presence. In other words, locations that have relatively higher vector populations may not have WNV present or may not have the highest intensity of WNV transmission [5].

Table 2
A sample mosquito surveillance data table

Pool number	Species	Date	Site	Collection method	Sex	Mosquito quantity			
						Empty	Gravid	Bloodfed	Half blood-fed/ half gravid
	Culex salinarius	7/1/2015	5th St.	Gravid Trap	Male	3			
1	*Culex salinarius*	7/1/2015	5th St.	Gravid Trap	Female	91	5	1	3
	Culex salinarius	7/1/2015	5th St.	Gravid Trap	Male	1			
2	*Culex salinarius*	7/1/2015	5th St.	Gravid Trap	Female	43	2		1
	Culex quinquefasciatus	7/3/2015	Joan St.	CDC-Light Trap	Male	5			
3	*Culex quinquefasciatus*	7/3/2015	Joan St.	CDC-Light Trap	Female	98	2		
4	*Culex restuans*	7/3/2015	Joan St.	CDC-Light Trap	Female	31	2		
	Culex restuans	7/5/2015	Bluebird St.	CDC-Light Trap	Male	11			
5	*Culex restuans*	7/5/2015	Bluebird St.	CDC-Light Trap	Female	6	2		

Specimens that are pooled for WNV testing are noted by their pool number. In this example male mosquitoes are quantified and the abdominal status of female mosquitoes is also recorded

Table 3
Sample mosquito pool data displaying the minimum required data to calculate the mosquito infection rate (MIR)

Pool number	Mosquito quantity	Test result
1	100	0
2	46	0
3	100	1
4	33	0
5	8	0

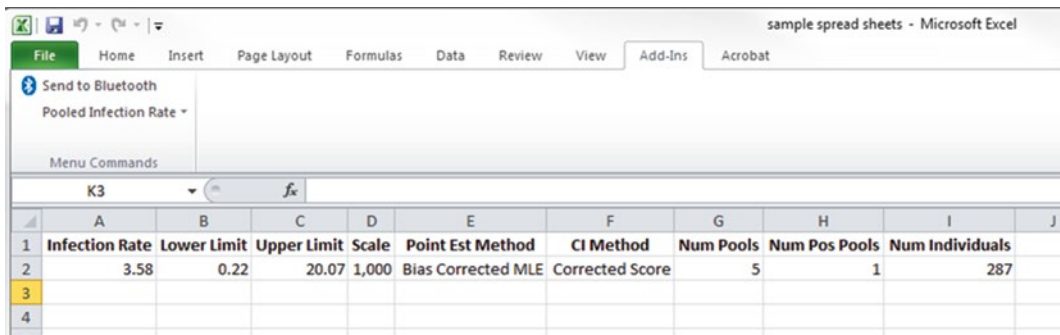


Fig. 2 Sample mosquito infection rate output. *Column A* displays the estimated mosquito infection rate (IR). *Column B* and *C* display the 95 % confidence intervals. *Columns D–I* present summary information for the estimation methods and the pooled data summary

This is due largely to the complexity of the animal host community and selectivity of mosquito vectors for certain host groups across geography [6, 7]. For this reason the presence and abundance of vector species populations alone provide a poor indication of imminent increases in human WNV infection risk.

3.2 Calculation of the Mosquito Infection Rate (IR)

The molecular detection of WNV in mosquito vectors was described in Chaps. 14 and 15. Though mosquitoes can be tested individually for the presence of WNV, they are often tested in groups or "pools" of multiple mosquitoes due to the relative rarity of the pathogen in mosquitoes in the wild. Subsampling from individual pools of specimens into "super" pools has also been shown to sensitively diagnose WNV infection in mosquitoes [8]. For individually tested specimens the mosquito infection rate (IR; number infected mosquitoes per 1000) is simply the number of infected mosquitoes divided by the number of mosquitoes tested scaled by 1000:

$$\mathrm{IR} = 1000\frac{x}{y}$$

Where x is the number of mosquitoes in which WNV was detected and y is the total number of individual mosquitoes tested.

When mosquitoes are tested in pools of multiple specimens the mosquito infection rate is based on an estimate of the number of infected individuals within the pool. The IR estimate varies with the number of individuals within the pool and with certain assumptions regarding the number of infected specimens within the pool.

The minimum infection rate (MIR) assumes that only one mosquito is infected in each pool that tests positive. The MIR can be calculated as the number of infected pools divided by the total number of mosquitoes tested:

$$\mathrm{MIR} = \frac{\#\ \text{pools testing positive}}{\text{total number of mosquitoes tested}}$$

The MIR gives a lower bound on mosquito infection rates and is a poor estimator when infection rates are high or pool sizes are large [9].

The maximum likelihood estimate (MLE) of the IR estimates the actual infection rate as well as confidence intervals, without requiring any additional data beyond the pool sizes and infection status of each pool [9]. The MLE takes pool size into account and is more accurate than the MIR when infection rates are high. While variable pool sizes lead to calculations requiring numerical tools, they may also lead to improved MLE estimates [10].

3.3 Calculation of Basic Mosquito Infection Rates

Here we present the step by step procedure for calculating mosquito infection rates:

1. Open or create the mosquito pooling data table (*see* Table 3). At a minimum this table must have individual mosquito pools listed as rows and include a column indicating the number of mosquitoes per pool and the test result (usually stored as a binary 1 = infected and 0 = uninfected). Additional information such as mosquito species designations, date collected, and site information are useful to create specific infection rates based on that information.
2. In the Add-Ins Tab on the MS Excel File bar click on Pooled Infection Rate and select "One-sample". A pop-up window will appear.
3. In the window for "Pool Size" select the column indicating the quantity of mosquitoes in each pool (*see* Fig. 1).
4. Next, designate the column that includes the binary (1 or 0) test result in the "Number Positive" window.
5. Leaving the default options checked for the bias-corrected MLE is recommended.
6. Clicking OK will produce a report of the infection rate that includes an estimate of the mosquito infection rate in column A, the lower 95 % Confidence Interval (CI) in column B, upper

95% CI in column C, scale (per 1000 mosquitoes) in column D, the method used to calculate the estimates in columns E and F, and a summary of the pools in columns G–I.

3.4 Calculate Mosquito Infection Rates by Group

To stratify mosquito infection rates across sites, dates, species, or other group requires the storage of this information along with the mosquito pooling data and selecting this information as a grouping criterion for infection rate calculations (Table 4). The Pooled Infection Rate 4.0 calculator only allows you to group infection rates by a single field. If you wish to create infection rates by two or more groups you must first stratify your dataset by these categories. For example, you may wish to stratify infection rates by species and by week—IR for *Culex pipiens* and (separately) *Culex salinarius* by week of the year. In MS Excel or other spreadsheet software this is easily accomplished by using either the data filtering or pivot table functions. Note that groups must have identical spelling and spacing—extra leading or trailing spaces or misspellings will be treated as separate groups. To illustrate the procedure for creating IR by groups we will create IRs for each calendar week in the dataset (Table 4):

1. Perform actions 1–4 in the above Subheading 3.2.
2. Designate a grouping criterion in the “Groups” window to calculate infection rates that are specific to that criterion. For instance, if you are interested in weekly trends of WNV infection rates, select the column storing the week number. TIP: To quickly calculate week numbers use the MS Excel WEEKNUM function = (WEEKNUM(specify cell with date, 1)). The number “1” in this function specifies the week to start on Sundays; a number “2” would specify the week to start on Mondays.
3. Leaving the default options checked for the bias-corrected MLE is recommended.

Table 4
A sample of a mosquito pooling data table with additional descriptive information

Pool number	Species	Sex	Mosquito quantity	Site	Collection method	Date collected	Week number	Test result
1	*Culex salinarius*	Female	100	5th St.	Gravid	7/1/2015	27	0
2	*Culex salinarius*	Female	46	5th St.	Gravid	7/1/2015	27	0
3	*Culex quinquefasciatus*	Female	100	Joan St.	CDC-light	7/3/2015	27	1
4	*Culex restuans*	Female	33	Joan St.	CDC-light	7/3/2015	27	0
5	*Culex restuans*	Female	8	Bluebird St.	CDC-light	7/5/2015	28	0

4. Click “Ok”.
5. An information pop-up or error message may appear to inform you that corrections of estimates are not defined for samples that are <= 0. This occurs because some of the groups you selected do not have any infected mosquito pools. Click through these messages by selecting “Ok”.

3.5 Vector Index (VI)

Though the IR is widely used and is an easily calculated WNV entomologic surveillance indicator, in certain instances it may not be well correlated with human risk. This is primarily due to the fact IRs may be high when vector abundances are low. In these situations the actual number of infected mosquitoes is often quite low, though the proportion of infected mosquitoes is high (Fig. 3). The Vector Index (VI), an estimate of the number of WNV infected mosquitoes derived from the IR and average mosquito abundance, has been shown to be more closely linked to imminent human WNV risk [11, 12]. The individual species VI can be calculated with the equation:

$$\mathrm{VI} = x\frac{y}{1000}$$

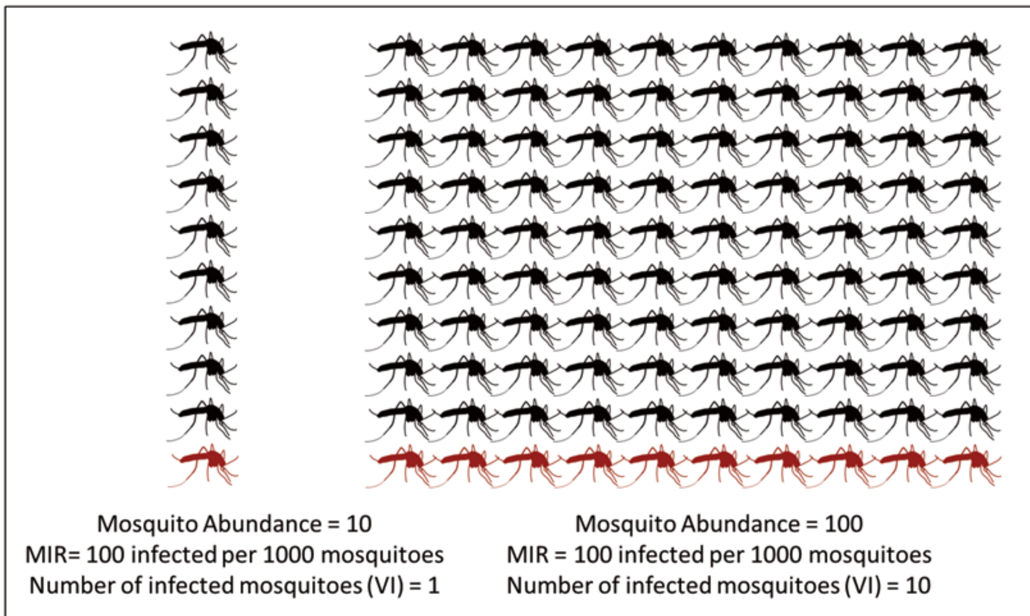


Fig. 3 A graphic demonstration of the effect of mosquito abundance on the interpretation of the mosquito infection rate (IR) and Vector Index (VI). At low mosquito abundances IR may be high, but the actual number of infected mosquitoes is low. Vector Index estimates the number of infected mosquitoes from the IR and the average mosquito abundance

where x is the IR and y is the abundance of the species. Created by Nasci et al. [13] as an indicator of human WNV risk, the VI was originally intended as an estimate of the number of infected mosquitoes (NIM) across multiple key vector mosquito species in an area. The VI calculation has been applied to describe the single species estimate of the number of infected mosquitoes [14–17] as well as the estimate of NIM across species within a genus [2, 13, 18]. Setting threshold triggers for mosquito control interventions based on VI is the current best practice for epidemic WNV management.

3.6 Calculation of the Species Vector Index and Multiple Species Vector Index Across Groups Such as Weeks or Sites

1. To calculate the species VI you must first compile the vector species abundance information. If you are calculating the VI for each individual trap location and for each night collected at this location, the mosquito abundance data is the raw number of female mosquitoes (regardless of abdominal status) collected in each trap for each night of collection. If you are creating an aggregated VI that represents data averaged from multiple collection nights at the same site or multiple sites during the same time period, you must first create the average abundance for the group.
2. Divide the IR for the group of interest or the individual trap by 1000 and multiply the product by the group average (multiple traps or time periods) or raw mosquito species count (individual trap).

3.7 Spatial Applications of the Vector Index

There have been relatively few reported geographic applications of the VI. Some studies report a single VI calculated for the entire area under surveillance (e.g., municipality or county) [2, 14]. Though VI estimates representing relatively large geographic areas, whether from individual trap sites or averaged from aggregated sites, may obscure the intensity of transmission in certain focal areas, the VI has been shown to be correlated with human WNV cases [2, 12, 17]. Jones et al. [19] created weekly and biweekly IR and VI values at the census tract level to provide a geographic and temporal illustration of entomologic WNV risk in order to guide preventative mosquito control activities. In their demonstration, Jones et al. [19] calculated IR and VI from individual mosquito traps. When mosquito abundance per trap is low, the few mosquitoes that are collected are often pooled into one pool resulting in high estimates of IRs (with large confidence intervals). By multiplying IR by mosquito abundance, the VI per individual trap night does to some extent account for the exaggeration of the IR, but care should be taken to include the 95 % confidence intervals of each IR.

3.8 Additional Measures of West Nile virus Transmission

Other indices and statistics have been created to give insight into the enzootic WNV transmission and resultant human WNV risk, but require computation that is beyond the scope of the methodology

presented in this chapter. We include a brief description of each the WNV host community index, force of infection, entomological inoculation rate (EIR), vectorial capacity (VC), and the basic reproduction number (R_0).

3.8.1 West Nile virus Host Community Competence Index and Force of Infection

The composition of reservoir hosts and vectors in a community is very important for determining levels of enzootic WNV transmission [5, 20–22]. Reservoir species are highly variable in their competence, or ability to transmit WNV [23]. Competence is a function of a host's susceptibility, infectivity, and duration of the infectious period. Competence (C) is defined as follows [23]:

$$C = SID$$

where S is the fraction of hosts susceptible to infection when challenged with WNV, I is the proportion of mosquitoes infected with WNV after feeding on the infected host, and D is the duration (in days) of viremia in the host at a sufficient level to infect mosquitoes feeding upon it. Some animal hosts never become infectious and therefore have a competence value of zero and are referred to as non competent or dead-end hosts [23]. Note the factors determining competence may be vector dependent as well as host dependent. Host diversity is frequently associated with an increased presence of non-competent hosts in a community. These hosts serve as sinks for WNV transmission as they absorb bites that might otherwise result in transmission of WNV if another competent host were bitten.

The host community competence index (CCI) is a measure of overall competence for an area [5]:

$$\mathrm{CCI} = \sum_{j} a_{\mathrm{j}} C_{\mathrm{j}}$$

where a_{j} is the abundance of species j and C_{j} is the laboratory derived competence value of species j [23]. The CCI for a community will decrease with the addition of non-competent species or species exhibiting lower WNV competence.

Vectors do not always bite hosts in proportion to their abundance; some species may be preferred hosts. Increased feeding on moderate or highly competent species will amplify transmission while preferential biting of noncompetent or low-competence species will result in the dilution of transmission [24]. A feeding preference index (P_{j}) can be calculated for each species as follows to quantify the heterogeneity in vector biting rates [6]:

$$P_{\mathrm{j}} = \frac{f_{\mathrm{j}}}{a_{\mathrm{j}}}$$

Where f_j represents the proportion of total blood meals from species j and a_j is the proportion of the population abundance comprised by species j. If a species is bitten in proportion to its abundance, then the feeding preference index will be 1. A value of P_j significantly different from 1 indicates a feeding preference for or against that species.

Kilpatrick et al. [6] used the product of the feeding preference index, host abundance, and competence measures to calculate the relative contribution of a single infected individual of each species to the total infectious mosquitoes in an area (F_j) as: $F_j = a_j P_j C_j$.

Hamer et al. [25] defined a similar measure, called the amplification fraction, to estimate the fraction of WNV infectious mosquitoes that were infected by feeding upon a single individual of a certain host species. They modify the above measure by multiplying by P_j to account for the likelihood the host species has been infected:

$$F_j = a_j P_j^2 C_j.$$

Since $P_j a_j = f_j$ this simplifies to

$$F_j = f_j P_j C_j.$$

Multiplying by the abundance of species j gives the fraction of infected mosquitoes infected by all individuals of that species [26]:

$$F_j = f_j^2 C_j.$$

Summing F_j over all species gives the community force of infection [7].

3.8.2 Entomological Inoculation Rate (EIR)

The Entomological Inoculation Rate (EIR), the number of infectious bites per human per day, provides a measure of human WNV risk. The EIR will depend not only on the number of infectious mosquitoes and overall vector biting rate, but also the proportion of bites going to human hosts, as opposed to avian or other hosts. For some important vector species, such as *Culex pipiens*, this proportion is not constant but can vary throughout the season [27].

3.8.3 Vectorial Capacity and R_0

The vectorial capacity (VC) is the total number of infectious bites resulting from vectors infected by one infectious host in 1 day [28].

$$\mathrm{VC} = \frac{V/H\, ba^2 p^n}{-\ln p}$$

where V is the number of vectors, H is the number of hosts, a is the per capita per day biting rate, b is the probability per bite of transmission from vertebrate host to mosquito (we note this term

is not always included), p is the daily survival rate, and n is the length of the extrinsic incubation period of the disease. The number of bites per day per host is aV/H. The number of mosquitoes infected in one day from biting the original infected host is baV/H. These infected mosquitoes survive the incubation period of the disease to become infectious with probability p^n, then bite a times per day for the duration of their infectious period.

The basic reproduction number, denoted by R_0, is defined as the expected number of secondary infections produced by a typical infected individual over the course of its lifetime in a completely susceptible population. It is used to measure transmission at the start of an outbreak; if R_0 is greater than 1, an outbreak will occur, whereas if R_0 is less than 1 the disease will die out in the population. R_0 can be calculated by multiplying the vectorial capacity (VC) by the probability an infectious bite results in vector to host transmission (c) and the length of the infectious period of the host ($1/r$) [29]:

$$R_0 = \frac{c\text{VC}}{r}.$$

Heterogeneity in vector biting rates can increase R_0 [30]. The presence of preferred hosts or host groups can result in increased levels of infection in the vector population and may even result in fewer overall infected hosts [29]. For vector-borne diseases like WNV, the amplification of the virus in the vector population still increases human risk due to increasing the EIR.

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Chapter 18

Role of Immune Aging in Susceptibility to West Nile Virus

Yi Yao and Ruth R. Montgomery

Abstract

West Nile virus (WNV) can cause severe neuroinvasive disease in humans and currently no vaccine or specific treatments are available. As aging is the most prominent risk factor for WNV, age-related immune dysregulation likely plays an essential role in host susceptibility to infection with WNV. In this review, we summarize recent findings in effects of aging on immune responses to WNV infection. In particular, we focus on the age-dependent dysregulation of innate immune cell types—neutrophils, macrophages, and dendritic cells—in response to WNV infection, as well as age-related alterations in NK cells and γδ T cells that may associate with increased WNV susceptibility in older people. We also highlight two advanced technologies, i.e., mass cytometry and microRNA profiling, which significantly contribute to systems-level study of immune dysregulation in aging and should facilitate new discoveries for therapeutic intervention against WNV.

Key words West Nile virus, Aging, Immune aging, MicroRNAs, Mass cytometry

1 West Nile Virus Infection in North America

West Nile virus (WNV) is a mosquito-borne enveloped positive-strand RNA virus belonging to the family Flaviviridae, which includes yellow fever, hepatitis C, and Dengue viruses [1]. WNV was first isolated in Uganda in 1937, and emerged into the USA in 1999. From 1999 to 2014, WNV spread across North America, South America, and the Caribbean leading to > 41,000 cases, including 1753 fatalities. While the majority of WNV infections are asymptomatic (~80 %), some infected patients develop mild symptoms of West Nile fever (~20 %), and a small subset (<1 %) develop severe neuroinvasive disease, including meningitis, encephalitis, and acute flaccid paralysis [2]. Currently, no vaccine or specific antiviral treatments against WNV are available. Notably, advanced age remains a dominant risk factor for WNV infection and elderly individuals are more susceptible to severe infection with neurological involvement [3, 4]. Among patients over 70 years of age, the case-fatality rate ranges from 15 to 29 % [5].

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The world's population is aging and the global human population over age 60 is predicted to increase to over two billion by 2050 [6]. With aging, elderly individuals are increasingly susceptible to infectious diseases and have reduced efficiency of responses to vaccination. While individuals over age 65 currently constitute approximately 15% of the population in the USA, the aged population accounts for a disproportionate use of medical resources. Age related changes in both innate and adaptive immune responses, termed immunosenescence, lead to inappropriate elevations, decreases, and dysregulated immune responses [7]. Here, we will review age-related immune dysregulation relevant to host susceptibility to WNV infection. We will also highlight novel areas for investigation and emerging technical approaches (e.g., mass cytometry and miRNA profiling) that promise to advance our understanding of the complexity of aging and foster discovery of novel therapeutic approaches.

2 Effects of Aging on Innate Immune Responses to WNV Infection

Numerous studies in elderly humans have revealed that aging has a profound impact on the phenotype and functions of innate immune cells [7, 8] and these cell types-neutrophils, monocytes/macrophages, and dendritic cells—have central roles in initiating immune responses to control WNV replication [9–11]. Dysregulation of two other innate immune cell types, natural killer (NK) and γδ T cells, although studied in aging, have not been examined for their role in immune susceptibility to WNV in elderly individuals. Here, we will summarize recent findings on age-dependent innate immune dysregulation of neutrophils, macrophages, dendritic cells in response to WNV infection, as well as age-related alterations in NK cells and γδ T cells that may contribute to WNV susceptibility in the elderly. *See* Fig. 1 for a schematic illustrating the effects of aging on immune cell response to WNV.

2.1 Impaired Neutrophil Function in Aging

Neutrophils are the most abundant leukocytes in human blood circulation and the first immune cells to arrive at the sites of inflammation [12]. At sites of inflammation, neutrophils exhibit potent antimicrobial activities by engulfing pathogens, generating reactive oxygen and nitrogen species, releasing granules containing proteolytic enzymes and antimicrobial peptides, and extruding neutrophil extracellular traps [13–15]. Once the invading pathogens are cleared, neutrophils undergo apoptosis [16]. A variety of neutrophil functions are impaired during aging, including chemotaxis, phagocytosis, superoxide production, NET formation, and apoptosis [17–20]. Alterations of neutrophil signaling pathways and receptors have also been observed in aged individuals. Prominent affected pathways are the MAP kinases, the Jak/STAT

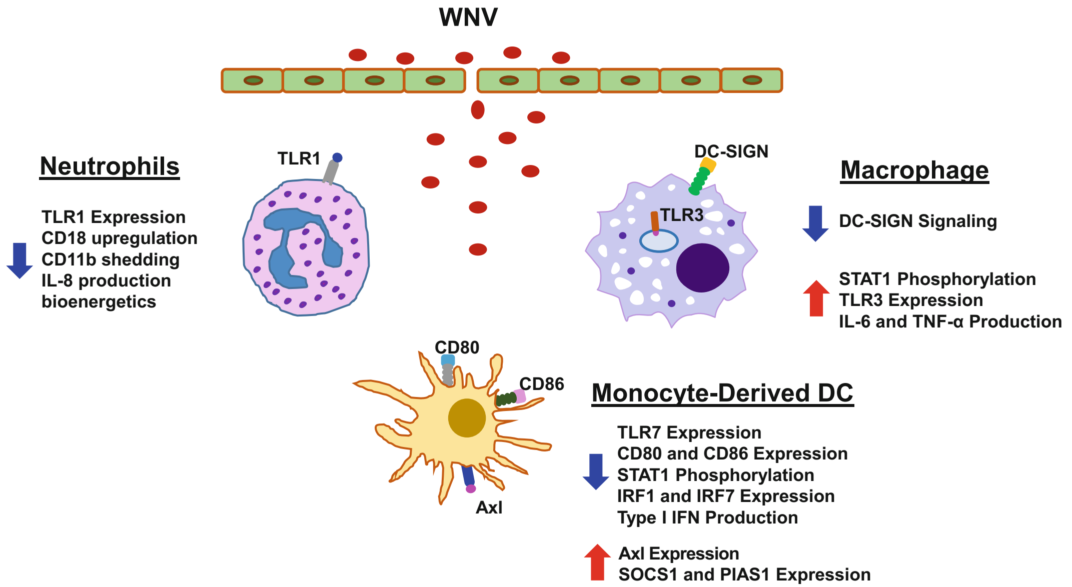

Fig. 1 Effect of aging on neutrophils, macrophages, and dendritic cells in response to WNV infection. The alterations are compared between cells from young and old subjects infected with WNV in vitro. Up- and down-regulation in the elderly is indicated by *red upward* and *blue downward arrows*, respectively. *DC-SIGN* DC-specific ICAM-3-grabbing nonintegrin, *IRF* interferon regulatory factor 1, *SOCS1* suppressor of cytokine signaling 1, *PIAS1* protein inhibitor of activated STAT1

and the PI3K-Akt pathways, which are important regulators of neutrophil functions [21, 22]. The decline of signal transduction in these pathways contributes to age-associated neutrophil dysfunction such as directional chemotaxis. Moreover, neutrophils in older adults have reduced bioenergetics, and lower expression of Toll-like receptor 1 (TLR1), leading to impairment of various neutrophil functions, including activation of integrins (CD18 and CD11b), and production of IL-8 [22].

Neutrophils play a dual functional role in response to WNV infection. Neutrophils serve as reservoirs for WNV replication and dissemination in the early stages of infection, but contribute to WNV clearance later in the infection process [9]. The shift in neutrophils from an early pro-viral state to a later anti-viral state may result from the effects of cellular context such as the robust production of type I interferon by macrophages in response to WNV infection. In vitro pretreatment of neutrophils with type I interferon significantly reduced their WNV viral load [9]. In spite of the supporting evidence in the role of neutrophils in WNV infection, the effects of aging on neutrophil functions in response to WNV remain unknown. Age-associated alterations in chemotaxis, phagocytosis, signal transduction, and expression of TLR receptors likely contribute to the reduced clearance of WNV infection in older subjects.

2.2 Reduced Macrophage Function in Aging

Macrophages are professional phagocytes and antigen-presenting cells and many of their functions become compromised in aged individuals, including chemotaxis, phagocytosis, intracellular killing, production of reactive oxygen species and cytokines (e.g., TNF-α and IL-12), as well as expression of MHC class II and co-stimulatory molecules (Table 1) [7, 8]. In addition, production of prostaglandin E2 is increased in activated macrophages from aged humans and mice, which suppresses MHC class II expression and IL-12 production, leading to impaired antigen presentation associated with age [8]. Alterations in TLR expression have been found in aged macrophages. The baseline level of TLR3 is lower in macrophages from elderly individuals [23]. A few studies have also shown an age-dependent reduction in the levels of p38 MAPK, NF-κB, and MyD88, as well as in the phosphorylation capacity of STAT-1α [24]. The changes of these key signaling molecules are critical factors in the decrease of macrophage activation and cytokine responses in aging.

Following mosquito inoculation of WNV in skin, macrophages are early responders from the innate immune system to control initial WNV replication [5]. They efficiently ingest WNV through receptor-mediated endocytosis, and become activated to produce a large amount of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, as well as type I interferons. These cytokines are critical for restriction of WNV replication and spread and for recruitment of more innate immune cells into the site of infection [25–27]. However, excessive inflammation and cytokine production upon WNV infection can increase the permeability of the blood–brain barrier, leading to viral infection of the central nervous system, and severe neurological disease [26]. Our recent studies indicate some interesting clues in this regard. In contrast to WNV-induced down-regulation of TLR3 expression in macrophages from young donors, in elderly donors the expression of TLR3 remains elevated in WNV-infected macrophages and leads to elevated production of proinflammatory cytokines [23]. This TLR3 dysregulation results from impaired signaling between DC-SIGN and STAT1, which also leads to an early and sustained elevation of IL-6 and IFN-β1 in the elderly [23]. This alteration of the macrophage response with aging detected in vitro may be relevant to cytokine-mediated elevated permeability of blood–brain barrier and increased severity of WNV infection in older individuals [26].

2.3 Dendritic Cell Function Is Diminished in Aging

Dendritic cells (DCs) are potent antigen presenting cells which act as a bridge between the innate and the adaptive immune systems [28]. Studies have shown dysregulation of several functions in both myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) with aging. DCs display an age-related decline in chemotaxis, endocytosis, and global reduction in expression of expression of Toll-like receptors (TLR) 1, 3, 5, 7, and 8, production of IL-12, and antigen

Table 1
Effects of aging on innate immune cells

Functional activity	Cell types				
	Neutrophils	**Macrophages**	**Dendritic cells**	**NK cells**	**Gamma-delta T cells**
Reduced	Chemotaxis Phagocytosis Superoxide production NET formation Apoptosis Signal transduction TLR1 expression TLR1-induced activation	Chemotaxis Phagocytosis Intracellular killing Reactive oxygen species Expression of MHC and co-stimulatory molecules DC-SIGN signaling Cytokine production Antigen presentation	Chemotaxis Endocytosis TLR 1,3,5,7, 8 expression TLR-induced cytokines type I IFN production PI3-K activity Antigen presentation	$CD56^{bright}$ subset frequency NCR expression DNAM-1 expression Granzyme A production Cytotoxicity per cell	Cell frequency and absolute number In vitro expansion capacity of Vδ2 T cells by IPP stimulation
Increased		STAT1 phosphorylation TLR3 expression PGE2 production	Basal expression of CD80, CD86 Basal NK-kB activity LPS, ssRNA-induced cytokines	$CD56^{-}CD16^{+}$ subset frequency CD57 expression	Apoptosis susceptibility of in vitro expanded Vδ2 T cells

presentation, leading to impaired activation of naïve T cells (Table 1) [8, 29]. Paradoxically, DCs from elderly individuals produce a higher basal level of cytokines (e.g., TNF-α, IL-6, and IL-23). In pDCs, reduced expression of TLR7, decreased production of IFN-α by TLR stimulation, and impaired phosphorylation of IRF-7 have been shown in older individuals [30, 31]. DCs from aged adults have reduced expression of co-stimulatory molecules CD80 and CD86, diminished induction of TLR7 expression, as well as decreased production of IFN-α and IFN-β following WNV infection. This dysregulation results from impaired STAT-1 phosphorylation, diminished induction of IRF-1 and IRF-7, and enhanced expression of negative signaling molecules Axl, SOCS1, and PIAS1 in DCs from elderly subjects [32]. These deficits in critical signaling pathways in DC antiviral responses may contribute to the increased susceptibility to WNV infections in the elderly.

2.4 Natural Killer Cell Antiviral Activity Wanes with Aging

Natural Killer (NK) cells are large granular lymphocytes, 10–15 % of the circulating lymphocyte pool, that specialize in early defense against viral infections and tumor cells [33]. NK cells recognize abnormal or infected cells through a complex recognition pathway involving both MHC and a repertoire of invariant activating and inhibitory NK receptors. NK cells maintain extraordinary their functional diversity determined from combinatorial expression of multiple activating and inhibitory receptors [33, 34]. NK cells are classically divided into two major functional subsets (immature and mature) based on the differential expression of surface markers CD56 and CD16: $CD56^{bright}CD16^{-}$ (immature) and $CD56^{dim}CD16^{+}$ (mature) [35, 36]. Immature NK subsets secrete cytokines and chemokines on activation and following maturation exhibit high cytotoxic capacity [37]. NK cells control viral replication by killing infected cells during the earliest stage of infection, and shape adaptive immune responses through cytokine release or by direct interaction with DCs [38–40]. An important role for NK cells has been noted previously in many viral infections, such as HIV-1, influenza virus, cytomegalovirus, and hepatitis C virus [41–43]. In aging, frequency of the immature $CD56^{bright}$ NK cell subset is reduced (Table 1) which may contribute to the impaired production of cytokines and chemokines observed in NK cells of aged subjects [44]. NK cells from older subjects show upregulation of the maturation marker CD57, reduced expression of activating receptors DNAM-1 and NKp30 and NKp46, as well as impaired cytotoxicity and decreased production of granzyme A [24, 45].

Primary human NK cell responses to WNV include activation following interaction of NKp44 receptor with WNV envelope protein [46, 47]; however deficiency of NK cells did not change morbidity in the murine model [48, 49]. It has been challenging to identify precise changes within the NK cell population in humans since current platforms of flow cytometry are limiting for interro-

gation of the more than 20 NK receptors expressed per cell. However, the recent development of mass cytometry (CyTOF) provides the first opportunity to simultaneously evaluate NK cell phenotype and function within the context of the overall immune response. Recent studies have used high-dimensional single-cell data to highlight the extreme diversity of the NK cell repertoire as well as to discover the functional significance of NK cell diversity in viral infection [50]. Indeed, the diversity of the NK repertoire increases following infection with either HIV or WNV, leading to terminal differentiation and reduced degranulation and an increased risk of viral acquisition [51]. Thus NK cell diversity may serve as a measure of immunological age and susceptibility, which may precede chronological aging.

2.5 Gamma-Delta T Cells in Aging

γδ T cells, an intriguing and enigmatic T cell subset, are present in humans at less than 10% of lymphocytes in the peripheral blood (Vδ1 subset) and in diverse tissues, such as skin, liver, gut epithelial tissue, and bronchial epithelia (Vδ2 subset) [52]. γδ T cells respond rapidly to antigens from bacteria, parasites, and viruses, do not require antigen processing and MHC presentation of peptide epitopes, and produce pro-inflammatory cytokines IFN-γ, TNF-α, and IL-17 [53–55]. Numbers of γδ T cells in the blood increase in patients with viral infections and potent antiviral responses include IFN-γ production and CCR5-mediated migration [53, 56–58]. In mouse models of WNV infections, although γδ T cells produce cytokines involved in inflammation and pathogenesis (IL-17, IL-10, and TGF-β), deficient mice ($TCR\delta^{-/-}$) are nevertheless more susceptible, showing elevated viremia and more severe encephalitis. This suggests an important role for γδ T cells in resistance to WNV infection which remains incompletely understood [54]. In aging, both the frequency and absolute number of γδ T cells are reduced (Table 1), stimulated expansion is reduced, and apoptosis is increased, which may contribute to increased susceptibility of older people to WNV infection [59–61].

3 Adaptive Immunity Shows Decreased Responses to WNV in Aging

Decline of the adaptive immunity with age has been well established. These changes include decreased pools of naïve T and B cells accompanied by increased memory and effector T and B cells, decreased diversity of antigen receptor repertoire, defective signal transduction in T cells with dysregulated cytokine production pattern, reduced class switching of B cells, and decreased clonal expansion and function of antigen-specific T and B cells [62, 63]. The age-associated deficits in the CD4 and CD8 T cell response against WNV include impaired production of cytokines and lytic granules, contributing to increased WNV viral titers in the brain of aged

mice [64]. Moreover, aged mice show lower levels of primary and memory T and B cell responses induced by vaccination with West Nile encephalitis vaccine, and repeated in vivo restimulation is needed to generate protective cellular and humoral immunity in older populations [65]. Collectively, these observations suggest age-related alterations of adaptive immunity are also relevant for increased WNV susceptibility in the elderly.

4 New Directions for Aging-Related Investigation

Recent advances in technology hold the promise for increasing our understanding of essential changes in immune cells associated with aging, and fostering new discoveries for prevention and therapeutic approaches to improve health. In particular, we highlight mass cytometry to characterize in depth phenotypic and functional changes in multiple cell types simultaneously; and micro RNA (miRNA) profiling to identify miRNAs that regulate expression of pivotal genes relevant to aging-associated conditions [66, 67].

4.1 Mass Cytometry (CyTOF): Novel Multidimensional Single Cell Phenotyping

Mass cytometry, or cytometry by time-of-flight (CyTOF), is a novel technology for multiparametric single cell analysis based on detection of metal-conjugated antibodies [68]. CyTOF improves on fluorescence flow cytometry and has greater dimensionality (40 parameters vs 8–10 by flow cytometry) and resolution of compensation issues. Furthermore, CyTOF can efficiently detect as few as 10,000 cells, which supports investigation from limited samples available through translational and clinical studies [69]. High dimensional data generated from CyTOF requires specialized computational methods for dimensionality reduction, clustering, visualization, and single cell resolution [70–72]. CyTOF technology is leading to advances in biology and medicine, such as cancer, autoimmune diseases, and infectious diseases [73–76]. Emerging studies have employed CyTOF to characterize single cell immune responses to viral infections and vaccination [77–79], and promising results to advance our understanding of age-associated changes in immune responses [80, 81].

4.2 MicroRNA Regulation of Gene Expression

Recent studies have identified an important role for noncoding short microRNAs (miRNAs, ~22 nucleotides) in posttranscriptional regulation of gene expression by binding to specific mRNA targets and facilitating their degradation and/or translational inhibition. The human genome is believed to encode ~1000 miRNAs, each of which may regulate expression of hundreds of genes [67]. Emerging evidence has shown that expression of dozens of miRNAs is altered with aging in different tissues and organisms, which may be associated with age-dependent diseases and disorders [82, 83]. Interestingly, several key immune-regulated miRNAs such as miR-

21, -146a, and -155, also show alterations during aging, suggesting that miRNAs may contribute to the age-associated basal inflammation [82, 84–86]. Cellular miRNAs have also been implicated in restriction or promotion of infection of various viruses, including hepatitis C virus (miR-122) and retrovirus primate foamy virus type 1 (miR-32) [87, 88]. Several miRNAs including miR-196a, -202-3p, -449c, and -125a-3p have been shown to be differentially expressed following WNV infection, suggesting their potential role in WNV resistance and pathogenesis [89, 90]. miRNA profiling will lead us to a better understanding of miRNA regulation in aging and viral infections as well as new discoveries for miRNA-based therapeutic intervention.

5 Concluding Remarks

Aging remains a dominant risk factor for susceptibility to infection with WNV [4], and aging-associated changes in innate and adaptive immunity may contribute to increased illness among the elderly. As reviewed here, dysregulation of TLR pathways in macrophages [23], reduced production of IFN by dendritic cells [32], and reduced efficiency of PMN clearance of virus [9] may contribute to the increased susceptibility to WNV infection in elderly individuals. In addition, in-depth investigations are needed to identify whether age-related differences in NK cells and γδ T cells may also be relevant to control of WNV infection in humans. Emerging technologies including single cell CyTOF and miRNA profiling provide multidimensional, high-throughput, genome- and proteome-wide analysis of age-associated changes in cell function and may offer new insights into pathogenesis of age-related diseases or disorders for development of promising preventive and therapeutic approaches.

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Chapter 19

An Overview of Current Approaches Toward the Treatment and Prevention of West Nile Virus Infection

Dhiraj Acharya and Fengwei Bai

Abstract

The persistence of West Nile virus (WNV) infections throughout the USA since its inception in 1999 and its continuous spread throughout the globe calls for an urgent need of effective treatments and prevention measures. Although the licensing of several WNV vaccines for veterinary use provides a proof of concept, similar efforts on the development of an effective vaccine for humans remain still unsuccessful. Increased understanding of biology and pathogenesis of WNV together with recent technological advancements have raised hope that an effective WNV vaccine may be available in the near future. In addition, rapid progress in the structural and functional characterization of WNV and other flaviviral proteins have provided a solid base for the design and development of several classes of inhibitors as potential WNV therapeutics. Moreover, the therapeutic monoclonal antibodies demonstrate an excellent efficacy against WNV in animal models and represent a promising class of WNV therapeutics. However, there are some challenges as to the design and development of a safe and efficient WNV vaccine or therapeutic. In this chapter, we discuss the current approaches, progress, and challenges toward the development of WNV vaccines, therapeutic antibodies, and antiviral drugs.

Key words West Nile virus, Vaccine, Antiviral drug, Therapeutic antibody

1 Human WNV Diseases and the Need for Antiviral Drug or Vaccine

West Nile virus (WNV), a neurotropic RNA virus belonging to the *FLAVIVIRIDAE* family, is generally transmitted to human by infected mosquito bites, primarily by *Culex* species [1, 2]. However, WNV can also be transmitted through other less frequent routes, including a transfusion of blood and blood components [3, 4], organ transplantation [5], breastfeeding [6], and congenital infections [7]. After an infected mosquito bite, WNV replicates in keratinocytes and skin-residential dendritic cells (Langerhans cells), and the latter cells carry the viruses to draining lymph nodes to cause viremia [8, 9]. Subsequently, WNV disseminates to the peripheral organs, such as spleen and liver, and possibly to spinal cord and brain. Human WNV infection

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may cause injury and death of neurons with various clinical manifestations, such as encephalitis, meningitis, flaccid paralysis, persistent neurologic sequelae, and possibly death, particularly in the elderly and immunocompromised individuals [1, 10, 11]. WNV strongly activates host immune responses, which play important roles in controlling viremia, viral dissemination to the central nervous system (CNS), and recovery from the disease [11]. However, the mechanism of WNV pathogenesis, including its tropism to neurons, CNS invasion, and viral or host factors that contribute to imbalance between viral pathology and host immunity still remain poorly understood.

Although WNV was first discovered in Uganda in 1937, it had been considered as a minor public health concern until its first appearance in the USA in 1999 [12]. Since then, it has dramatically spread to all the continental states of the USA and became an endemic disease throughout North America within a few years [13–16]. In the USA alone, there have been over 40,000 reported cases of WNV between 1999 and 2014, of which ~45 % were classified as neuroinvasive and claimed lives of nearly two thousand people [17]. However, the actual WNV burden is likely much higher than previously thought because only about 20 % of infected individual develop a clinical WNV disease [13]. It has been estimated that over three million individuals have been infected with WNV in the USA, of which about 780,000 had a symptomatic disease [18]. WNV also has potential to develop unusual clinical manifestations [19–21] and may involve in renal diseases [22, 23], myasthenia gravis [24], and myocarditis [25], suggesting that the range and severity of WNV disease may be even worse than previously believed. Importantly, increasing numbers of WNV outbreaks during the last 15 years have been associated with greater number of neuroinvasive cases and a higher rate of fatalities [16, 17]. However, no vaccine or antiviral therapeutic is currently available, which limits current treatments to only supportive care measures, such as intravenous fluids, antipyretics, respiratory support, and prevention of secondary infections. Considering the worldwide distribution of this virus and evidence of its potential to change in pathogenicity and transmission [26–30], there is an urgent need to develop safe and effective antiviral drugs or vaccines against WNV infection [31]. Intensive research during past decades has made significant progress in the design and development of several treatment and prevention methods for WNV infection (reviewed by [32–34]). Here, we discuss the current approaches and recent progress toward the development of vaccines, therapeutic antibodies, and antiviral drugs against WNV infection in humans.

2 WNV Structure and Therapeutic Targets

WNV is a spherical virus with 50 nm in diameter, which comprises an icosahedral nucleocapsid surrounded by a lipid envelope [35]. The virus contains a single-stranded, capped, and plus-sensed RNA genome of approximately 11 kb in size. The viral genome encodes a polyprotein precursor, which undergoes posttranslational processing by cellular and viral proteases to generate three structural proteins (capsid [C], premembrane [PrM], and envelope [E]), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins form virion structure, whereas the non-structural proteins play essential roles in the replication of viral genome, assembly of virion, and viral pathogenesis [36–38]. Thus, the structural and non-structural proteins of WNV may be potential targets for developing vaccines and antiviral therapeutics (Table 1).

In a WNV virion structure, C protein encapsulates viral genomic RNA to form a nucleocapsid that is enveloped by a lipid bilayer into which trimmers of prM-E heterodimers form the spike-like projections. Among these structural proteins, E protein mediates crucial roles in binding to cellular receptors, membrane fusion, and entry of WNV into host cells, making it a key target for the development of vaccines, neutralizing antibodies, and entry inhibitors. Crystal structure analysis has confirmed that E protein folds into three structurally distinct ectodomains (EDs) termed EDI, EDII and EDIII [39–43]. Among these, the EDIII consists of the major neutralizing epitopes and is an antigen of choice to elicit production of neutralizing antibodies [43–46]. Based on the structural characterization of antiflaviviral monoclonal antibodies from both human and nonhuman primate, it appears that the epitopes of flaviviral E protein are more complex and diverse than previously thought [47–50]. In addition, mapping of B-cell and T-cell epitopes has led to the identification of many immunodominant epitopes in both structural and nonstructural proteins of WNV [51, 52].

Among the non-structural proteins, NS3 and NS5 are best-characterized, multifunctional proteins, both of which contain enzymatic activities that are essential for viral replication [53–55]. Such enzymatic functions of NS3 and NS5 have received considerable attention as potential targets for antiviral drug development [34, 54]. The NS3 protein contains two distinct functional domains. The N-terminal domain of NS3 (184 amino acid residues) has serine protease activity that requires a polypeptide cofactor NS2B for activation [54, 56, 57]. Recent X-ray crystallographic studies have shown that the conformation of β-loop of NS2B controls the substrate binding by NS2B/NS3 protease [58, 59]. In contrast, the C-terminal domain of NS3 functions as an RNA helicase,

Table 1
Therapeutic targets of WNV structural and non-structural proteins

Targets	Structural features	Functions	Targeting approaches
Envelope (E)	Contains a central domain I, a extended finger-like domain II, a immunoglobin-like domain III and a hydrophobic pocket between domain I and II; domain II contains a fusion loop; contains the major epitopes for B and T cells	Mediates virus binding to host cellular receptors and membrane fusion	Drug targeting to block viral entry, disrupt membrane fusion, and produce neutralizing monoclonal antibodies
Capsid (C)	Alpha-helical structural protein	Encapsidates viral RNA, induces host cell apoptosis [326], and to disrupt nucleosome formation	Internal deletion in capsid gene results in deficient replication and reduced pathogenicity [113]
Membrane (PrM)	Forms heterodimer with E protein	Virion assembly and fusion modulation [38]	Antigen for production of antibodies
NS1	Secreted glycoprotein, contains epitopes for antibody production	Immune evasion activities [37]	Potent antigen for antibody production; ablation of NS1 glycosylation attenuates WNV
NS2A	Transmembrane protein, associates with endoplasmic reticulum membrane; component of replication complex	Virion assembly/maturation; antagonizes host immune responses	Alanine to proline substitution at position 30 of NS2A attenuates viral virulence [36]
NS2B	Consists of a 40-amino acid hydrophobic region, transmembrane protein, component of replication complex	The 40-amino acid hydrophobic region serves as cofactor for NS3	NS2B cofactor activity can be targeted by inhibitors
NS3	Multifunctional protein containing two functional domains; contains a shallow ATP binding pocket and an additional domain that is not present in human helicases	Serine protease (N-terminal); Helicase (C-terminal)	Substrate-based inhibitors can target NS3 protease; small-molecule inhibitors may target helicase domain
NS4B	Transmembrane protein, component of replication complex	Inhibits NS3 ATPase activity	NS4B forms ATP-binding site that may be targeted by a drug
NS4B	Transmembrane protein, component of replication complex	Participates in viral replication and immune evasion	Selected mutations [365] in NS4B attenuate WNV
NS5	Multifunctional protein containing two functional domains	Methyltransferase and guanyltransferase (N-terminal); RNA-dependent RNA polymerase (C-terminal)	NS5 functions can be targeted by various inhibitors

nucleoside triphosphatase, and RNA triphosphatase [60, 61]. Although the ATPase and helicase activities of NS3 function independently, NS4A protein has been suggested to regulate both of these activities [62]. Besides its role in cleaving the viral protein, the protease activity of NS3/NS2B may also contribute to host cell apoptosis and neuropathogenesis by cleaving host proteins [63]. Similarly, NS5 is another multifunctional protein containing N-terminal methyltransferase/guanyltransferase, and C-terminal RNA-dependent RNA polymerase (RdRp) activity [64, 65]. The N-terminal methyltransferase and guanylyltransferase activities of NS5 are essential for the formation of a cap structure in viral mRNA [66]. Thus, the functions of NS5 are crucial for both protection of viral genome and efficient translation of viral polyprotein. The N-terminal domain of NS5 contains multiple residues that can be phosphorylated by host protein kinases [67]. Besides its function in viral replication, NS5 also plays a role in viral pathogenesis by antagonizing host's interferon response [28]. Other nonstructural proteins NS2A, NS2B, NS4A, and NS4B form the scaffold for the viral replication complex and also have roles in the replication of viral genome and host immune evasion [68–70]. Mutations in NS4B protein may attenuate WNV and other flaviviruses [68, 69]. In addition, a recent successful clinical trial of a hepatitis C virus NS5A inhibitor suggests that targeting non-structural proteins may be an ideal strategy to develop therapeutics against other flaviviruses, including WNV [71].

3 Current Approaches and Progress in WNV Vaccine Development

WNV infection induces potent activation of host immune responses that is critical for controlling viremia, viral dissemination into the CNS, and recovery from WNV diseases [1, 11]. Studies of WNV pathogenesis in animal models have demonstrated that humoral responses (antiviral antibodies) are essential in limiting viremia and neuroinvasive diseases [72, 73]. Thus, development of a vaccine that produces high titer of neutralizing antibodies would offer efficient protection against WNV infection [44]. Several epitopes for both B and T cells have been characterized in WNV proteins [52, 74–78]. In particular, the C-terminal EDIII of E protein that contains critical neutralizing epitopes is the major target for neutralizing antibodies against WNV infection [40, 44, 78, 79]. In addition to humoral immune response, cell mediated immunity by $CD4^+$ and $CD8^+$ T cells play critical roles in recovery from WNV infections [80–83]. Thus, the efficient generation of vaccine-induced immunity against WNV may also require activating and shaping of multiple effectors of adaptive immune response by early innate signaling pathways [84–86].

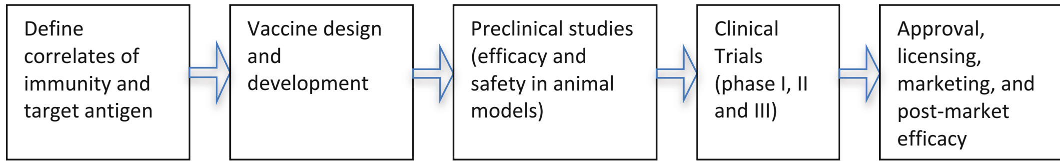

Fig. 1 Overview of vaccine development process

3.1 Vaccine Developments and Testing

Development of an effective vaccine requires multiple steps from design and development to rigorous evaluation of both safety and efficacy (Fig. 1). Since the biology of vaccine-induced immunity and principles in vaccine development and testing have been extensively discussed before, we will not discuss these topics here. Immunization of the laboratory animals and subsequent challenge of those animals with a pathogen under controlled conditions is the common method for early evaluation of vaccine effectiveness. Various factors such as route of administration, immunogen dose, and type of adjuvant greatly influence the effectiveness of a vaccine candidate. Mouse models of WNV infection partially mimic the clinical course of WNV disease in humans, which not only help with the understanding of WNV pathogenesis but also facilitate the development and testing of WNV vaccines. In animal studies of vaccine efficacy, infected animals are observed for mortality and monitored for survival, pathology, seroconversion, immune responses, and vaccine safety. Plaque reduction neutralization test (PNRT) is a gold standard method to assess whether a candidate WNV vaccine induces neutralizing antibodies in both animals and humans, for which standard guidelines and methods are available [87]. Neutralizing antibody titer is correlated with protection against disease for other licensed flavivirus vaccines and is considered a key marker to assess vaccine efficacy [88]. For example, the protective threshold for a Japanese encephalitis virus vaccine is correlated with the titers of neutralizing antibodies, with a serum $PRNT_{50} \geq 1:10$ considered protective [88]. Although WNV lineage 1 strains are commonly involved in human disease and were used in vaccine efficacy testing, the recent emergence of pathogenic lineage 2 strains in Europe has raised additional concerns in WNV vaccine efficacy studies, because WNV vaccine candidates based on lineage 1 strains may not protect against the lineage 2 strains. After promising results from animal studies, human WNV vaccines are further evaluated in terms of protection against natural challenge, as well as their safety and immunogenicity during series of clinical trials (phases I–III).

3.2 Vaccine Development Approaches

Both traditional and modern approaches have been used for the development of WNV vaccines, and the most common approaches are listed below.

(a) *DNA-based vaccine*: By using the power of modern genetic tools, viral protein(s) can be expressed in a suitable vector to develop DNA vaccines against WNV infection [89, 90]. However, this strategy is sometimes hampered by poor immunogenicity and potential safety concerns, such as integration of foreign DNA into the host genome.

(b) *Chimeric/recombinant vaccine*: This approach relies on the replacement of gene(s) of the established viral vaccine strain by equivalent WNV genes. Several live attenuated vaccines and viral vectors may be used as a backbone for developing recombinant WNV vaccine candidates, such as yellow fever virus (YFV) vaccine (YFV-17D) [91, 92], attenuated DENV serotype 4 (DENV4) [93, 94], HIV-based lentivirus vector vaccine [95], Schwarz strain of attenuated measles virus [96], vesicular stomatitis virus (VSV) vaccine vector [97, 98], and adenovirus A [98].

(c) *Live-attenuated vaccine*: Attenuation of WNV can be achieved by classic cell culture passage or animal passage and targeted genetic mutations. However, several potential challenges including residual pathogenicity, reversion to virulent strain, relative short self-life, and the demand of a safe biological production system need to be overcome.

(d) *Inactivated (killed) vaccine*: Chemical inactivation of live viruses may be used to develop inactivated or killed virus particles. The limitations are the possibility of incomplete inactivation, short-lived immunity, and the requirement of multiple doses for efficient immunization.

(e) *Subunit or recombinant protein vaccine*: This approach uses soluble recombinant protein(s) or protein expressed in virus-like particle (VLP) platform as a vaccine candidate. Success of this approach relies on optimum immunogenicity of a vaccine, as the protein vaccines generally require multiple boosts with strong adjuvants to provide acceptable efficacy.

3.3 Anti-WNV Vaccine Candidates Currently in Development and Clinical Trial

Although no vaccine is currently available against WNV infection in human, several WNV equine vaccines are available (Table 2). Several human vaccines are under development, and some are in clinical trials (Table 3). Their current status and approaches used for development are discussed below.

3.3.1 DNA-Based Vaccine

West Nile Innovator® DNA is the first licensed DNA vaccine for veterinary use following a successful demonstration of vaccine induced B and T cell-based immunity after immunization of mice and horses with DNA vaccines expressing *prM* and *E* genes of WNV [89] or the domain III (DIII) region of *E* gene [99]. This approach has been widely used do develop various DNA vaccines against WNV. One of the first DNA vaccines introduced in phase

Table 2
Licensed WNV vaccines in veterinary use

Vaccine name	Company	Vaccine approach	Design/features	Status	References
West Nile-Innovator® DNA	Fort Dodge Animal Health/Pfizer	DNA	Plasmid DNA encoding WNV prM-E	Licensed (discontinued)	Davis et al. [89]
Vetera™ West Nile vaccine	Boehringer Ingelheim	Killed	Whole virus	Commercialized	
West Nile-Innovator®	Pfizer	Formalin inactivated	Whole virus	Commercialized	Ng et al. [127]
RecombiTek®	Merial	Recombinant vaccine	WNV prM-E in canarypox virus	Commercialized	Karaca et al. (2005)
PreveNile®	Intervet	Recombinant vaccine	WNV prM-E in yellow fever vaccine (17D) backbone	Licensed in 2006 (recalled in 2010 after severe reaction)	

Table 3
Human WNV vaccine candidates in clinical trial

Vaccine name (company)	Vaccine approach	Design/features	Status of development	Reference(s)
Chimeri-Vax-WN (Acambis, Sanofi-Pasteur)	Recombinant	WNV prM-E and E replacing capsid and non-structural protein of yellow fever vaccine strain (17D)	Phase I clinical trial completed, Phase II trial ongoing	Biedenbender et al. [366]
WN-DEN4	Recombinant	WNV prM gene in a backbone of attenuated DEN-4	Phase I clinical trial completed, Phase II trial ongoing	Pletnev et al. [94]
WN-80E	Subunit protein	Recombinant E protein lacking transmembrane domain	Phase I clinical trial completed	Lieberman et al. [136, 147]
WNVDNA017-00-VP (VRC in collaboration with Vical)	Plasmid based DNA vaccine	Plasmid DNA vector that express WNV-NY99 prM-Env under a cytomegalovirus promoter	Phase I clinical trial completed	Martin et al. [90]; Ledgerwood et al. [100]
HydroVax-001 (OHSU, NIH funded)	Inactivated	Chemical inactivation by H_2O_2	Phase I clinical trial ongoing	http://www.nih.gov/news/health/jul2015/niaid-06.htm

I clinical trial was based on a circular plasmid DNA vector incorporating a cytomegalovirus (CMV) promoter to express the WNV-NY99 *prM* and *E* coding sequences in downstream of a modified JEV signal sequence (VRC-WNVDNA017-00-VP) [90]. Although no serious adverse effects were reported, its low immunogenicity hampered further development [90]. In an effort to improve immunogenicity of this vaccine, an additional regulatory element from human T-cell leukemia virus type 1 (HTLV-1) was incorporated in conjunction with the previously used CMV promoter [100] but without significant success when tested in the clinical trial [100]. Several studies have also tested carrier-conjugation and different inoculation routes [101, 102] to improve immunogenicity and efficient delivery of DNA vaccines. For example, a DNA vaccine expressing full length of truncated WNV *E* gene derivatives conjugated to the P28 region of C3d (a complement protein) induced strong IgG titers and efficient protection of mice when vaccinated by gene gun method [103]. In another study, a plasmid DNA vector expressing the ectodomain of WNV E protein into linear polyethyleneimine (lPEI) nanoparticles covalently bound to mannose was developed. However, this conjugation failed to generate sufficient E-protein specific humoral responses, despite the boosting of the vaccinated mice with recombinant E protein induced a significant increase in neutralizing antibodies [104].

Large deletions of capsid gene in the flaviviral RNAs result in a failure to produce infectious virions but retain the ability to replicate viral RNA genome and express prM and E proteins [105, 106]. This novel property has been used to develop several plasmids DNA (pDNA) vectors that after transfection produce single-round infectious particles (SRIPs), which in turn produce virus-like particles (VLP) containing viral surface proteins without viral genome. This strategy has been used to develop several candidate DNA vaccines against flavivirus by expressing E and prM proteins in a plasmid vector and forming VLPs. Using this approach, several plasmid-DNA constructs were developed as candidate DNA vaccines against WNV. This type of DNA vaccines encode for single-round infectious particles expressing E/prM [107–109], a full-length cDNA copy of attenuated WNV Kunjin strain [110], or ectodomain of E protein [111].

3.3.2 Live Attenuated Vaccine

Cell culture or animal passage used to be conventional methods to develop live-attenuated vaccines. The advents of genetic manipulation techniques make it feasible to introduce targeted mutations into the viral genome and attenuate viruses. Using this approach, a WNV vaccine (RepliVAX WN) developed by an internal deletion of a region in capsid gene [112, 113] has been shown to induce neutralizing antibodies and protective immune responses in mice

[114], hamsters [108], and nonhuman primates [109]. RepliVAX WN strongly activates B cell population secreting anti-NS1 IgG antibody and induces prolonged activation of memory CD8⁺, CD4⁺, and NS1 specific plasma cells [115]. Innate immune signaling pathways, such as TLR3 and MyD88-dependent signaling pathway are involved in strong activation of B cell response, development of germinal center, generation of long-lived plasma cells, and production of antibodies following immunization with RelpiVax WN vaccine [116]. In addition, another live attenuated WNV vaccine developed by generating mutations in glycosylation sites of E and NS1 proteins induces neutralizing antibodies and protective immune responses in mice [117]. Similarly, approaches of introduction of mutations in *NS4B* [69], *NS2A* [36], or *E* gene that were previously characterized to attenuate JEV-SA-14142 [118] have also been used to develop attenuated WNV vaccine candidates.

3.3.3 Chimeric/Recombinant Vaccine

Using this approach, a recombinant live attenuated WNV vaccine for veterinary use was developed and licensed in 2004 by Merial (RecombiTEK). This vaccine expresses WNV prM and E proteins in a canarypox virus backbone [119, 120]. Using a similar approach, a chimeric vaccine (ChimeriVax-WN02) has been developed by replacing *prM* and *E* genes in YFV vaccine strain (YFV-17D) with WNV-NY99 *prM* and *E* genes [91, 121]. ChimeriVax-WN02 was the first recombinant WNV vaccine candidate tested in clinical trial. Introduction of three mutations responsible for attenuation of JEV (SA14-14-2) in equivalent positions of WNV *E* gene further attenuated ChimeriVax-WN02. Similarly, DENV4 vaccine candidate (rDEN4Δ30), attenuated through a 30-nucleotide deletion in the 3′ untranslated region (UTR) of the viral genome, was further engineered to express WNV-NY99 *prM* and *E* [122, 123]. After preclinical evaluation in mice, geese, and monkeys, rDEN4Δ30 showed strong immunogenicity in the clinical trial [94]. In addition, a chimeric DENV2 vaccine candidate expressing the WNV NY99 prM and E proteins has been shown to protect mice from infection with WNV NY99 strain [124]. Another recombinant WNV vaccine based on influenza vaccine (FLU-NA-DIII) was developed by cloning DIII of WNV E into the N-terminal region of neuraminidase of influenza virus. This vaccine candidate induced WNV-specific neutralizing IgG and protected mice against lethal WNV infection [125]. Similarly, a recombinant adenoviral vaccine vector (CAdVax-WNVII) expressing all three structural proteins (C, prM, and E) along with NS1 of WNV induced neutralizing antibodies in mice [98]. Several other recombinant WNV vaccines have also been developed by expressing WNV protein in the backbones of attenuated measles virus [96], vesicular stomatitis virus [97], and herpes virus-1 [126].

3.3.4 Inactivated (Killed) Vaccine

The most common approach to develop non-replicating inactivated viral vaccines is to inactivate entire virus particles by using chemicals. A formalin-inactivated WNV vaccine based on WNV-NY99 strain was the first successful veterinary vaccine (marketed by Pfizer as West Nile Innovator®) licensed in 2003 [127]. Another veterinary WNV vaccine using killed virus was also licensed by USDA (marketed by Boehringer Ingelheim as Vetera™ WNV). Recently, an inactivated WNV vaccine (WN-VAX) based on WNV NY99 protects mice against lethal WNV infection and exhibits immunogenicity in monkeys [128]. In addition to WNV NY99, formalin inactivation of WNV IRS98 strain induces neutralizing antibody and protects immunized geese [129]. As an alternative to traditional formalin-based vaccines, a novel hydrogen peroxide (H_2O_2) inactivation approach has been recently used to produce a whole-virus vaccine against WNV [130, 131]. Mice immunized with H_2O_2-inactivated WNV vaccine candidate developed high serum neutralizing titers, and offered complete protection of vaccinated mice against lethal WNV challenge [130]. One of such H_2O_2 inactivated vaccine (HydroVax-001) has been recently introduced into phase I clinical trial. Although inactivation of virulent WNV virus strain has been successfully achieved by chemical-inactivation method [127, 129], use of a naturally attenuated Kunjin strain of WNV [132, 133], or chemically synthesized virus by cDNA system [134] as starting material has also been proposed.

3.3.5 Subunit, VLP, or Recombinant Protein Vaccine

Several studies demonstrated that soluble recombinant protein or VLP based approach could serve to develop WNV vaccines [135–139]. VLP are specialized subviral particles that lack of viral genome and solely contain viral structural proteins [140, 141] or express viral proteins on envelope membranes [142, 143]. Different vectors and production system were evaluated for development of various subunit vaccines against WNV. For example, a recombinant truncated form of WNV E protein produced in *Escherichia coli* induced neutralizing antibodies and protected mice from lethal WNV challenge [46, 144]. In addition, a recombinant truncated WNV E protein produced in the SF+ insect cells via baculovirus infection induced neutralizing antibodies and protected mice and hamsters from WNV infection [145]. Recombinant baculovirus was also used to express WNV prM and E proteins in mammalian cells under the CMV promoter, with or without vesicular stomatitis virus glycoprotein (Bac-G-prM/E). Such vaccines induced robust immune responses when inoculated in mice and produced both neutralizing antibodies and inflammatory cytokines [146]. In a recently proposed novel approach, known as pseudotyping, a retrovirus Gag polyprotein forms a VLP scaffold to display the ectodomain of human membrane glycoprotein (CD16) that was fused to the high affinity IgE receptor gamma chain (RIgE).

Using this retrovirus based VLPs platform, a WNV vaccine was generated by replacement of the CD16 ectodomain in CD16-RIgE glycoprotein with EDIII of WNV, which induced neutralizing antibodies in mice [139].

A recombinant E protein of WNV-NY99 produced in *Drosophila* S2 cells (WNV-80E, developed by Hawaii Biotech) is the only WNV subunit vaccine candidate that has been tested in phase I clinical trial. Although preclinical studies revealed WNV-specific neutralizing antibody responses in vaccinated animals [136, 147, 148], the immunogenicity of this vaccine in humans was low. To increase immunogenicity, conjugation of recombinant proteins with nanoparticles or pathogen associated molecular patterns (PAMPs) as carrier/adjuvant have been tested. For instance, a recombinant WNV E protein administered with unmethylated CpG oligonucleotide adjuvant or loaded onto CpG-modified nanoparticles strongly activated dendritic cells and lymphocytes and elicited Th1-dominant immune responses by producing high titers of IgG2a and IgG2b in immunized mice [149, 150]. Similarly, mice injected with DIII of WNV E conjugated with bacterial flagellin (STF2Δ.EIII) [137] or VLP derived from bacteriophage AP205 engineered to express DIII of WNV E (DIII-C-AP205) [138] also significantly increased neutralizing antibody production and protected the immunized mice.

3.4 Potential Novel Approaches for WNV Vaccine Development

Despite the intensive efforts in development of WNV vaccines, only a few reached the clinical trial stages. Of those in clinical trials, most candidate vaccines fail to demonstrate efficient immunity and safety. Development of new tools for antigen screening, expanded understanding of immunological correlates of vaccine induced-immunity, and discovery of novel adjuvants for vaccine delivery may facilitate the design and the development of WNV vaccines. For example, knowledge of genomic information and bioinformatics has been used for in silico identification of candidate antigens and development of vaccines by a novel method called “reverse vaccinology” [151, 152]. This comprehensive tool can quickly identify all potential antigens coded in the genome and may be used to develop a novel viral vaccine [153]. Development of vaccine against group B streptococci proved the potential of this approach [151]. Similarly, a “structural approach” that improves antigenicity of vaccines by rational designing has been developed by utilizing the knowledge of immunology, structural biology, and bioinformatics [154]. In addition, increased understanding of immunogenetics and role of environmental and host factors that determine the variation of vaccine immunity may offer new approaches to design a more effective vaccine against WNV infection in humans.

4 Antibody-Based Therapy: A Promising WNV Therapeutic

Therapeutic monoclonal antibodies (mAbs) or hyperimmune sera have been successfully used for prophylaxis of a number of infectious and noninfectious diseases, including WNV infection. In recent years, the number of mAbs in preclinical development and clinical trials has been increased significantly [155]. So far, nearly 50 mAbs have been approved for therapeutics by US FDA, including a humanized mAb Synagis (palivizumab) for preventive use against respiratory syncytial virus (RSV) infection in neonates and immunocompromised individuals [156–158]. Many mAbs have been developed against viruses, such as SARS-CoV, influenza, HIV-1, and other (re)emerging viruses including WNV [155, 159–163]. Some of them showed excellent therapeutic potential for clinical use in humans.

WNV infection induces a potent humoral immune response, which is essential in controlling viremia and limiting WNV dissemination to the CNS [72, 73]. Hyperimmune sera, γ-globulin, or affinity-purified antibodies harvested from WNV-infected humans and animals protect both wild-type and immunocompromised micefrom WNV challenge in laboratory conditions [164–166]. In addition, WNV patients who received antibodies from the WNV seropositive donors recovered from WNV infection [167–171]. These studies not only encouraged the efforts toward the development of human or humanized monoclonal antibodies against WNV, but also led to the discovery of several potent monoclonal antibodies that showed efficient protection of mice and hamsters from WNV infection [172–175]. Among these, a humanized anti-WNV mAb (Hu-E16) that binds to a highly conserved epitope in WNV E protein blocks viral fusion and provide post-exposure therapeutic potential [172]. This antibody is currently being assessed for its potential use as a WNV therapeutic antibody [176]. The phase I clinical trial showed that another humanized recombinant antibody targeting E protein of WNV (known as MGAWN1) has a good safety and tolerance profile in healthy humans [177], however, the phase II trial to assess its efficacy in WNV infection failed due to poor enrollment of participants. Besides the development of whole antibody, recombinant fusion proteins are also generated from single-chain antibody fragment of the variable region. Such antibody fragments that target E protein may be potential candidates for immunoprophylaxis and therapy of WNV infections. A recombinant human single-chain variable region antibody fragments (Fv-Fc) fusion protein has a protective role against WNV infection in mice [178].

One of the potential limitations of this approach is antibody-dependent enhancement (ADE), a phenomenon by which infection of some viruses is enhanced by virus-reactive antibodies resulting in more efficient virus entry through Fc receptor-mediated

pathways. This phenomenon plays a role in the pathology of severe dengue infection and has also been observed in WNV in vitro [179]. Although the role of ADE in WNV disease in unclear, the development of a therapeutic antibody against WNV should address this potential issue. Another limitation of antibody-based therapeutics is high production cost, which limits mAb scalability. Producing therapeutic proteins, including antibodies in plants, may be a promising solution. Feasibility of this approach has been affirmed by the successful production of anti-WNV monoclonal antibody Hu-E16 in plants (*Nicotiana benthamiana*) (MAb-pE16) [180, 181]. The plant-derived MAb-pE16 confers a potent neutralizing activity in vitro without ADE, efficiently binds to complement and Fc receptors, and protects mice against lethal WNV-challenge with similar potency as their mammalian-cell counterparts [180, 182, 183].

Antibodies employ several mechanisms to control WNV and other viral infections, including blockage of viral entry, Fc-dependent viral clearance, complement-mediated viral lysis, and antibody-dependent cytotoxicity of infected cells. Most of the current researches in the development of therapeutic antibodies against WNV are designed and tested for efficient neutralization potential [184, 185]. Increased understandings of the biology of antibody Fc regions, in particular, the roles of glycan in Fc mediated functions may facilitate the design and development of high-quality antibody through glycoengineering [186, 187]. Such engineering of antibody Fc region may be used to overcome ADE, modulate pharmacokinetics, and enhance Fc mediated effector functions, such as enhancement of antibody-dependent cell mediated cytotoxicity (ADCC), complement binding, and phagocytosis [188].

Recent technological advancement not only in development, production, and purification but also in ease of achieving desirable quality, efficacy, and safety required for the FDA approval makes monoclonal antibodies a promising therapeutic option. Thus, monoclonal antibodies may prove useful for WNV prophylaxis and therapy particularly in the elderly and immunocompromised individuals with limited ability to respond to a vaccine. To meet its therapeutic goal, a controlled clinical trial of therapeutic antibody should ensure its prophylactic and therapeutic efficacy along with optimal dose and timing of administration across the range of patient groups.

5 Moving Towards Anti-WNV Drug Discovery: Recent Approaches and Future Directions

Development of effective therapeutics have been successful in treating many viral diseases including influenza, HIV-1, hepatitis C virus (HCV), and hepatitis B virus (HBV). WNV causes transient viremia in human and animal models that is associated with

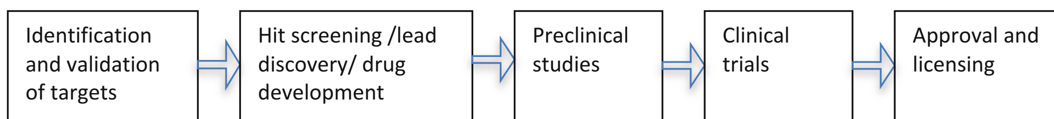

Fig. 2 Steps in antiviral drug development

its dissemination to brain and development of more severe disease [73, 189–192], suggesting that reducing viral loads by an antiviral drug during the early phase of infection may offer efficient control of WNV or lessen the chances of progression to neuroinvasive diseases. In addition, antiviral drugs are particularly useful for the elderly and the immunocompromised patients who may fail to develop efficient vaccine-induced immunity. Recent progress in the structural characterization of WNV and other flaviviruses broadens the understanding of WNV biology and provides a foundation for the development of small molecule inhibitors for WNV therapeutics [34, 193]. In addition, better understanding of the pathogenesis of WNV and other flaviviruses has offered new opportunities for designing many different classes of promising antiviral therapeutics by targeting both viral replication and the host cell metabolism.

5.1 Approaches for Drug Discovery

The development of an antiviral drug is a multistep process that takes years before it reaches the market. A general overview of a drug development process is outlined in Fig. 2. Hit-to-lead is an initial stage in a drug discovery, where small molecule hits are screened and further evaluated to identify promising lead compounds with a therapeutic potential. Recent progress in the development of multiple approaches for designing, screening, identification, and validation of hit compound (reviewed by [194–199]) have witnessed growing interests in the field of drug development. Significant progress in structural and functional characterization of both structural and nonstructural proteins of WNV and other flaviviruses has facilitated identification of therapeutic targets and hit-to-lead screening. For example, characterization of pseudo-atomic structure of mature and immature WNV [35], atomic resolution structure of WNV and other flaviviral protein by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy [39, 41, 48, 200–203], and structural characterization of binding of a neutralizing monoclonal antibody to E protein of WNV [48] have greatly increased our understanding of both structural and functional aspects of potential therapeutic targets. Two approaches have been commonly used for small molecule inhibitor screening include target-based approach and cell-based approach. For target-based screening, several methods can be applied, including enzyme activity-based screening, fragment-based screening, affinity-based screening, structure-based rational

designing, and in silico docking [197, 198, 204–207]. In contrast, cell-based approaches use viral infection and replication-dependent assays to identify inhibitors [208]. Each of these approaches presents their unique sets of merits and challenges. For instance, it is generally difficult to identify a target and also achieve specificity by cell-based assay because such identified inhibitors may potentially affect multiple steps of viral infection cycles and may target both viral and host proteins. Although inhibitors identified by cell-based assay may prove useful as antiviral drug candidates, these compounds could also act nonspecifically; thus further elucidation of their mechanism of action is required. The target-based approaches are highly efficient in screening process, however, an inhibitor screened by such method may require further modification for effective cellular permeability and validation for its antiviral activity, selectivity and toxicity by using cell-based assays. In the final stages, lead compounds are selected for in vitro and in vivo pharmacokinetic profiling, including efficacy, plasma stability (half-life), exposure, bioavailability, and preclinical toxicity before planning a clinical trial.

5.2 Current Status of Anti-WNV Drug Development

The most common strategies for antiviral drug development include blocking virus attachment or entry into host cells and inhibiting viral replication, either by targeting on viral components or host cells metabolism. There has been a significant progress in development and testing of potential antivirals against flaviviruses including WNV (reviewed by [34, 209, 210]). Several natural and synthetic compounds, antiviral peptides and siRNAs have been identified to target both structural and nonstructural proteins of WNV and evaluated for their potential therapeutic roles. Other approaches include targeting host cell metabolism and physiology and modulating host immune system by using antiviral cytokines as potential therapeutics against WNV. Current status and strategy used to develop antiviral drugs targeting WNV and other flaviviruses are described below.

5.2.1 Natural and Synthetic Compounds as Small Molecule Inhibitors of WNV

Several natural and synthetic compounds have been identified to target both structural and non-structural proteins of flaviviruses. Most of these compounds are designed to target DENV and some of them also show antiviral activities against WNV and other flaviviruses. As these compounds are diverse in their chemistry, they are discussed below based on their modes of action.

Viral Entry/Fusion Inhibitors

The E protein of WNV and other flaviviruses play key roles in viral entry into host cells by mediating viral attachment to host cell receptors and subsequent membrane fusion [35, 41, 211, 212]. After binding to host cell receptors, WNV enters into cells through a clathrin-independent endocytosis process followed by a low-pH-dependent viral uncoating in the endosome to release viral genome

into the cytoplasm for replication [213]. Inhibitors that disrupt the interaction of E protein with cell receptors or inhibit membrane fusion would be a potential antiviral against WNV. Two successful HIV drug Maraviroc (CCR5 antagonist) and Enfuvirtide (a peptide inhibitor) that respectively block viral entry [214] and membrane fusion [215] attest to this antiviral strategy. The flaviviral E glycoprotein contains several functional sites such as a hydrophobic pocket, the receptor-binding domain and stem domain that may be targeted by inhibitors. Among these, the hydrophobic ligand-binding pocket in a hinge region between domain I and II of E protein plays an important role in low-pH-mediated membrane fusion process and is a unique target for developing small-molecule inhibitors against flaviviruses [216, 217]. Various screening approaches were used to identify inhibitors against DENV and other flaviviruses that bind into this hydrophobic pocket and interfere with the conformational changes of E protein [207, 218–221], Most of these inhibitors were designed and tested against DENV, some of which were reported to exhibit antiviral activities against WNV [207, 220]. However, these compounds failed in the further drug development due to their undesirable properties, such as low solubility and cytotoxicity.

The domain III of E glycoprotein that mediates receptor-binding can be potentially targeted by developing inhibitors that can disrupt the viral attachment to host cell receptors. Neutralizing antibodies against E protein have proven the potential of this strategy. Several compounds have also been shown to interfere with the binding of flaviviruses to host cell receptors [222–226]. However, lack of understanding of cell receptor for WNV has hampered the success of this approach. Identification of cellular receptors for WNV and understanding of virus–receptor interaction may provide new opportunities to identify small molecule inhibitors that interrupt the binding of WNV to host cell receptors.

NS3 Protease Inhibitors

Viral proteases are essential for WNV life cycle for they cleave the viral polyprotein precursors into functional proteins. Successful development and licensing of protease inhibitor against HIV-1 [227, 228] and HCV [229, 230] provides the proof of concept and feasibility for similar targeting of proteases of other viruses. The N-terminal domain of flaviviral NS3 (amino acids 1–169) has serine protease activity whereas a hydrophobic region of NS2B protein serves as cofactor to activate the enzymatic activity of NS3 [53, 231, 232]. The NS3 protease of WNV processes the viral polyprotein precursor into structural and non-structural proteins and disruption of this activity is lethal for WNV [54, 57]. In addition, WNV NS2B/NS3 protease can also cleave host proteins and may contribute to neuropathogenesis [63]. Recent progress in the expression of stable NS2B/NS3 and identification of the high-affinity substrate for this viral enzyme has promoted large-scale

screening of protease inhibitors for WNV and other flaviviruses. A wide range of assays, such as conventional enzyme-substrate based detection, HPLC, ELISA, and high-throughput fluorescence-based detection methods have been developed for screening of viral protease inhibitors [233–236]. Thus, NS3 protease is an attractive target for the development of antiviral against WNV and other flaviviruses (reviewed by [237]).

Except for aprotinin, a pancreatic trypsin inhibitor, most of the classical inhibitors of serine protease do not inhibit flaviviral NS2B/NS3 protease activity [238, 239]. Although aprotinin is a potent inhibitor of flaviviral protease, this compound was withdrawn from the market in 2008 due to safety issues [240]. To screen and identify small molecule inhibitors of flavivirus protease, both high throughput screening and structure-based drug designing have been used. These strategies are based on the identification of allosteric inhibitors that target the interface of NS2B-NS3 protease, or the active site of NS3 protease. The former strategy may overcome nonspecificity of the latter due to the largely conserved active sites of the human and viral serine proteinases. A number of inhibitors for WNV NS2B/NS3 protease have been identified by in silico docking or high-throughput screen using in vitro enzyme activity-based assays [34, 59, 206, 241–249]. However, most of these compounds failed to demonstrate potent antiviral activity in cell culture. Although a few compounds identified by these approaches show anti-WNV activity in cell-based assays [62, 206, 242], none of the inhibitors has progressed beyond the hit optimization stage. Discovery of NS2B/NS3 protease inhibitors has been hampered largely due to the difficulties in obtaining co-crystal structures of inhibitor-protease complexes. Moreover, because of the weak binding affinity of NS2B/NS3 active site due to its flat and charged nature, the design of potent small molecule inhibitors by structure-based method becomes difficult [53, 242, 250, 251].

NS3 Helicase/Nuclease Inhibitors

The helicases are enzymes that unwind nucleic acid by using energy derived from hydrolysis of NTP. The C-terminal domain of NS3 of WNV contains helicase/nuclease activities and plays important roles in virulence and pathogenesis [252–254]. High throughput assays that measure helicase activity by monitoring helicase-catalyzed strand separation in real-time by using radioactive or fluorescent-labeled oligonucleotides have been developed to screen helicase inhibitors [255–258].

Several small molecule inhibitors targeting helicase of HCV and HIV-1 have been developed [259]. By using the substrate-based assay, a few compounds have been identified and evaluated in vitro against NTPases/helicases of WNV and other flaviviruses [260–262]. However, inhibitory effects of WNV helicase by these compounds are specific to either DNA or RNA substrate. For example, a compound named 4,5,6,7-tetrabromobenzotriazole

(TBBT), a halogenated benzotriazole, inhibits NS3 helicase, but not NTPase activity [260]. A series of ring-expanded nucleoside/nucleotide analogs (RENs) also inhibit NTPases/helicases activities of flaviviruses, including WNV, HCV, and JEV [263, 264], however, these compounds did not show any promising anti-WNV activity in cell culture. A nucleoside analog imidazo[4,5-d]pyridazine nucleosides [265], and a broad-spectrum antiparasitic drug named ivermectin [266] inhibit NS3 helicase and also show anti-WNV activity in cell culture.

RNA-Dependent RNA Polymerase Inhibitors

The RNA-dependent RNA polymerase (RdRp) activity of C-terminal NS5 protein of WNV and other flaviviruses is an attractive target for developing antiviral agents [55, 267–269]. Two approaches used to target WNV RdRp include nucleoside inhibitors (NIs) or non-nucleosides inhibitors (NNIs). NIs (also known as type 1 inhibitors) are nucleoside/nucleotide analogs that target the active sites of the polymerase and generally compete with natural NTP substrates of RdRp to block their incorporation into viral genome during replication and lead to incomplete replication or mutations of viral genome. The success of NIs against several viruses including HIV-1, herpesviruses, HBV, and HCV has already proved the therapeutic potential of this class of compounds [270–272]. In addition, NI generally displays broad-spectrum antiviral activities across related RNA viruses suggesting its potential as pan-flaviviral therapeutics. Various cell-based and cell-free assays have been developed for high-throughput screening of flaviviral RdRp inhibitors [273–276].

So far, several NIs that inhibit WNV, DENV, and other RNA viruses have been identified [34]. For example, favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) and related compounds selectively inhibit viral RNA-dependent RNA polymerase and have potent anti-influenza activity [277, 278]. This antiviral drug is currently being evaluated in clinical trials against influenza virus. In addition, favipiravir also blocks replication of many other RNA viruses, including WNV and are promising drug candidate against a broad range of RNA viral diseases [279]. Two other nucleoside analogs called 7-deaza-2′-C-methyl-adenosine and 5-aza-7-deazaguanosine (ZX-2401), which are the derivatives of triphosphates of 2′-C-methyl-adenosine and 2′-C-methyl-guanosine, respectively, are also broad-spectrum antiviral compounds targeting viral RdRp that inhibit DENV, HCV and WNV [280–282]. Similarly, two other NI inhibitors, NITD-008 (beta-D-2′-ethynyl-7-deaza-adenosine triphosphate) and NITD203 (3′,5′-O-diisobutyryl-2′-C-acetylene-7-deaza-7-carbamoyladenosine) inhibit all four of DENV serotypes and WNV.

In contrast to NI inhibitors, antiviral NNI inhibitors (also known as type 2 inhibitors) interfere with the function of viral polymerase by occupying its allosteric sites, thus preventing viral

RNA synthesis. Analysis of RdRp crystal structure of WNV and DENV3 revealed a cavity that plays a critical role in viral replication, suggesting a potential target for screening of structure-based allosteric inhibitors [55, 283]. N-sulfonylanthranilic acids derivatives identified by high-throughput screening are examples of allosteric inhibitors of RdRp activity of DENV [284]. However, these compound were specific to DENV and did not show any activity against WNV RdRp. A recent study demonstrated that a conformational change occurred in DENV-3 polymerase after binding with an inhibitor [285]. However, a similar antiviral activity of NNI inhibitors targeting polymerase of WNV has not been reported yet.

Methyltransferase Inhibitor

Messenger RNA (mRNA) of WNV possesses a 5′ cap that plays important roles in stability of mRNA and its translation. The methyltransferase (MTase) activity of the N-terminal domain of NS5 is responsible for N-7 and 2′ O-methylation of the viral RNA cap [64, 286] [287]. In addition, MTase activity is also responsible for evading host's antiviral interferon response and plays an important role in WNV pathogenesis [288]. Several structural and functional studies along with identification of several potential inhibitors suggest that targeting MTase represents a novel approach for the development of novel therapeutics against WNV and other flaviviruses [289–294]. Flaviviruses MTase catalyzes sequential methylations of the viral RNA cap using S-adenosyl-L-methionine (SAM) as the methyl donor and contains a single binding site for SAM in its crystal structure [289, 294]. In addition to MTase activity, binding of GTP has been shown in MTase domain of several members of flavivirus [295]. Several assays have been developed for high-throughput screening of methyltransferase inhibitors by structural-based and ligand-based methods [296, 297]. Rational design of SAM analogs has identified several inhibitors targeting MTase activity of DENV and WNV [34, 298].

Nonspecific inhibition of host MTase is one of the potential drawbacks of SAM analogs. A specific inhibition of flaviviral, but not host, MTase can be achieved by targeting a pocket near the SAM-binding site [290, 298]. Two nucleoside analogs were identified that potently inhibited the MTase of WNV without inhibiting human MTase. One of these compounds (GRL-003) showed antiviral activity against WNV in cell culture [299]. In addition, several screening studies against YFV and DENV NS5 have identified hits targeting MTase activity, some of which showed antiviral activity against WNV in cell culture [204, 300, 301]. However, an extensive multistage molecular docking approach to screen a library of about 5 millions of commercial compounds against two active sites of DENV MTase/GTase failed to identify any specific hits [302]. Recently, 5′-silylated nucleoside scaffold derived from 3′-azidothymidine (AZT) demonstrated antiviral activity against WNV and DENV, which binds MTase [303].

5.2.2 Antiviral Peptides

Several potential tools, including rational design and phase display library, have been developed for high-throughput screening of specific antiviral peptides [304, 305]. Enfuvirtide, a 36-amino-acid peptide based on the stem region of the HIV gp41, exemplifies an efficient antiviral peptide currently in clinical use [306]. Thus, antiviral peptides may serve as a novel therapeutic measure against WNV. Several antiviral peptides targeting both structural and non-structural protein of WNV and other flavivirus have been identified.

Targeting WNV E protein by antiviral peptides is a potential strategy that blocks virus attachment and entry into the host cells. Several short antiviral peptides (13–16 amino acid residuals) that bind to WNV E protein have been identified by screening of a murine brain cDNA phage display library [307]. One of those peptides (P9) reduces viremia and fatality after WNV infection in mice. P9 can efficiently penetrate the murine blood–brain barrier, implying that it may have antiviral activity in the CNS [307]. Similarly, a peptide inhibitor (WN83) targeting domain II of WNV E protein designed by using a physicochemical algorithm approach potently inhibits WNV infectivity [308]. Another peptide designed to target domain II of DENV E shows antiviral activity against both DENV and WNV [308]. In addition, a rational drug design approach has been used to identify a peptidomimetic that mimic NS2B/NS3 protease substrate and inhibits its activity. The mechanism proposed for the peptidomimetic is that NS2B/NS3 cleaves between P_1 and P_1' in a peptide substrate consisting of $P_2P_1P_1'$, where P_1 and P_2 are basic amino acids (Arg or Lys) and P1′ is a side-chain amino acid (Gly, Ser, or Ala) [56, 231]. Thus, a preferred peptide substrate contains several positively charged amino acids. A common method for screening peptide inhibitors of NS2B/NS3 protease employs a fluorophore conjugated peptide substrate containing basic amino acids at the P1 and P2 positions. Cleavage of peptide substrate by NS2B/NS3 protease results in a release of fluorophore and increase of fluorescence [242, 243]. Several peptide inhibitors of NS2B/NS3 protease have been identified against WNV [62, 309–311]. A novel agmatine dipeptide inhibitor with improved inhibitory activity against WNV NS2B/NS3 has been recently identified [309]. In addition, a recombinant peptide called retrocyclin-1 (RC-1) has been shown to inhibit NS2B/NS3 protease [312]. However, most of these peptides showed poor activity in the cell-based assay and has not been tested for their in vivo efficacy. Thus, all of the peptide inhibitor of NS2B/NS3 protease that has been identified so far failed at the early development stages. Potential limitations of this approach include poor pharmacokinetic properties due to charged nature of peptide, lack of specificity, requirement of intravenous delivery, rapid degradation in plasma, and costly production.

5.2.3 Small Interfering RNAs (siRNA)

RNA interference (RNAi) is a cellular process first described in the nematode *Caenorhabditis elegans* [313, 314]. This process specifically degrades RNA in a sequence-specific manner and is conserved in mammalian cells [315, 316]. RNAi is a natural defense of eukaryotic cells against viral infections, and may be a promising strategy for developing a potential antiviral therapeutic. Numerous siRNA targets were identified in the genomic region of WNV encoding both structural and non-structural proteins, and siRNA targeting these proteins effectively inhibits WNV replication [191, 317–321]. Besides the siRNA targeting coding regions, siRNA that targets noncoding regions have also been identified to inhibit WNV replication in a sequence-specific manner [191]. Although anti-WNV siRNAs efficiently block viral replication in cell cultures, similar successes are difficult to achieve in animal models [191]. Quick degradation by serum nucleases, failure to reach target cells, and rapid renal excretion due to their small size and anionic character are hindering the clinical application of antiviral siRNAs. Several delivery systems, including cell-penetrating peptide [322, 323], nanoparticles [324, 325], and viral vectors [326], may improve siRNA stability and enhance delivery efficiency. Despite many challenges, use of antiviral siRNA as anti-WNV therapeutics remains promising.

5.2.4 Targeting Cytokines/Chemokine Signaling as Therapeutics

Cytokines signaling controls diverse immune functions during infection, autoimmune disease, and cancer. Various immunomodulatory or immunostimulatory cytokines and chemokines have been identified to play a protective or pathological role in WNV infection. For examples, type-I interferons (IFNs) [327, 328], interleukin (IL)-23 [192], interferon-γ (IFN-γ) [83], IL-1β [329], macrophage migration inhibitory factor (MIF) [330], CXCL10 [331], and CCL5 [332, 333] protect against WNV infection, whereas IL-10 [190] and IL-22 [334] favor WNV pathogenicity. Pharmacological blockade of IL-10 by neutralizing antibody has been shown to protect mice against WNV challenge [190]. Type I IFNs (IFNα/β) inhibit many flaviviruses including WNV and have been used as therapeutics against hepatitis C virus [335]. Although the therapeutic effect of type I IFNs in WNV has yet to be evaluated, its application may be limited due to the antagonistic role of WNV NS5 protein in IFN signaling [336, 337]. Interestingly, treatment with pegylated IFN-λ, also known as a type III interferon, has been recently shown to protect mice against lethal WNV infection by decreasing blood–brain barrier permeability [338]. Thus, strategies targeting the expression of cytokine and chemokine, blocking their signaling, or direct use of recombinant cytokines may be novel approaches for treating WNV infection or controlling its pathology.

5.2.5 Inhibitors Targeting the Host

Viruses utilize host cellular system for entry, genome replication, transcription, synthesis of viral proteins, and production of viral progenies. In addition, interactions of viral proteins with cellular proteins may evade host immune defense and favor viral replication and pathogenesis. Several host pathways and enzymes including clathrin-mediated endocytosis cyclophilins [339], ubiquitin-proteasome system [340], unfolded protein response [341], nucleotide biosynthesis [342, 343], post-translational protein modification [344–346], and lipid metabolism [347–349] have been suggested in flavivirus replication and pathogenesis. Targeting host factors may be used as a strategy for developing antiviral therapeutic against flaviviruses, including WNV infection [350–352].

So far, many inhibitors targeting host proteins have been developed and tested against WNV and other flaviviruses, such as HCV. Host cyclophilin, a family of cellular peptidyl-prolyl isomerases, may serve as a component of flavivirus replication complex and play a role in flaviviral replication. Targeting this enzyme by cyclosporine inhibits replication of WNV [339]. Targeting lipid signaling and metabolism by a bioactive lipid signaling modulator 4-hydroxyphenyl retinamide (4-HPR, fenretinide) also inhibits replication of WNV and other flaviviruses. Similarly, ribavirin and mycophenolic acid target inosine monophosphate dehydrogenase (IMPDH), an enzyme in purine biosynthesis, and thereby inhibit replication of flaviviruses [342, 343]. In addition, NITD-982 and brequinar that block pyrimidine biosynthesis also inhibit replication of broad range of RNA viruses, including WNV and other flaviviruses [353, 354]. Besides blocking viral genome replication, antiviral targeting ofother steps, such as virus maturation, assembly, and viral dissemination into brain has also been suggested and tested against WNV. For instance, inhibitors of alpha-glucosidase I and II, enzymes that play a role in processing of N-linked oligosaccharides of the viral glycoproteins, also inhibit WNV and other flaviviruses [205, 355]. Although the mechanism by which WNV enters the brain is still poorly understood, the two potential routes include axonal retrograde transport (ART) from the peripheral nervous system and direct hematogenous diffusion via a breakdown in the blood–choroid plexus barrier [356]. Nocodazole, a microtubule inhibitor that blocks ART, delays WNV entry into brain [357]. The 3′ or the 5′ terminal stem-loop in flaviviral RNA contains essential cis-acting elements and plays important roles in viral replication [358, 359]. Interestingly, a range of cellular proteins have been identified to interact with 3′ stem-loop of flaviviral RNAs [352, 359–362], suggesting a potential strategy to design inhibitors targeting this virus–host protein interaction.

Targeting host factors may raise a higher barrier to viral resistance emergence and provide broad-spectrum antiviral effects.

However, current understanding of virus–host cell interaction and research on targeting of host factors to block viral infections are still limited. In addition, there are some potential drawbacks of this approach, including undesirable drug-induced side-effects and difficulties for drug delivery into brain to control WNV encephalitis. Further understanding of virus–host interaction will facilitate identification of novel antiviral agents.

6 Conclusions and Perspectives

Development and testing of various methods for treatment and prevention of WNV infection, such as protective vaccines, therapeutic antibodies, antiviral compounds, peptides, and siRNA have been proposed and intensively studied. Although a number of WNV veterinary vaccines have already been licensed and are in use for years, human vaccine candidates are still in various stages of development and testing. Some therapeutic antibodies that show excellent efficacy in small animal models and are currently being tested in clinical trials represent a promising class of WNV therapeutic. Recent technological advancement and increased understanding of the biology of WNV and other flaviviruses along with structural/functional characterization of viral proteins have provided a solid foundation for the development of small molecule inhibitors as future WNV therapeutics. However, efforts for development of an effective drug for prevention or control of WNV infection in human still remain unsuccessful. Some of the reasons include a low incidence of diseases, low commercial interest by pharmaceutical companies, high cost of mass vaccination, and difficulties with running clinical trials due to unpredictable and sporadic nature of WNV outbreaks [363, 364]. Another challenge for developing successful WNV therapeutics is to ensure safety and efficacy in target populations that mostly include children, elderly, and immune-compromised individuals. Despite all these difficulties, the quest for development of effective treatment and prevention methods against WNV infection are likely to be facilitated by recent technological advancement and should continue to meet the public health needs.

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Index

A

Additive ... 171
Aedes ... 152, 155, 156, 172
Agarose ... 31–35, 39, 40, 52, 57, 72, 76, 87, 92, 98, 180, 184, 191, 201
Aging ... 62, 210, 218, 235–243
Amplification ... 30, 42, 47, 51, 52, 57, 160, 176, 191–193, 207, 209, 221, 232, 233
Anesthetics ... 72, 75, 80
Animal models ... 19, 62, 129, 253, 263, 271, 273
Antibody ... 48, 55, 56, 59, 79, 94, 95, 99, 100, 118, 122, 123, 125, 129–140, 154, 159, 162, 171, 172, 177, 183, 185, 194–196, 198–200, 203, 207, 208, 215, 216, 219, 254, 259–264, 271
Antigen ... 22, 62, 88, 94, 95, 99, 100, 131, 135, 140, 168, 177, 182–185, 194–196, 198–200, 202, 203, 238, 241, 251, 261
Antiviral therapy ... 250, 251, 264, 271, 272
Apoptosis ... 8, 89, 96, 97, 236, 241, 253
Arthropod ... 143, 146–148, 152
Arthropod containment laboratory (ACL) ... 146, 148
Avian ... 19, 143, 176, 179, 185, 187, 188, 199, 200, 202, 207–219, 231, 232

B

Biohazard/biohazardous ... 86, 90, 147, 213
Biosafety cabinet (BSC) ... 21, 77, 79, 87, 90, 119, 120, 135, 148, 217
Biosafety level 3 (BSL3) ... 19, 63, 75, 143, 178
Birds ... 6, 15, 16, 61, 151, 165, 173, 175–204, 208–218, 231
Blood brain barrier (BBB) ... 6, 64, 83, 103–112, 238, 270, 271
Brain tissue ... 84, 88, 90, 91, 93–98, 111, 112, 145, 186, 217, 218
Buffer ... 31, 34–36, 38, 72, 74, 75, 79, 80, 86–91, 95–97, 117–119, 122, 123, 130, 133–135, 138, 153, 154, 157, 159, 161, 171, 179–182, 185–191, 199, 201, 202, 211, 217, 218

C

C6/36 cells ... 63, 80, 152, 154, 160
Cell culture ... 19, 21, 23, 80, 86, 87, 92, 93, 97, 104, 105, 117, 119–121, 132, 135, 143, 152–155, 177, 183, 184, 186, 193, 194, 202, 255, 258, 267–269, 271
Central nervous system (CNS) ... 61, 71, 83–101, 104, 238, 250
Centrifuge ... 23, 34–36, 74, 76–79, 111, 124, 154, 155, 157, 171, 178, 181, 184, 188, 189, 192, 194, 198, 199, 201, 216
Challenge ... 46, 67, 129, 136, 254, 255, 260, 262, 263, 265, 271, 273
Chemokine ... 46, 240, 271
Clone ... 29–42, 51, 53, 79
Collection ... 36, 37, 41, 75, 84, 86, 90–91, 110, 169, 170, 172, 173, 179, 185, 188, 189, 202, 204, 207–219, 224, 230
Complementary (cDNA) ... 42, 47, 51, 52, 57, 86, 91, 92, 153, 157, 190, 258, 260, 270
Containment ... 19, 84, 90, 138, 146, 147, 178
Cryopreservation ... 26
Cryostat ... 88, 93, 94, 96
Culex ... 15, 16, 67, 146, 148, 149, 151, 176, 228, 232, 249
Cytokines ... 46, 64, 84, 237, 238, 240, 241, 260, 265, 271
Cytopathic effect (CPE) ... 22, 41, 154, 160, 193

D

Dead-end hosts ... 16, 231
Detection ... 9, 17, 55, 56, 84, 86, 94, 96, 99, 100, 118, 126, 130, 147, 157, 160, 161, 165, 166, 168–172, 175–204, 207–209, 217, 218, 226, 242, 267
Diagnosis ... 94, 177, 196, 219
Digestion ... 39, 86, 91
Dissection ... 86, 90, 105, 110, 149, 162, 211, 217
DNA ... 30, 32–34, 37–39, 47, 48, 50, 52, 54, 57–59, 86, 96, 153, 161, 182, 190, 191, 201, 202, 255, 258, 267

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E

Electrophoresis 52, 180, 181, 190, 191
Electroporation
Elongation 3, 191
Emergence 254, 272
Encephalitis 15, 61, 64, 71, 79, 83, 103, 151, 152, 176, 177, 208, 235, 241, 242, 250, 254, 273
Endocytosis 7, 238, 265, 272
Enzyme-linked immunosorbent assay (ELISA) 177
Epidemiology 15–18, 175, 176

F

Feather 187, 188, 193, 196, 202, 210, 214, 215, 218
Field collection 129, 165, 178, 185, 207–219
5' cap 2, 269
Fixation 74, 87, 94, 98–100
Flavivirus 3, 4, 6–8, 15, 19, 29, 46, 61, 103, 115, 131, 132, 152, 175, 177, 198, 207, 208, 253, 254, 258, 264–273
Formaldehyde 87, 89, 100

G

Genome 1–3, 6, 8, 29, 37, 38, 42, 45, 46, 51, 115–117, 119, 126, 152, 175, 242, 243, 251, 253, 255, 258–261, 265, 268, 272
Green fluorescent protein (GFP) 46–48, 51, 53, 54, 56–58

H

HeLa cells 117, 118, 120–123
Hematoxylin&Eosin (H&E) 84
Hemotek 153, 158, 162
High-throughput screen 243, 267–270
Histology 87, 88
Homogenization 91, 97, 153, 186, 189, 202
Horse 15, 16, 61, 83, 143, 151, 166, 176, 177, 255
Host 2, 4–7, 9, 48, 62, 64, 66, 67, 71, 92, 98, 115–126, 172, 175, 207, 223, 226, 231–233, 236, 250, 251, 253, 255, 261, 264–266, 269, 270, 272, 273
Host susceptibility factors (HSFs) 115, 126

I

Immune evasion 6–8, 253
Immunofluorescent antibody assay (IFA) 177
Immunoglobulin (Ig) 4
Immunohistochemistry (IHC) 87, 88, 91, 94, 95
Immunopathology 8, 71, 72, 79
Immunostaining 48, 99, 100, 118, 125, 137, 138, 140, 153, 154
Incidental hosts 151, 176
Incubator 22–24, 36, 37, 47, 48, 51, 54, 74, 75, 87, 88, 92–94, 105, 117, 120–122, 152, 153, 156–159, 182–184, 216, 221, 223, 224
Infectious clone 29–42
Infiltration 72, 99, 104
Inhibitor 104, 182, 251, 253, 264–270, 272, 273
Inoculation 21, 62, 63, 66, 67, 84, 86, 90–91, 97, 109, 110, 112, 146, 154, 159, 223, 231, 232, 238, 258
Insect 6, 18, 131, 143–149
Insectary 143–149

K

Knock-out 124–126

L

Leukocytes 71, 79, 236
Ligation 33, 34, 39, 40
Light trap 168, 169, 172

M

Mammal 6, 16, 175–204
Media 24, 36, 47, 54, 56, 59, 72, 77, 88, 92–94, 98, 104–106, 108, 109, 112, 131, 132, 136, 137, 139, 193, 194
Membrane feeding 162
Meningitis 61, 71, 151, 235, 250
Methylation 2, 6, 269
Microarray 117, 119, 126
Microcentrifuge 48, 51, 86, 90, 92, 179, 210, 211, 215, 216
Microinjection 148, 152, 153, 155–157, 159, 160
Microsphere immunoassay (MIA) 177
Migratory patterns 7
Monolayer 21, 24, 25, 75, 92, 93, 98, 106, 108, 137, 138, 183–185, 193, 194, 197, 199, 203, 204
Mosquito 6, 16, 18, 19, 21, 61, 62, 67, 80, 83, 103, 143–149, 151–162, 165, 166, 169–173, 175–204, 207, 209, 216, 223, 224, 226–230, 232, 233, 249
Mosquito infection rate 224, 226–229
Mouse models 71–81, 84, 104, 143, 241, 254

N

Neuroinvasion 83–101, 104
Neuronal death 87–89, 93–97
Neuropathogenesis 83–101, 104, 253, 266
Non-structural protein 4–6, 84, 129, 251–253, 265, 266, 270, 271

O

Optical density (OD) 112, 200
Organ 17, 21, 71, 110

P

Paraffin 88, 89, 93, 94, 96, 99
Pathogen 7, 8, 61, 146, 147, 221, 223, 224, 226, 254, 261

Pathogenesis 9, 46, 62, 64, 67, 71, 79, 103–112, 152, 155, 241, 243, 250, 251, 253, 254, 264, 267, 269, 272
Persistence 66
Personal protective equipment (PPE) 147
Phosphate buffered saline (PBS) 20, 48, 63, 72, 86, 87, 89, 105, 118, 130, 132, 210, 215
Plaque reduction neutralization test (PRNT) 177, 184, 196–198, 208, 254
Plasmid 29, 30, 32, 33, 37–40, 46–48, 53, 54, 58, 258
Poly-A tail 2
Propagation 19–27, 132

Q

Quantification 19, 38, 72, 123, 126, 192
Quantitative real-time polymerase chain reaction (qRTPCR)

R

Replication 2, 3, 5–9, 38, 45, 58, 64, 93, 103, 115, 236–238, 240, 251, 253, 265, 266, 268, 269, 271, 272
Reservoir 15, 16, 117, 119–122, 153, 158, 207, 221, 237
RNA
  interference (RNAi, dsRNA) 5, 7, 116, 271
  sequencing 57

S

Selection 124, 126, 207, 209
Sentinel animal 165, 166
Sequelae 62, 66, 143, 250
Serum 19, 41, 47, 48, 54, 59, 64, 66, 72, 80, 86, 88, 92, 95, 104, 109, 117, 119–121, 125, 129, 130, 132, 133, 135–138, 147, 152, 153, 160, 171, 177, 178, 183–185, 197–200, 203, 208, 215, 219, 254, 260, 271
Severity 62, 238, 250
Structural protein 2–4, 27, 45, 175, 251, 252, 259, 260
Supernatant 19, 21, 23, 36, 37, 41, 51, 76, 77, 137, 154, 157, 158, 160, 186, 189, 190, 194, 198
Surveillance 8, 16, 18, 147, 165–173, 177, 190, 193, 207–219, 221, 223, 224, 229, 230
Susceptibility 66, 71, 208, 231, 235–243

T

Therapeutics 71, 115, 151, 253, 262, 263, 265, 268, 269, 271, 273
Thermocycler 180, 190, 192
Thoracic infection 156, 159
Tissue 6, 19, 21, 24, 31, 32, 36, 64, 66, 67, 75, 80, 83, 86, 88, 91, 93–101, 104–106, 111, 112, 130, 136, 149, 152, 153, 159, 173, 182, 186, 187, 190, 196, 202, 217, 218, 241, 242
Titer 16, 19, 21, 36, 41, 42, 80, 84, 92, 93, 97, 100, 104, 109, 130, 135, 136, 138–140, 182, 184, 185, 198, 199, 202, 208, 219, 241, 253, 254, 258, 260, 261
Titration 19–27, 72, 112, 129, 132, 134, 140, 157, 197–199, 203
Transcription 8, 31, 32, 34, 37–40, 42, 86, 272
Transfection 32, 35, 36, 38, 40, 41, 48, 53–55, 58, 59, 117–121, 125, 258
Transfection 41
Transmission 4, 9, 15–18, 144–147, 152, 155, 165, 166, 168, 169, 207, 209, 216, 221–233, 250
Transmission cycle 16–18, 151, 208, 221
Tropical disease 146
Tropism 6, 7, 66, 83, 250
TUNEL assay 84, 89, 96, 97, 100

U

Untranslated regions (UTRs) 5, 33, 259

V

Vaccine 4, 15, 62, 64, 71, 103, 115, 129, 136, 139, 143, 145, 151, 197, 223, 235, 242, 249–251, 253–261, 263, 264, 273
Vector 15, 16, 18, 38, 47–48, 51, 53, 57, 58, 62, 89, 133, 146, 147, 151, 152, 155, 165, 166, 168, 172, 173, 176, 183, 184, 188, 195, 196, 202, 207, 216, 223, 224, 229–233, 255, 258–260, 271
Vero cells 19–27, 31, 36, 37, 40, 47, 50, 63, 72, 76, 80, 92, 93, 97, 132, 136, 153–155, 158, 160, 184, 193, 197, 198
Vertebrate host 3, 151
Viral replication 3–6, 27, 240, 251, 253, 264, 265, 269, 271, 272

W

West Nile virus (WNV) 1–9, 15–27, 29–42, 45–59, 61–67, 71–81, 83–101, 103–112, 115–126, 129–140, 143–149, 151–162, 165–173, 175–204, 207–219, 221–233, 235–243, 249–273
Western blot 84, 94

X

Xylene 30, 88, 89, 93, 95, 96, 99