

DEVELOPMENT, OPTIMIZATION AND VALIDATION OF MICROSPHERE
BASED LUMINEX ASSAYS FOR IDENTIFICATION OF WEST NILE VIRUS
AND DENGUE VIRUS INFECTIONS

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ABSTRACT

Purpose: West Nile virus (WNV) and dengue virus (DENV) are emerging arthropod-borne flaviviruses that represent an immense global health problem. Therefore, there is need for improved diagnosis of these infections for timely patient management, to prevent the spread of flaviviral infections as well as to understand the virus and antibody dynamics in humans as well as animal models. In this study, Luminex-based WNV E-protein microsphere immunoassay (MIA) was developed and optimized for detection of anti-WNV IgG and IgM antibodies in mice using assay parameters such as serum heat-inactivation (HI) and-dilution. In addition, an in-house newly developed Luminex-based assay for detection of anti-DENV IgG and IgM antibodies was further validated using different serum panels and was further optimized for improved sensitivity and specificity which is critical for accurate diagnosis of DENV infection. Similarly, in-house newly developed Polymerase Chain Reaction-Microsphere Bead Assay (PCR-MBA) was further validated for detection of DENV serotypes and WNV.

Methods: Magnetic carboxylated microspheres were coupled to purified rWNV-E protein and WNV E-MIA was conducted using serial dilutions of HI and non-HI (NHI) serum samples collected at days 0, 3, 6, 8, 10 and 24 from mice inoculated with WNV. In-house newly developed DENV MIA was validated using different serum panels from Hawaii, Vietnam and Niue as compared to gold standard PRNT assay and in-house DENV IgM Capture ELISA (MAC-ELISA) and a U.S. FDA approved InBios DENV IgM Capture ELISA. To improve the specificity and

sensitivity of DENV MIA, the effect of assay parameters such as serum dilution, the use of alternative blocking agents such as PVA, PVP and 5-10% animal serum in serum diluents as well as assay buffers and the type and concentration of secondary antibody on the non-specific binding was studied. An in-house newly developed PCR-MBA was further validated for differential detection of DENV serotypes using CDC DENV panel samples as compared to gold standard CDC DENV 1-4 real-time RT-PCR assay. For validation of PCR-MBA for detection of WNV, WNV cDNA was diluted serially (10-fold dilutions) from 3×10^7 PFU/ml to 0.3 PFU/ml and WNV specific qRT-PCR and PCR-MBA was conducted. Data was analyzed to assess the sensitivity and specificity of PCR-MBA for detection of WNV as well as for differential detection of DENV serotypes.

Results: In WNV E-MIA, serum HI significantly enhanced detection of IgM and IgG antibodies as compared to NHI serum. Anti-WNV IgM and IgG antibodies in HI sera were detected earlier at day 3 and IgM antibodies persisted up to day 24 after infection. HI serum at 1:20 dilution was found to be optimal for detection of both IgM and IgG antibodies as compared to higher serum dilutions. Further, addition of exogenous complement to the HI serum decreased the WNV E-MIA sensitivity. Results of validation of DENV MIA demonstrated that DENV MIA is 100% and 71-100% sensitive for detection of anti-DENV IgG and IgM antibodies, respectively from human serum samples. However, the specificity of DENV MIA was found to be 33-100% and 11-98% for detection of anti-DENV IgG and IgM

antibodies respectively. The low specificity of DENV MIA for samples from Vietnam was not due to syphilis antibodies present in these serum samples. Use of alternative blocking agents such as PVA and PVP, 5-10% animal serum in serum diluents did not increase the specificity of DENV IgM MIA. Higher serum dilution as well as pretreatment of serum samples with high concentration of BSA or BSA-coated beads reduced the assay interference to some extent in DENV IgM MIA. The use of monoclonal IgM secondary antibody for detection of anti-DENV IgM antibodies resulted in low BSA IgM MFI for IgM false-positive samples as compared to polyclonal IgM secondary antibody in DENV MIA. However, the IgM MFI for true IgM positive samples was reduced using monoclonal antibody as compared to polyclonal secondary antibody. The specificity of PCR-MBA for detection of four DENV serotypes as compared to 'gold standard' CDC DENV 1-4 real time RT-PCR assay was found to be 100%, whereas the sensitivity of PCR-MBA was found to be varied from 50-100% for DENV-1, 29-100% for DENV-2, 100% for DENV-3 and 80-100% for DENV-4 using CDC DENV panel samples. PCR-MBA for detection of WNV is specific and more sensitive as compared to WNV-specific qRT-PCR. Moreover, low background MFI was observed for the other arboviruses included in PCR-MBA and thus indicated negligible probe cross reactivity for the DENV serotypes and WNV.

Conclusions: Serum-HI and optimal dilution enhanced WNV E-MIA sensitivity by eliminating the complement interference. This optimized WNV E-MIA can be used for detecting low-titer anti-WNV antibodies during early and late phase of

infection in mice. Validation of DENV MIA using different serum panels demonstrated that sensitivity and specificity of immunoassays may differ according to the origin of samples. Minimizing the serum concentration as well as the absorption of sera with high concentration of BSA or BSA coated beads decreased the non-specific binding in DENV IgM MIA. Moreover, the results of this study conclude that sample pretreatment or the use of alternative blocking agents will vary for immunoassays. Therefore, it is important to optimize the blocking conditions for newly developed immunoassays to avoid the false-positive results. PCR-MBA efficiently detected and differentiated the DENV serotypes from CDC DENV panel samples as well as WNV. PCR-MBA can be further optimized for improved sensitivity for detection of dengue serotypes and WNV by changing the concentration of probe coupled, concentration of the input RNA, hybridization temperature and time.

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LIST OF ABBREVIATIONS

BSA, Bovine Serum Albumin; **BSL-3**, Biosafety Level 3; **CDC**, Centers for Disease Control and Prevention; **cdNA**, Complementary DNA; **Ct**, Cycle Threshold; **CSF**, Cerebrospinal Fluid; **CNS**, Central Nervous System; **DENV**, Dengue Virus; **DF**, Dengue Fever; **DHF**, Dengue Hemorrhagic Fever; **DSS**, Dengue Shock Syndrome; **E**, Envelope; **EDC**, 1-Ethyl-3-[3 Dimethylaminopropyl] Carbodiimide; **EIA**, Enzyme Immunoassay; **ELISA**, Enzyme-linked immunosorbent assay; **ER**, Endoplasmic Reticulum; **Fc-receptor**, Fragment, Crystallizable region, a cell receptor; **FDA**, Federal Drug Administration; **HI**, Hemagglutination Inhibition; **HI**, Heat-Inactivation; **IgG**, Immunoglobulin G; **IgM**, Immunoglobulin M; **IgA**, Immunoglobulin A; **JEV**, Japanese Encephalitis Virus; **LOD**, Limit of Detection; **MIA**, Microsphere-based Immunoassay; **MAC-ELISA**, IgM Antibody Capture Enzyme-Linked Immunosorbent Assay; **MBA**, Microsphere-Bead Assay; **MFI**, Median Fluorescent Intensity; **NHI**, Non-Heat-Inactivated; **NS1**, Non-structural Protein 1; **PBS**, Phosphate Buffered Saline; **PCR**, Polymerase Chain Reaction; **PE**, Phycoerythrin; **PFU**, Plaque Forming Units; **PRNT**, Plaque Reduction Neutralization Test; **PVA**, Polyvinyl Alcohol; **PVP**, Polyvinyl Pyrrolidone; **qRT-PCR**, Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction; **rpm**, rotations per minute; **RNA**, Ribonucleic acid; **RT-PCR**, Reverse Transcriptase-Polymerase Chain Reaction; **Sulfo-NHS**, N-Hydroxysulfosuccinimide; **SLEV**, St. Louis Encephalitis Virus; **TBEV**, Tick-borne encephalitis virus; **YFV**, Yellow Fever Virus; **WHO**, World Health organization; **WNV**, West Nile Virus.

Chapter 1

INTRODUCTION

1.1 Flaviviruses

Flaviviruses belong to genus *flavivirus* (family Flaviviridae) [1]. The family was named “flavi” which means “yellow after the family’s prototype, the yellow fever virus (YFV; flavi means yellow) and the associated jaundice that occurs during infection [2]. Many flaviviruses are major emerging human pathogens, including the Dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis (JEV), and Tick-borne encephalitis virus (TBEV), affecting millions of individuals worldwide [3]. These viruses are transmitted to humans predominantly after a bite from an infected arthropod vector such as mosquitoes or ticks.

Flavivirus virions are spherical in shape, with a diameter of approximately 50 nm. The nucleocapsid of approximately 30 nm in diameter consists of capsid and genomic RNA. It is surrounded by a lipid bilayer in which the viral envelope and membrane proteins are embedded. The flavivirus genome is a single-stranded RNA of approximately 11 kb with a positive polarity [4]. This RNA encodes three structural proteins, capsid (C) protein, membrane/matrix (M) protein, envelope (E) protein and seven nonstructural (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) proteins (Figure 1). The structural proteins constitute the viral particle whereas the non structural proteins are involved in viral RNA replication, viral assembly and evasion of host immune response [5].

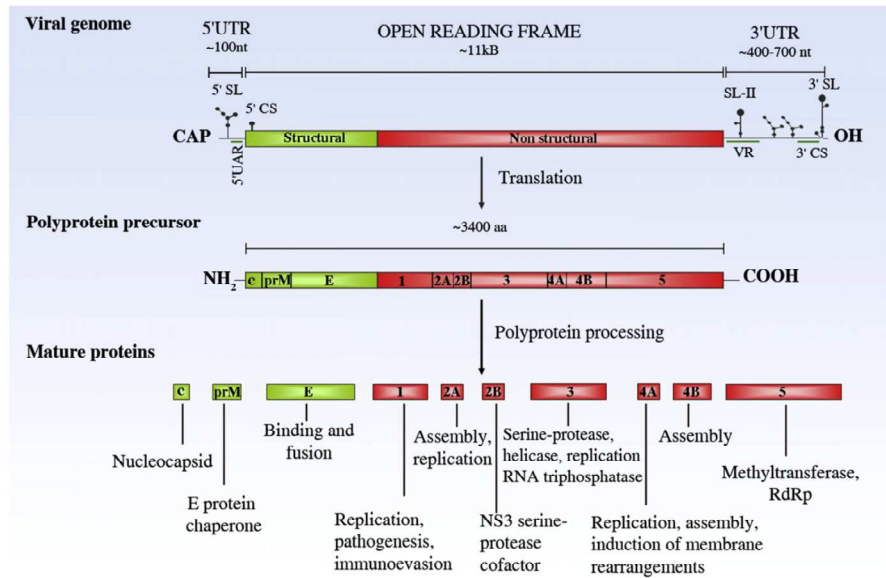


Figure 1: Flavivirus Genome organization and viral protein functions. The single open reading frame encodes a polyprotein precursor that is co- and posttranslationally cleaved into three structural proteins (in green) that make up virion particle and seven nonstructural proteins (in red) that are necessary for virus replication and immune evasion.

[Fernandez-Garcia et al., *Cell Host & Microbe* 5, 2009]

Flaviviruses enter target cells by receptor-mediated endocytosis. The acidic environment in early endosomes triggers major conformational changes in their envelope glycoprotein (E) protein that induce fusion of the viral and host cell membranes and results in release of genome in to the cytoplasm. Viral RNA is transcribed in to messenger RNA (mRNA). This is followed by translation into various proteins, including a large polyprotein which is then processed by host and virus-encoded proteases into ten or more separate proteins. Virus particles are assembled on the surface of the ER. The resultant immature virions are transported to the trans-Golgi where furin-mediated cleavage of prM to M

generates mature infectious particles that are released from the host cell by exocytosis [5].

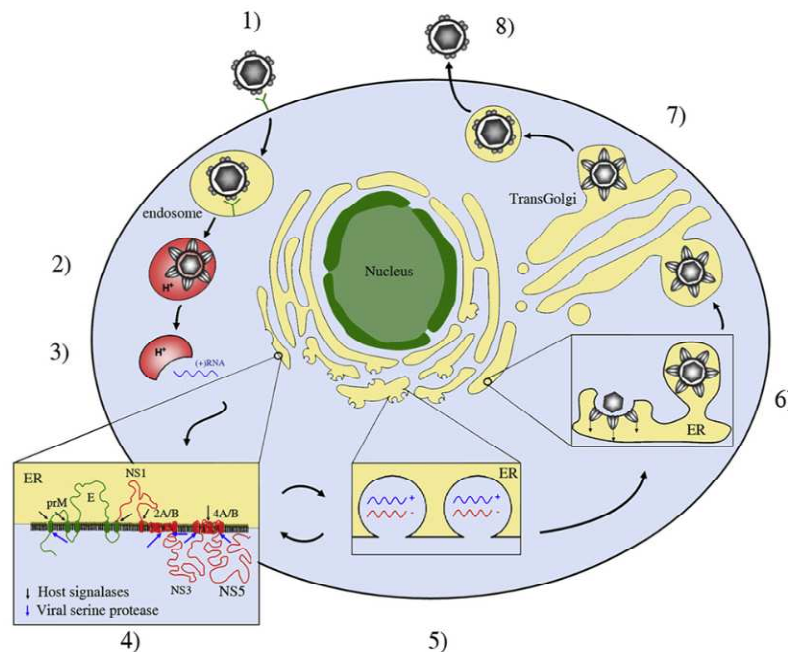


Figure 2: Flavivirus infectious life cycle. Flaviviruses are internalized by receptor-mediated endocytosis (1) and trafficked to early endosomes, where the acidic environment induces fusion (2) between the virus and the host membrane resulting in genome release (3). Translation of viral RNA is followed by processing of the resulting polyprotein by host and virus-encoded proteins (4). The cleavage sites and topology of structural (in green) and nonstructural (NS) (in red) proteins at the endoplasmic reticulum (ER) membrane are illustrated schematically. Following translation, a replication complex is assembled and associated to virus-induced membranes where viral replication takes place (5). The replication complex starts to transcribe the RNA (+) template into RNA (-), which then serves as template for new RNA (+) synthesis. Progeny RNA (+) strands can either initiate a new translation cycle or be assembled into virions (6). Packaging occurs on the surface of the ER, followed by budding of the structural proteins and newly synthesized RNA into the lumen of the ER. The resultant immature virions are transported to the trans-Golgi where furin-mediated cleavage of prM to M generates mature infectious particles (7) that are released by exocytosis (8).

[Fernandez-Garcia et al., *Cell Host & Microbe* 5, 2009]

1.2 West Nile virus and Dengue virus

West Nile virus (WNV) and dengue virus are mosquito-borne flaviviruses and are classified as significant human pathogens of global epidemiological importance [6-10]. WNV is maintained in an enzootic cycle between mosquitoes and birds. Human and horses are incidental hosts. WNV infects the central nervous system (CNS) and can cause lethal encephalitis [11]. Dengue is an acute viral disease caused by any of the four dengue serotypes, DENV-1,-2,-3 and -4. Dengue virus is unusual among arboviruses in that humans are the natural hosts [12].

In case of WNV infection, viremia is usually low and short-lived (Figure 2). It is undetectable in many cases by day 1 of illness onset [4, 13]. Immunoglobulin M (IgM) antibodies can be detected within 4 to 7 days after the initial exposure and may persist for more than one year [14], whereas immunoglobulin G (IgG) antibodies are detected around day 8 after the onset of symptoms and thus are not very useful in the initial diagnosis of WNV infection [15].

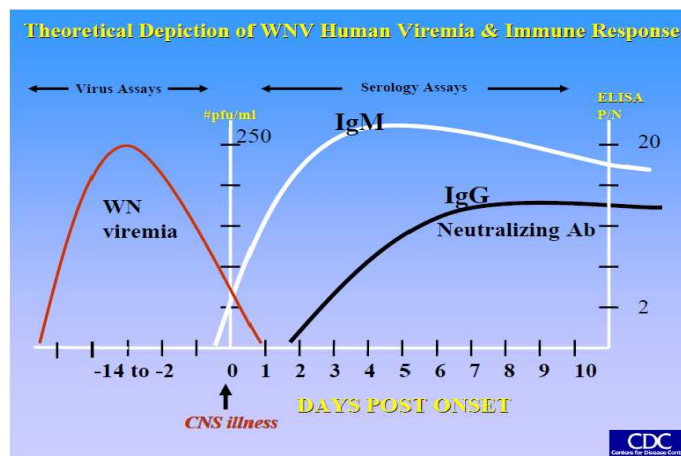


Figure 3: The course of WNV infection in humans depicting the WNV viremia and immune response.

During the course of dengue infection, around days 3 to 4 after the mosquito bite, there is onset of fever accompanied by viraemia which lasts for 4 to 5 days (Figure 3). However, during a secondary or subsequent infection, the duration of viraemia can be 2 or 3 days [16]. Antibody response also differs during primary and secondary dengue infection. In primary infections, IgM antibodies appear at around 5 to 6 days after the onset of fever in majority of the infected individuals, which remain detectable for over 90 days. Anti-dengue IgG antibodies are detected at around days 10-15 after the onset of illness [17]. In secondary infections, IgM antibodies appear earlier or in the same time frame but are usually at lower titers than in primary infection. IgG antibodies are present from the previous infection and the titer increases rapidly [18].

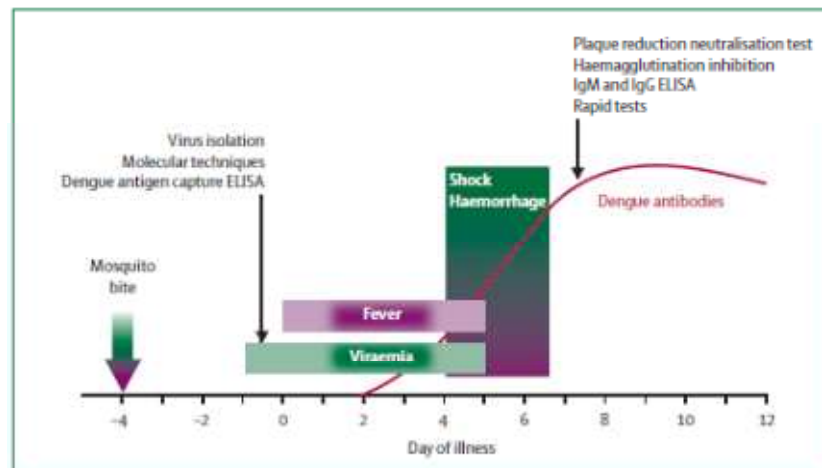


Figure 4: Course of dengue infection and timings of diagnosis. [Halstead, *Lancet* 2007; 370: 1644–52]

1.2.1 Epidemiology

WNV was first isolated in the West Nile region of Uganda in 1937 from the blood of a febrile patient [19]. Since 1996, WNV outbreaks have occurred with a

high incidence of severe disease in both humans and horses [20, 21]. WNV is endemic in parts of Africa, Europe, the Middle East, and Asia [22]. It was first detected in the United States in 1999 during an outbreak of encephalitis in New York City. Since then, it has rapidly spread across North America, and has recently been reported in Mexico, South America, and the Caribbean [11]. So far, in the United States between 1999 and 2010, 30 662 cases were confirmed and associated with 1163 deaths [23]. WNV is mainly transmitted by *Culex* mosquitoes, but the mosquito species from other genera are also susceptible to infection [23]. Humans and other mammals are considered as dead-end hosts because the concentration of virus in the blood is too low to infect a feeding naïve mosquito [10]. Besides natural transmission by mosquitoes, WNV can also be transmitted through blood transfusion and organ transplantation [24]. Therefore, there is urgent need for the development of a rapid and specific serologic assay for diagnosis of WNV infection [4]. Currently, no specific therapy or vaccine has been approved for use against WNV infection in humans [25].

Dengue virus infection is a major health problem in tropical and subtropical areas of the world [26]. More than two billion people are at risk globally of infection by dengue virus [7]. Worldwide, approximately 100-200 million infections, 34 million cases of dengue fever (DF), 2 million cases of dengue hemorrhagic fever (DHF) occur each year, causing roughly 20,000 to 30,000 deaths [27]. The disease is now endemic in more than 100 countries in Asia, Africa, and the Americas [28]. Most of the reported dengue cases in the continental United States are acquired by travelers or immigrants [29]. It has re-

emerged in southern Florida in 2009-2011 and also in Hawaii in 2011 [30]. Dengue virus is mainly transmitted to humans through the bite of an infected *Aedes aegypti* mosquito (less commonly *Aedes albopictus*) [31]. There is a sylvatic nonhuman primate cycle of DENV transmission, it rarely crosses to humans, and antibodies to the sylvatic virus appear to protect against human dengue [31].

1.2.2 Clinical manifestations

WNV infection remains asymptomatic in the majority of cases or results in West Nile fever (a mild flu-like illness) in approximately 20 to 30% of infected cases [32]. Symptoms are of sudden onset and may include malaise, eye pain, headache, myalgia, gastro-intestinal discomfort and rash [33]. About 1 in 150 people infected with WNV develop a neuroinvasive disease and long-term neurological sequelae are common in more than 50% of these cases [34]. Disease manifestation is explained by neuronal damage in several regions of the brain [35]. The mortality rate in neuroinvasive disease cases is approximately 10%, with increased risk for patients with compromised immune systems, advanced age and with underlying conditions such as diabetes mellitus [11, 35].

The clinical manifestations associated with dengue infection range from asymptomatic infection to mild disease and severe, life-threatening disease [36]. According to World Health Organization (WHO), dengue-related symptomatic infections are classified into probable dengue, dengue without warning signs (previously dengue fever), dengue with warning signs (previously dengue

hemorrhagic fever [DHF]), and severe dengue (also known as dengue shock syndrome [DSS]) [(WHO, 2009) [37]]. Dengue without warning signs is the most common presentation of primary infection and typically occurs after a short incubation period of 4–7 days (range 3–14 days) [36]. The most common manifestations are fever, headache with or without retroorbital pain, myalgia, arthralgia, and a maculopapular to petechial rash [7]. No symptom or sign can clearly discriminate between patients with primary infection or secondary infection [38]. Less commonly, patients progress to more severe forms of illness (WHO 2009 classifications: dengue with warning signs and severe dengue) that include thrombocytopenia, hemorrhage, plasma extravasation, shock, and even death. These forms of severe disease are manifested in an estimated 5–10% of patients and are most commonly associated with secondary infection by a serotype other than the one that caused the initial infection. Mortality rates associated with more severe forms have been estimated to be from 1–26% but maybe as high as 47% in some areas [[7] and WHO, 2009].

1.2.3 Immunopathogenesis

The host immune response is critical for determining the outcome of human flavivirus infection [39]. Both innate and adaptive immune responses are important for control of viral infection and dissemination. This control is mainly provided by neutralizing antibodies generated against viral E glycoprotein. The E protein consists of three distinct structural domains, DI, DII, DIII, involved in virion assembly, membrane fusion and receptor binding, respectively. All three

domains contain epitopes for neutralizing antibodies, but DIII elicits the strongest neutralizing antibody responses [40]. These antibodies inhibit viral attachment, internalization, and/or replication within cells [41]. In human WNV infections, most antibodies produced against the E protein are non-neutralizing or only poorly neutralizing and target DII [42]. Nonstructural protein 1 (NS1) is a secreted protein that is highly conserved among the flaviviruses. High levels of NS1 protein in serum from dengue-infected patients are found to be correlated with severe disease [43]. Antibodies generated against this protein protect from flavivirus induced disease and these anti-NS1 antibodies can be used both prophylactically and therapeutically in animal models [44].

After the bite of an infected mosquito, WNV replicates peripherally resulting in transient viremia. In the absence of sufficient immune response, virus can cross the blood brain-barrier. The immune response especially antibody-mediated response play important role in spread of WNV infection to the CNS [45]. This response is characterized by early production of IgM antibodies in both serum and CSF, followed by production of IgG [46]. IgM antibodies limit viral dissemination in the host whereas, IgG antibodies effectively clear the virus and mediate protective immunity in the long term. IgM antibodies do not cross blood brain barrier, hence their presence in CSF reflects intrathecal production or damage to the blood brain barrier itself [15]. A failure of antibody production is associated with increased likelihood of a fatal outcome in WNV encephalitic patients [47]. Patients on immunosuppressive drugs were especially vulnerable to severe disease caused by WNV in recent outbreaks in North America [39].

The T cell response, mediated mostly by CD8+ lymphocytes, also plays a crucial role in recovery, particularly in viral clearance [48]. Humoral immune response in conjunction with cellular immune responses not only prevents the dissemination and persistence of WNV infection, but also resolves CNS infection [41].

Interferons also play an important role in the immune response against flaviviruses. They inhibit the infection *in vitro* [49] and mice deficient in interferon γ show a higher mortality rate after a WNV infection [50].

The adaptive, protective immune response to dengue infection is driven by virus neutralizing antibodies and T-cell responses involved in helping antibody synthesis. Upon dengue infection, IgM, IgG and IgA antibodies are produced. Infection by one dengue serotype induces life-long immunity against reinfection by the same serotype, but only transient and partial protection against infection with the other serotypes [51, 52]. Several seroepidemiologic studies suggest that the presence of heterotypic antibodies from a primary dengue infection was a risk factor for developing DHF/DSS during secondary dengue infection [53-55]. Also infants born to dengue immune mothers are at risk of developing more severe dengue disease during a primary infection [56]. This suggests the role of possible immunopathological activity in the humoral immune response to dengue virus infection, known as antibody-dependent enhancement of infection, or ADE [57, 58]. It is believed that ADE occurs when viruses are bound by non-neutralizing or too few neutralizing antibodies and these antibody-virus complexes are internalized into cells carrying Fc gamma receptors (FC γ Rs) such as monocytes, macrophages and dendritic cells [41]. This leads to enhanced infection of these

cells and thus, to high viral loads, resulting in excessive T cell activation early in the infection process [59]. These T cells produce high amounts of cytokines and chemical mediators which results in endothelial cell damage and subsequent plasma leakage [60, 61]. ADE has not yet been associated with human WNV cases [62]. One of the possible reason for this is that complement opsonin C1q binds to anti-WNV IgG antibodies, thereby reduces the number of antibodies required for neutralizing WNV infectivity [63].

1.2.4 Control and prevention strategies

Effective human vaccines are in use for the prophylaxis of yellow fever (live attenuated), Japanese encephalitis (live attenuated and inactivated whole virus), and tick-borne encephalitis (inactivated whole virus). [64]. However, there are no vaccines or specific antiviral drugs available yet for prevention and treatment of WNV and dengue virus infections in humans [23, 65]. A wide range of vaccines, antiviral drugs and immunotherapeutic products are currently in various stages of the development and approval pipeline for use in humans [64] [25].

Currently, the only accepted therapy for WNV infection is supportive care. Clinical trials are undergoing to determine the efficacy of treatment with antivirals, immunoglobulins, and novel vaccines for WNV [66]. DNA vaccines have been tested in animals and shown to produce an effective antibody response [67]. A second-generation DNA vaccine using an improved vector was evaluated in a phase I clinical trial [68]. A live, attenuated chimeric WNV vaccine has been

developed. Phase I clinical trials have demonstrated that the chimeric vaccine is safe, well tolerated, and highly immunogenic [69]. This same technology has been used to produce a successful equine vaccine. Other effective inactivated/killed and DNA vaccines have also been approved for use in equines [8]. Equine WNV vaccines in the USA led to a significant decrease in the number of severe clinical signs associated with WNV infections among horses [70].

Similar to WNV, the treatment of dengue is supportive and nonspecific. It consists of rest, fluids, and medications to reduce fever, such as acetaminophen or ibuprofen [3]. Aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs) are contraindicated, since they are associated with the increase risk of hemorrhagic manifestations. The mortality in severe dengue cases may be reduced to less than 1% with adequate intensive supportive therapy. Attempts to develop safe and effective vaccines against dengue make use of several approaches that include attenuation of natural strains by serial culture, development of chimeric viruses based on the yellow fever-17D vaccine in which the envelope protein of the dengue virus replaces the yellow fever-17D envelope protein, and production of engineered proteins using recombinant virus expression systems that elicit specific immune responses to dengue virus [71]. The development and use of vaccines has been hampered by the risk of vaccine-related adverse events such as immune enhancement of infection and the requirement to induce a long-lasting protective immune response against all four dengue serotypes simultaneously. Currently, several kinds of dengue vaccines are in development, but only one of these candidates, a chimeric dengue-yellow fever liver attenuated

vaccine (ChimeriVax manufactured by Sanofi Pasteur) has reached the stage of phase 3 clinical trials [64, 72, 73]. Other candidates in early phases of clinical development include vaccines containing live attenuated dengue viruses and recombinant subunit vaccines.[74]

Until effective antivirals, vaccines or other therapeutics become available, prevention of WNV and dengue virus infections in humans is focused on education, control and reduction of mosquito populations, elimination of breeding sites, and prevention of mosquito bites. However, vector control, through chemical or biologic targeting of mosquitoes and removal of their breeding sites has failed to stop disease transmission in almost all countries where dengue is endemic [75]. New vector-control approaches include the release of genetically modified male mosquitoes that sterilize the wild-type female population, thereby reducing egg output and the population size of the next generation that would be available for potential transmission of the dengue virus.[76]. An alternative strategy involves embryonic introduction of strains of the obligate intracellular bacterium *wolbachia* into *A. aegypti*. Strikingly, *wolbachia*-infected *A. aegypti* are partially resistant to dengue virus infection and can invade natural *A. aegypti* populations, suggesting the possibility of induction of widespread biologic resistance to dengue viruses in *A. aegypti* populations [75, 77]. However, public health, medical, and vector-control communities must collaborate to prevent and control disease spread. [78].

1.3 Serologic diagnosis of flaviviral infections

Serological assay is the dominant method for diagnosis of flavivirus infections in humans [4]. Serological methods predominantly used for detection of flaviviral infections include hemagglutination inhibition antibody test (HAI), IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA), and plaque reduction neutralization test (PRNT).

1.3.1 Plaque reduction neutralization test

The Plaque Reduction Neutralization Test (PRNT) is the gold standard for the serological diagnosis of flaviviral infections [79]. It relies on the presence of antibodies within serum samples that neutralize the virus and is evidenced by a reduction in the number of plaques [79]. PRNT assay is conducted in Vero (African green monkey kidney) cell cultures and utilizes live infectious virus [80]. It is still used as the reference assay for specific diagnosis of flaviviral infections as flavivirus-infected sera show cross-reactivity in serodiagnosis with heterologous flavivirus infections [23]. However, PRNT assays are expensive, time consuming, require skilled personnel and must be carried out in a biosafety level 3 (BSL-3) facility for diagnosis of WNV infection [80].

1.3.2 Hemagglutination inhibition assay

Hemagglutination Inhibition assay (HI) have been widely used for the detection of the antibodies developed to arthropod-borne viruses [81]. These assays exploit the ability of the envelope glycoprotein to bind and agglutinate avian erythrocytes so that they form a visible lattice in a U-bottom microtitre

plate. In the HI assay, antibodies from infected individuals prevent the agglutination of the erythrocytes, which subsequently form a pellet [79]. The highest dilution of serum that completely inhibits agglutination of the avian erythrocytes is taken as the HI titer of the serum [4]. The advantages of HI assays are that providing avian red blood cells are available, the assays can be conducted with minimal training and equipment and the antigen used can be inactivated by a simple extraction process. It also has the advantage that it does not require antispecies conjugate so it can be used to detect antibodies using serum samples from any species. However, HI test exhibits extensive cross-reactivity among the flaviviruses. In addition, HI test cannot differentiate between IgM and IgG antibodies [82].

1.3.3 Immunofluorescence assay

The Immunofluorescence assay (IFA) can be used to detect the IgM, IgG or total antibodies against flaviviral infections [4]. It involves incubating patient serum with glass slides, upon which are fixed flavivirus-infected cells. The patient's virus-specific antibodies are then detected with a fluorophore-conjugated anti-species IgM or IgG antibodies. The advantage of this assay is that prefixed slides can be stored at 4 °C and a BSL-3 facility is not required to perform the assay and results can be obtained quickly, particularly due to the commercialization of IFA kits (e.g., Focus Diagnostics Arbovirus IFA). However, cross reactivity of immune antibodies with closely related flaviviruses can impair

the accuracy of the diagnosis and there is a requirement for a fluorescent microscope to evaluate the results [79].

1.3.4 Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assays used for detection of flaviviruses can be classified into three types: IgG ELISA, Immunoglobulin M (IgM)-enzyme linked immunosorbent assay (MAC-ELISA) and epitope blocking ELISA. The recommended immunoassays are the MAC-ELISA and the indirect IgG ELISA. MAC-ELISA is the front line serologic test employed by most state health departments and testing facilities for diagnosis of recent flavivirus infections [83]. The MAC-ELISA involves the capturing of test-serum IgM antibodies with immobilized anti-species IgM antibodies, followed by the addition of virus specific antigen and detection with a flavivirus-specific, horseradish peroxidase-conjugated monoclonal antibody [83]. MAC-ELISA and IgG ELISAs for WNV were successfully used to confirm WNV encephalitis in the US epidemic in 2002 [84]. Processing of clinical samples by the CDC established MAC-ELISA protocol can take up to 1.5 - 3 days depending on whether plates are incubated overnight or not [8, 85]. At present, there are several commercially available ELISA kits available that have comparable sensitivity and specificity that can be completed in one day [86, 87]. Epitope blocking ELISAs have also been developed and used to increase the specificity of WNV serodiagnosis and have been useful for differentiating flaviviral infections through targeting epitopes on NS1 or E protein

[88]. These ELISAs have also been used extensively for surveillance for WNV in North America [89].

1.3.5 *Microneutralization assay*

The microneutralization assay is based on the same principle as PRNT [90]. However, this method uses a colorimetric measurement of the virus induced cell lysis to determine the end-point dilution instead of counting the number of plaques per well the assay [90]. An automated colorimetric microneutralization assay has recently been developed for the detection of, and differentiation between, WNV and SLEV infections in humans [91]. Of the 152 PRNT-confirmed negative, WNV-positive or SLEV-positive sera, there was concordance between PRNT and the rapid microneutralization assay for all samples except one. The advantages of this assay over traditional PRNT are that the testing duration is significantly reduced and the assay can be conducted in a 96-well format, enabling 8 dilutions of each sample to be analyzed simultaneously. Like PRNT, neutral red is used to stain live cells; however, in this automated assay, the stained live cells are solubilized and the optical density quantified using a plate reader. The disadvantage of this assay is that the handling of live virus is still a necessity [79].

1.3.6 *Luminex-based microsphere immunoassay (MIA)*

Serologic assays have been developed that utilize xMAP technology (Luminex Corp., Austin, TX) [83]. This technology is based on the covalent

bonding of antigen or antibody to 5.6- μm -diameter polystyrene microspheres or beads [92]. Detection methods include two lasers to elicit fluorescence of varying wavelengths. The first laser excites the fluochrome mixture intrinsic to the microspheres, enabling the bead identity to be determined as the beads pass single file through the laser path in the flow cell. The second laser excites the extrinsic fluochrome (R-PE) that is covalently attached to the secondary antibodies. The dual lasers allow the operator to mix beads with different antigens together in a single well of a 96 well plate, thus enabling multiplex analysis of different antibody specificities at one time. Orange and red fluorescence are used for microsphere classification, and green fluorescence is used for analyte measurement. These Luminex-based microsphere immunoassays (MIA) are more rapid, sensitive, more cost effective as compared to traditional ELISA techniques and allows multiplex analysis to detect against multiple antigens in a single tube [4].

MIA are becoming increasingly popular as a serological option for the laboratory diagnosis of many diseases [93]. MIA has been also developed for detection of antibodies to specific human leukocyte antigens (HLA) [94]. Multiplexed MIAs for quantitation of various cytokines, chemokines and growth factors have been developed and used extensively [95]. Diagnosis of infectious diseases requires testing for multiple antibodies. The xMAP technology has been used for detection of antibodies against various viral and bacterial diseases such as a panel of seven respiratory viruses, including influenza A and B viruses; adenovirus; parainfluenza viruses 1, 2, and 3; and respiratory syncytial virus [96],

simultaneous measurement of antibodies to 23 pneumococcal capsular polysaccharides (PnPs) [97] and multiplexed detection of serotype-specific *Streptococcus pneumoniae* antigen in urine samples [98]. In addition, the Luminex technology was used to simultaneously measure antibodies to HIV-1 p24, gp160, and gp120 eluted from dried blood-spot specimens from newborns[99]. A multiplex assay was also developed for detection of strain-specific antibodies against the two variable regions of the G protein of respiratory syncytial virus (RSV), a single most important lower respiratory tract pathogen of infants and young children worldwide [100].

Recently, MIA platform also has been used for the detection of anti-WNV E-protein IgM and IgG antibodies both in human serum and CSF samples [92, 101]. It involves the covalent coupling of WNV E-protein to the microspheres and can be completed within 2.5 h. However, this assay cannot differentiate anti-WNV antibodies from other anti-flavivirus antibodies. The WNV NS5 protein-based MIA can differentiate between WNV infections and dengue virus or SLE virus [101] infections and can also be used for the differentiation between flavivirus vaccination and natural WNV-infection and between recent and old infections. This technique has been further developed for the detection of anti-WN and anti-SLE IgM [102]. It is based on the reactivity of serum antibodies to antigen captured by the anti-E monoclonal antibody, 6B6C-1 [102]. Many laboratories in the USA have adopted this assay for WNV diagnosis [103]. One major disadvantage of the MIA platform is the requirement for expensive, specialized equipment.

1.4 Nucleic acid based tests for detection of flaviviruses

Nucleic acid based amplification assays (NAAT) offer a sensitive and relatively rapid method for detection and identification of flaviviruses than virus isolation methods. NAAT assays can detect flaviviruses during the acute/viremic phase prior to antibody production and also can provide virus quantitation which may be an important predictor of disease severity [7, 104]. These tests can also be used to complement IgM testing in the diagnosis of acute flavivirus infection if the patient plasma sample is tested within the first few days of clinical illness [8].

1.4.1 Reverse transcriptase PCR (RT-PCR)

The reverse transcriptase PCR (RT-PCR) is rapid and sensitive assay for early detection of flaviviruses. It involves the amplification of target nucleic acid sequences. The PCR assay routinely used for identification of dengue virus is the nested RT-PCR assay developed by Lanciotti et al. [105]. This involves a two step PCR reaction involving an initial reverse transcription and amplification step using universal dengue primers targeting a region of the virus genome (C-PrM) followed by a second amplification that is serotype specific. Dengue serotypes are identified by the size of their bands observed in agarose gel electrophoresis. Nested RT-PCR also has been used for high-throughput detection of WNV RNA [106]. Though these methods are sensitive, they cannot be used for large scale testing as they require multiple handling steps and thus increase the risk of false-positive results due to amplicon contamination.

1.4.2 Real-time RT-PCR

The real-time RT-PCR assay is rapid and reliable virus detection assay from a wide variety (tissues and animal species) of samples. It can be used for large-scale diagnosis [107]. It is a one step assay system using primer pairs and probes that are specific to the respective virus. The use of a fluorescent probe enables the detection of the reaction products in real time without need for electrophoresis. Many real-time RT-PCR assays have been developed either as 'singleplex' (only detecting one serotype at a time) or 'multiplex' (able to identify all four serotypes from a single sample [108]. The multiplex assays for detection of dengue virus have the advantage that a single reaction can be used to determine all four serotypes without the potential for introduction of contamination during manipulation of the sample [109]. However, the fourplex real-time RT-PCR assays are often less sensitive than nested RT-PCR assay but are faster. A TaqMan qRT-PCR has been developed for rapid detection of WNV in human clinical specimens [110]. Compared to the traditional RT-PCR assay, this TaqMan RT-PCR was more sensitive and could detect less than 1 PFU of virus whereas the traditional RT-PCR had a detection limit of 1 PFU of virus [110]. Since 2003, real-time RT-PCR has been used for screening of blood donors in the USA [110]. About 30 million blood donations have already been tested, out of which 1500 were detected positive [107]. It is also used as a screening method for large-scale surveillance of mosquitoes/birds in the USA [111]. An advantage of real-time RT-PCR assay is the ability to determine viral load in a given sample, which is important in determining the severity of dengue disease [18]. However, false-negative results can occur in real-time RT-PCR due

to a mismatch in sequences between the amplimer and probe and also due to the continual evolution of the viral RNA [108].

1.4.3 Nucleic acid-sequence based amplification assay (NASBA)

Nucleic acid sequence-based amplification (NASBA) is a single-step isothermal RNA-specific amplification process which involves the use of silica to extract nucleic acid which is then amplified without thermocycling [112]. The amplified product is then detected by electrochemiluminescence (ECL). The NASBA assay has been successfully used for the detection of a number of RNA viruses including WNV, dengue and SLE viruses [112, 113]. Lanciotti et al. developed the NASBA assays for the detection of WNV and Saint Louis encephalitis (SLE) [113, 114]. With these newly developed NASBA assays, a diagnosis can be made within a hour and their sensitivity is similar or even greater than the sensitivity of their previously developed TaqMan assay [66].

1.5 Antigen detection

The flavivirus non-structural protein NS1 is a highly conserved, ~48-kDa glycoprotein that is essential for viral replication and viability [2]. It exists in the cell as a heat-labile homodimer that associates with cellular organelle membranes and is transported to the mammalian cell surface where it is vulnerable to immunological recognition. NS1 is also secreted by flavivirus-infected mammalian cells as a soluble hexamer [115]. The presence of secreted NS1 (sNS1) in the bloodstream stimulates a strong humoral response and anti-NS1 antibodies are generated.

Many studies have investigated the utility of sNS1 antigen detection as a diagnostic tool during the acute phase of a dengue infection [116]. NS1 antigen can be detected in patient serum 1-9 days after the onset of fever [117]. However, antigen detection in the acute stage of secondary infections can be compromised by pre-existing virus-IgG immunocomplexes [116]. *In vivo*, NS1 protein has been detected in high amounts in sera of patients infected with WNV and dengue virus [118, 119]. Increased level of soluble NS1 (free sNS1) in serum have been shown to correlate with viremia levels, and disease severity in dengue virus-infected patients. [120]. Microvascular leakage in such patients has been linked to complement activation by NS1-antibody complexes [121]. Similarly, NS1 antigen has also been detected early during the course of experimental WNV infection in hamsters where NS1 abundance also correlates with disease severity [118] .

Currently, several NS1 antigen detection commercial kits are now available. Many investigators have evaluated these assays for sensitivity and specificity [122-124]. The sensitivity observed for these assays varied from 63% to 94%. Additional independent studies are needed to confirm the performance of these kits and to further validate these assays for NS1 antigen detection. Serotype-specific MAb-based NS1 antigen-capture ELISA has recently been developed and shows good serotype specificity [125]. This test can differentiate between primary and secondary dengue virus infections. There is a good correlation between NS1 serotype-specific IgG as determined by ELISA and plaque reduction neutralization test (PRNT) results, but the performance and

utility of these NS1-based tests require additional evaluation. Furthermore, the advantage of NS1 antigen detection based assays is that they may also be useful for differential diagnostics between flaviviruses because of the specificity of the assay [17].

1.6 Quality control and validation parameters for diagnostic assays

Several rapid diagnostic kits are commercially available and many in-house assays have been developed for detection of flaviviruses [17, 126]. However, the performance characteristics of many of these tests have not been adequately evaluated. Laboratory-based evaluations using archived serum panels can be used to determine the ability of these assays to detect flaviviral infections compared to an existing methods or gold standard techniques [116] [126]. The parameters used to compare different serologic tests as well as NAAT assays are sensitivity, specificity and predictive values (Figure 5). The specificity and sensitivity of these assays are important for accurate diagnosis of specific flaviviral infections with respect to other infectious agents that often co-circulate in a endemic region [116].

A diagnostic assay should accurately classify serum samples as positive or negative for a given infectious agent; unfortunately, for assays in the real world, there is an overlapping continuum of assay responses from negative to positive (Figure 5). It is possible to measure how well an assay classifies serum samples as positive or negative in a given population by calculating the assay's

diagnostic sensitivity (DSn) and diagnostic specificity (DSp). The diagnostic sensitivity (DSn) is the probability that an assay correctly identifies positive status (infected or diseased), and the diagnostic specificity (DSp) is the probability that an assay correctly identifies negative status (normal) [127].

		Infection status		
		Infected	Uninfected	
Test status	Positive	TP	FP	Positive predictive value $PPV = \frac{TP}{TP + FP}$
	Negative	FN	TN	Negative predictive value $NPV = \frac{TN}{FN + TN}$
		Diagnostic sensitivity $DSn = \frac{TP}{TP + FN}$	Diagnostic specificity $DSp = \frac{TN}{TN + FP}$	

Figure 5. A 2 × 2 contingency table used to classify serum samples and to assess population-specific assay performance characteristics (DSn and DSp) as well as the predictive values for the assay in the test population. DSn, diagnostic sensitivity; DSp, diagnostic specificity; FN, false negative; FP, false positive; NPV, negative predictive value; PPV, positive predictive value; TN, true negative; TP, true positive.

[Simmons, 2008 ILAR J 49 (2): 157-69]

The DSn and DSp of an assay vary according to where the assay cutoff is set. There are a variety of ways to determine assay cutoff values. The most common method for determining the cutoff values is the mean value obtained from a large number of known negative serum samples plus two or three standard deviations (SD). Samples with values below the cut off are considered negative, whereas those above the cutoff values are considered positive [128]. However, serological data do not typically follow a normal (Gaussian) distribution

pattern. For example, data from negative sera (Figure 6) are typically skewed to the right (positive skew)[127].

Another statistical method for determining assay cutoffs is to plot the data on a receiver operating characteristics (ROC) curve. On a traditional ROC curve, the true positive rate or diagnostic sensitivity (DSn) is plotted on the ordinate of a graph versus the false positive rate (1-DSp) on the abscissa i.e., the *benefit* of the true positive result is plotted versus the *cost* of a false positive result. Unfortunately, the results of the ROC curve can be difficult for the unfamiliar to interpret.

A final method of assay cutoff determination is empirical in which the data are plotted as a frequency distribution pattern and a line or lines are drawn to maximize the assay performance characteristics that are consistent with the results of other gold standard techniques. Many laboratories draw two cutoff lines, results to the left of line (A) are classified as negative, results to the right of line (B) are classified as positive, and results between the two lines are classified as intermediate or equivocal (Figure 6). Intermediate results could represent a response that is either a “noisy” negative or an early positive result. For samples classified as intermediate, either an additional sample obtained one to several weeks later or additional diagnostic testing using other assays on the original serum sample may help to determine whether the sample should be classified as positive or negative [127].

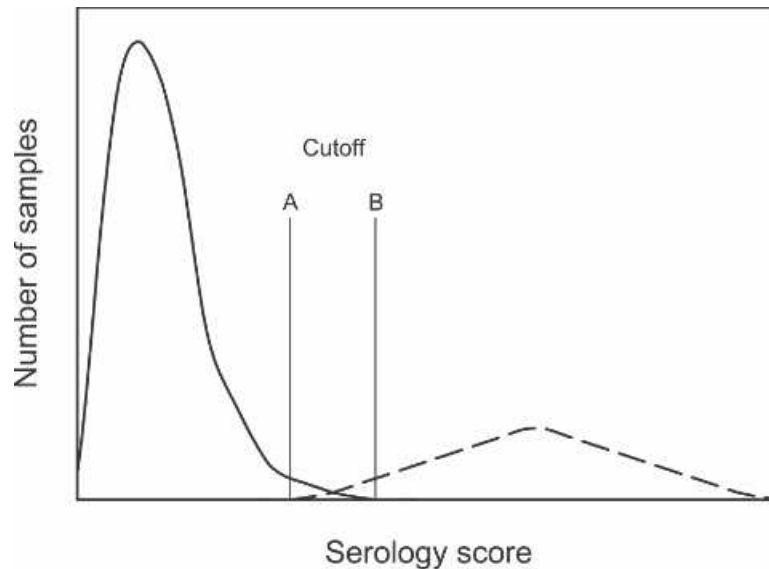


Figure 6. Frequency distribution for a hypothetical set of reference sera for serodiagnostic testing. The curve on the left represents known negative sera and the curve on the right, positive sera. An assay cutoff at line A would properly classify all positive serum samples (high diagnostic sensitivity, or D_{Sn}), and at line B would properly classify all negative serum samples (high diagnostic specificity, or D_{Sp}).

[Simmons, 2008 ILAR J 49 (2): 157-69]

Quality control and quality assurance of newly developed diagnostic assays is also critical for the accurate diagnosis of flaviviral infections [17, 126]. Variation in parameters and conditions used in these assays are main concerns in the quality assurance and validation of laboratory diagnosis [129]. The goal of a quality assurance program is to enhance the confidence of both the laboratory and the consumer in the reported diagnostic test results. The reliability of these results depends on the constant monitoring of serodiagnostic assays during routine use to verify that they are performing within predetermined assay performance specifications. It is therefore useful to develop a quality assurance (QA) program that monitors as many steps as possible in reporting assay results,

including sample processing, sample- and assay-specific components, and data analysis and reporting. System and sample suitability controls may indicate that the results are valid but not necessarily that they are correct, as all assays are prone to both false-positive and false-negative results. To limit day-to-day variation in reported assay results, the diagnostic laboratory should implement a process for tracking and monitoring QC results over time [127].

The need for a validated diagnostic test for dengue virus infection for clinical and epidemiological use was recommended by the International Expert Meetings on dengue diagnostics held at the WHO in Geneva in October 2004 and April 2005 [116]. Assay validation is a formal process for determining the suitability of a given laboratory method for generating the data necessary to calculate assay performance characteristics [127]. Careful selections of positive and negative controls as well as the samples selected for testing are the most important factors in validation of diagnostic assays [126]. The data that result from assay validation are used to calculate population specific assay performance characteristics such as sensitivity and specificity (where the “population” is the samples collected for assay validation)[127].

Field trials are also needed to determine the performance and utility of diagnostic tests during epidemic situation. A complete analysis of the data should be provided to each commercial company that supplied the kit along with recommendations for any areas of improvement. In addition, the evaluation should be published in a peer-reviewed journal as well as a WHO bulletin to allow other public health and private laboratories that perform these testing to

access this information for future testing. Recommendations should include acceptable sensitivity and specificity values for a given test [17].

1.6 References

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Chapter 2

Thesis Scope

2.1 Background and research question

Luminex-based assays can be used for rapid laboratory diagnosis of several diseases [1]. Luminex-based microsphere immunoassays (MIA) have been developed and used for detection of anti-WNV antibodies in humans [2, 3] and mice model [4, 5] using purified recombinant proteins (E, NS3 and NS5) of WNV. WNV E-MIA is sensitive, cost-effective and requires less time than traditional ELISA and PRNT assays for detection of anti-WNV antibodies [2, 3]. However, several assay parameters such as serum heat-inactivation (HI), serum dilution and removal of interfering IgG can alter the sensitivity of WNV E-MIA. Therefore, optimization of newly developed MIAs is important, otherwise it can give false-positive or false-negative results and high levels of non-specific background with low dynamic range of detection [6]. In our laboratory, Luminex-based DENV microsphere immunoassay (MIA) was developed for detection of dengue IgM and IgG antibodies. Similarly, we also have in-house developed Luminex-based Polymerase Chain Reaction-Microsphere Bead Assay (PCR-MBA) for detection of four DENV serotypes and other arboviruses. Validation and optimization of these assays for improved sensitivity and specificity is critical for accurate diagnosis of DENV infection.

2.2 Objective and Hypothesis

The **objective** of this study is to develop, optimize and validate the rapid, specific and sensitive Luminex-based assays for diagnosis of WNV and DENV infections. Based on our preliminary data and published literature, we **hypothesize** that in-house PCR-MBA and optimized WNV E-MIA as well as in-house DENV MIA for detection of WNV and DENV infections will be rapid, specific and sensitive as compared to traditional serological and nucleic acid based assays such as PRNT, ELISA, and qRT-PCR, respectively.

2.3 Specific Aims

2.3.1 Specific Aim 1: To develop and optimize the Luminex-based WNV E-MIA for rapid, specific and sensitive detection of anti-WNV IgM and IgG antibodies in mice.

Gap: Immunopathogenesis studies employing WNV mice model are important for the development of antivirals and vaccines against WNV. Since, antibodies produced in mice early during WNV infection are essential for clearing virus from the periphery, it is important to detect early and persistent anti-WNV IgM and IgG antibodies. Several assay parameters such as serum heat-inactivation (HI), serum dilution and removal of interfering IgG from serum can alter the sensitivity of WNV E-MIA for detection of low-titer anti-WNV IgM and IgG antibodies in mice.

Hypothesis: We *hypothesize* that serum heat-inactivation (HI) will improve the sensitivity of WNV E-MIA for detection of anti-WNV IgM and -IgG antibodies in mice and optimal serum dilution will provide low background signal and high

dynamic range of detection. Furthermore, interfering IgG removal will improve the sensitivity of WNV E-MIA for detection of anti-WNV IgM antibodies.

Rationale: The effect of serum heat-inactivation, serum dilution and interfering IgG removal from serum on the sensitivity of Luminex-based WNV E-MIA for detection of anti-WNV IgM and IgG antibodies in mice has not been studied.

Objective: To develop and optimize the Luminex-based WNV E-MIA for detection of early and persistent anti-WNV IgM and IgG antibodies in mice.

Approach:

- i. Couple the magnetic microspheres with the WNV E-protein (L2 diagnostics) using standard Luminex protocol and develop the WNV E-MIA for detection of anti-WNV IgM and IgG antibodies from mice sera.
- ii. Optimize the WNV E-MIA by studying the effect of serum heat-inactivation (HI), serum dilution and removal of interfering IgG from serum on the sensitivity of the WNV E-MIA for detection of anti-WNV IgM and IgG antibodies.
- iii. Confirm the complement interference in WNV E-MIA by adding exogenous complement to HI serum and further verify it by re heat-inactivating the HI serum with complement.
- iv. Use the optimized WNV E-MIA to study the anti-WNV IgM and IgG response in mice models such as diabetic and ASC knockout mice as well as to detect anti-WNV IgG antibodies in mouse hybridomas.

2.3.2 Specific Aim 2: To validate and optimize the newly developed in-house DENV MIA for detection of dengue-IgM and IgG antibodies in human serum.

Gap: Our laboratory has developed Luminex-based DENV MIA for detection of dengue-IgM and -IgG antibodies. Further validation using dengue positive serum samples from different patient cohorts is critical for assessing the sensitivity and specificity of the newly developed DENV MIA for accurate diagnosis of dengue infection. Moreover, further optimization of DENV IgM MIA is important for improved specificity and sensitivity of this newly developed MIA.

Hypothesis: Based on the published data and our preliminary data, we *hypothesize* that the sensitivity and specificity of DENV IgM MIA can be further improved by using synthetic blockers such as polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP).

Rationale: Non-specific binding can occur in DENV MIA as a result of insufficient blocking and thus can lead to decreased specificity for detection of dengue IgM and IgG antibodies. This problem can be addressed by using synthetic blockers such as PVA and PVP which, are currently used to conduct various MIAs [7, 8].

Objective: To validate the newly developed in-house Luminex-based DENV MIA using serum samples from different cohorts (Hawaii, Vietnam and Niue) and to further optimize the DENV MIA for improved sensitivity and specificity for detection of dengue-IgM and IgG antibodies in human serum.

Approach:

- i. Conduct DENV MIA on serum samples collected in Hawaii, Vietnam and Niue. Confirm some of these samples by IgM Capture ELISA and PRNT assays.
- ii. Confirm IgM false-positive results in DENV MIA by using FDA approved InBios DENV Detect™ IgM Capture ELISA kit.
- iii. Evaluate InBios DENV Detect™ IgM Capture ELISA as compared to in-house MAC-ELISA for diagnosis of acute dengue virus infection.
- iv. Serum from syphilis patients containing syphilis antibodies demonstrated high frequency of false-positive results in rWNV E-MIA [3]. Conduct syphilis IgG EIA using CAPTIA Syphilis IgG kit (Trinity biotech) on Vietnam samples that demonstrated IgM false-positive results in DENV MIA to investigate if the syphilis antibodies are interfering in DENV MIA.
- v. Use synthetic blockers such as PVA and PVP to eliminate the IgM false-positive results in DENV MIA and improve the specificity and sensitivity of DENV IgM MIA.
- vi. Optimize DENV MIA using assay parameters such as serum dilution, concentration of PE conjugate antibody and using StabliGuard (Surmodics Inc.) as BSA-free alternative blocker during washing and storage of beads coupled with DENV antigen.

2.3.3 Specific Aim 3: To validate the newly developed in-house polymerase chain reaction-microsphere bead assay (PCR-MBA) for detection of West Nile virus (WNV) and dengue virus (DENV) serotypes.

Gap: Our laboratory has developed Luminex-based PCR-MBA for differential detection of WNV, DENV serotypes and other arboviruses. Further validation of PCR-MBA is critical to assess the sensitivity and specificity for detection of WNV and four DENV serotypes.

Hypothesis: Based on our preliminary data, we *hypothesize* that PCR-MBA will efficiently detect WNV and four DENV serotypes and will be a rapid, specific and sensitive alternative to currently available qRT-PCR techniques for early detection of WNV and DENV serotypes.

Rationale: Luminex-based assays available for rapid, specific and sensitive detection of WNV are still under development and no Luminex-based assay is available for differentiation of DENV serotypes. Multiple primer sets used in fourplex qRT-PCR assays for detection of DENV serotypes, tend to increase the false-positive results.

Objective: To determine the sensitivity and specificity of PCR-MBA for detection and differentiation of WNV and DENV serotypes respectively, as compared to qRT-PCR.

Approach:

- i. Dilute WNV-cDNA of known titer serially (10 fold dilutions) from 3×10^7 PFU to 0.3 PFU and conduct WNV-specific qRT-PCR and PCR MBA. Analyze the WNV qRT-PCR and PCR-MBA data to assess the sensitivity and specificity of PCR-MBA for WNV detection.
- ii. Extract RNA from serum samples positive for DENV 1 to 4 serotypes (10 samples for each serotype), proficiency panel specimens, Limit of

detection (LOD) panel samples received from CDC (San Juan) and conduct the PCR-MBA and CDC singleplex Quanta qRT-PCR. Analyze the PCR-MBA and qRT-PCR data to assess the sensitivity and specificity of PCR-MBA.

2.3 Significance and Impact

West Nile virus (WNV) and Dengue virus (DENV) are classified as significant human pathogens of global epidemiological importance [9-13]. To date, there is no vaccine or specific antiviral treatment for WNV and dengue infections in humans [14, 15]. Dengue virus infection is a serious health problem in tropical and subtropical areas of the world [11]. Effective treatment of the patients with dengue fever and dengue hemorrhagic fever depends on rapid diagnosis [16]. Since its introduction into the United States in 1999, WNV has spread throughout most of the country and has caused major epidemics of neuroinvasive disease [12]. Serologic-based assays as well as various nucleic acid amplification technology (NAAT) based assays can be used for diagnosis of flavivirus infections in human [16-21]. Current serological methods for detection of flaviviral infections include hemagglutination inhibition antibody test (HAI), IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA), and plaque reduction neutralization test (PRNT)[19]. These serological assays may take over three weeks to generate a final result. Various NAAT assays have been developed for detection of DENV serotypes and WNV such as RT-PCR [22, 23], TaqMan real-time RT-PCR [20, 24, 25] and nucleic acid sequence-based

amplification [26, 27]. Though these assays are sensitive, there is chance of nonspecific amplification as multiple primer sets are used and thus can result in false-positives. Moreover, these assays are labor intensive and require high technical expertise. The development of specific and sensitive Luminex-based diagnostic assays for detection of flavivirus infections will be valuable for early diagnosis and thus it will greatly enhance the ability to treat diseases caused by these viruses [19]. Further optimization of Luminex-based assays for detection of low titer anti-WNV antibodies and low levels of WNV will also provide better understanding of virus and antibody dynamics in humans as well as animal models [28]. Detection of these early and persistent IgM and IgG antibodies during WNV infection is important to study WNV immunopathogenesis in mice model that may further influence development of antivirals and vaccines against WNV infection in humans [28]. In addition, validation and optimization of in-house developed DENV MIA and PCR-MBA is critical for accurate diagnosis of DENV and WNV infections in humans. This optimized and validated Luminex-based assays for detection of WNV and dengue virus infections will be a rapid, specific and sensitive alternative to time-consuming ELISA and PRNT assays.

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Chapter 3

Development, optimization and validation of the Luminex-based assay for rapid, specific and sensitive detection of WNV infection in mice

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3.1 Abstract

Immunopathogenesis studies employing West Nile virus (WNV) mice model are important for the development of antivirals and vaccines against WNV. Since, antibodies produced in mice early during WNV infection are essential for clearing virus from the periphery, it is important to detect early and persistent anti-WNV antibodies. ELISA and plaque reduction neutralization test are traditionally used for detection of anti-WNV antibodies and WNV-neutralizing antibodies, respectively. Although these assays are sensitive and specific, they are expensive and time consuming. Microsphere immunoassays (MIA) are sensitive, specific, allow for high throughput, are cost effective, require less time to perform than other methods, and require low serum volumes. Several assay parameters such as serum heat-inactivation, serum dilution and removal of interfering IgG from serum can alter WNV MIA sensitivity. We examined the effect of these parameters on WNV E-protein MIA (WNV E-MIA) for the enhanced detection of anti-WNV IgM and IgG antibodies. WNV E-MIA was conducted using serial dilutions of HI and non-HI (NHI) serum collected at various time points from mice inoculated with WNV. HI significantly enhanced detection of IgM and IgG antibodies as compared to NHI serum. WNV IgM and IgG antibodies in HI sera were detected earlier at day 3 and IgM antibodies persisted up to day 24 after infection. HI serum at 1:20 dilution was found to be optimal for detection of both IgM and IgG antibodies as compared to higher serum dilutions. Further, addition of exogenous complement to the HI serum decreased the WNV E-MIA sensitivity. Anti-mouse IgG treatment of mice serum did not improve the

detection of IgM antibodies. Altogether, these results suggest that serum-HI and optimal dilution enhance WNV E-MIA sensitivity by eliminating the complement interference, thereby detecting low-titer anti-WNV antibodies during early and late phase of infection. This improved MIA can also be readily employed for detection of low-titer antibodies for detection of other infectious agents and host proteins.

3.2 Introduction

West Nile virus (WNV), a mosquito-borne flavivirus that causes lethal encephalitis, has emerged as a significant cause of viral encephalitis in the United States [1]. Although, WNV infection in humans is mainly acquired after mosquito bite, human-to-human transmission can occur through blood transfusion, organ transplantation and breastfeeding [2,3]. Currently, no antiviral or vaccine is available to counteract or protect against WNV infection in humans [4]. WNV immunopathogenesis studies in animal models such as mice provide important information for the development of antivirals and vaccines against WNV infection in humans. In WNV-infected mice, IgM and IgG antibodies are produced early after the infection and persist for a long time. These WNV-specific antibodies limit viremia and dissemination of virus into the CNS and provide protection against lethal infection [5]. Induction of these antibodies is also a critical determinant for the efficacy of WNV vaccines [4]. Therefore, it is important to detect low levels of both anti-WNV IgM and IgG antibodies during early and late phase of the infection. WNV E-protein enzyme linked immunosorbent assay

(ELISA) and plaque reduction neutralization test (PRNT) have been used for detection of both anti-WNV IgM and IgG antibodies and WNV-neutralizing antibodies, respectively, in mice [6]. Though these assays are sensitive and specific, they are expensive and time consuming. Luminex-based microsphere immunoassays (MIA) have been developed and used for detection of anti-WNV antibodies in humans and in mice model using purified recombinant proteins (E, NS3 and NS5) of WNV [7-11]. WNV E-MIA is sensitive, cost-effective and requires less time than traditional ELISA and PRNT assays for detection of anti-WNV antibodies [7,8]. MIA has also been used for improved serological detection of several other viruses such as respiratory syncytial virus [12], HIV [13], WNV [7,8,14], human papillomaviruses [15], equine arteritis virus [16], and avian influenza virus [17].

Several assay parameters such as heat-inactivation (HI) of serum, serum dilution and presence of interfering IgG in serum can affect the MIA results. Heat-inactivation of serum at 56°C for 30 min is a standard procedure in diagnostic laboratories to conduct neutralization test for the purpose of inactivation of complement [18]. Complement components present in serum are known to react with multi-molecular immune complexes or immunoglobulin aggregates [19,20]. Serum heat-inactivation decreased the number of false-positives in multiplexed immunoassay for detection of antibodies against human papilloma viruses [15]. In another Luminex based assay for detection of human leukocyte antigen (HLA) antibodies, HI serum decreased the frequency of false-negative results by

eliminating the complement interference or prozone effect [21]. In contrast, HI of the cattle serum had little effect on the performance of the liquid array multiplexed assay for detection of antibodies against foot and mouth disease virus [22]. Two recent WNV persistence studies have employed WNV E-MIA to study the anti-WNV antibody response in the mice after infection [10,11]. In the first study, MIA was conducted using non heat-inactivated (NHI) sera [10], whereas in the second study, sera were HI at 56°C for 1 hour prior to testing [11]. These two studies detected total anti-WNV antibodies (IgG, IgA and IgM). Therefore, the effect of HI sera on the WNV E-MIA for detection of low-titer anti-WNV IgM and IgG during early and late phase of infection cannot be deduced. In addition to HI, serum dilution is another important parameter that can affect the sensitivity of MIA. Serum dilution, to some extent can eliminate the complement interference or prozone effect observed in the case of high antibody titers, thus can improve the sensitivity of MIA [21]. On the other hand increased serum dilution can lead to decreased sensitivity [23]. WNV E-MIA was conducted at 1:100 dilution of serum [8,10,11], whereas other MIA employed lower serum dilution (1:20), as it resulted in the low levels of non-specific background with high dynamic range of signal intensities [24,25]. In addition, immune IgG present in the serum more effectively competes with immune IgM for available binding sites on an antigen substrate. This can result in false-negative results in serum samples with low IgM titers [26]. Removal of murine IgG antibodies resulted in enhanced detection of allergen-specific mouse IgE by ELISA [27]. IgG antibodies were also removed from human serum samples in Luminex-based MIA for

detection of anti-flavivirus IgM antibodies [8,28]. Therefore, HI of serum, serum dilution and presence of interfering IgG in serum are the critical parameters that can affect MIA results and thus should be considered as important part of the development and optimization of MIA.

In this study, we examined the effect of serum HI, serum dilution and removal of interfering IgG from serum on the sensitivity of WNV E-MIA for the detection of anti-WNV IgM and IgG antibodies in mice.

3.3 Materials and Methods

Ethics statement

This study was specifically approved by the University of Hawaii Institutional Animal Care and Use Committee (protocol number 10-948) and conducted in strict accordance with animal use protocols in the animal biosafety level-3 laboratory. Mice that exhibited severe disease were euthanized to limit suffering.

WNV infection

Nine-week old C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbour, ME). All infection experiments were conducted using the lineage I WNV strain (NY 99) as described previously [29]. Lineage I WNV strain NY99 used in all experiments was originally isolated from a crow in New York and further propagated in Vero cells as described previously [30]. Mice were inoculated via the footpad route with 100 PFU of WNV or with PBS (mock). On days 0, 3, 6, 8,

10 and 24 after infection, 100 μ L blood was collected from tail vein, from which serum was separated and frozen at -80°C for future analyses.

Coupling of microspheres with rWNV-E antigen

Magnetic carboxylated microspheres (MagPlexTM-C) were obtained from Luminex Corporation (Austin, TX, USA). A two-step carbodiimide process recommended by Luminex Corporation (Austin, TX.) was used to link 10 μ g of purified rWNV-E (L2 Diagnostics) to the surface of 1.25×10^6 microspheres as described previously [8]. *Drosophila* S2 expression system was employed by L2 Diagnostics to make the rWNV-E antigen [31]. The antigen-conjugated microspheres were stored in 250 μ L of PBN buffer (PBS with 1% bovine serum albumin Fraction V, OmniPur, and 0.05% Ultra sodium azide, Sigma Aldrich) at 4°C .

WNV E-MIA

Serum samples were diluted in PBS-1% BSA. A total of 50 μ L of PBS-1% BSA containing approximately 1250 coupled microspheres was added to each well of a flat-bottom 96-well plate. 50 μ L of diluted serum was added to the beads and incubated for 30 min in the dark. The plates were then washed twice with 200 μ L of PBS-1% BSA and 50 μ L of diluted red-phycoerythrin (R-PE) conjugated secondary antibody (2 μ g/mL) was added to test wells and incubated for 45 min in the dark. The plates were washed twice with 200 μ L of PBS-1% BSA. Microspheres were then resuspended in 100 μ L of PBS-1% BSA per well and

incubated for 5 min before analysis on the Luminex 100 machine (Qiagen, Valencia, CA). The median fluorescence intensity (MFI) was quantitated for 100 microspheres and recorded for each well. The secondary antibodies used were R-PE conjugated F(ab')₂ fragment goat anti-mouse IgG, Fcγ fragment specific and R-PE conjugated F(ab')₂ fragment goat anti-mouse IgM, μ chain specific (Jackson ImmunoResearch, West Grove, PA). All assays were done in duplicate and all incubations were conducted on a plate shaker at 700 rpm and the wash steps were conducted using 96-well magnetic plate separator (Millipore Corp., Billerica, MA).

To study the effect of HI on the sensitivity of WNV E-MIA, serum samples were HI at 56°C for 30 min in water bath. To optimize serum dilution, WNV E-MIA was conducted using HI and NHI mice sera serially diluted from 1:20 to 1:160 using PBS-1% BSA.

Cutoff determinations

Cutoff values were calculated as the average MFI of 25 serum samples from mock-infected C57BL/6 mice plus three standard deviations (Microsoft® Office Excel). Negative cutoff values for HI and NHI serum for IgG MFI were 131 and 85, and for IgM MFI were 366 and 314, respectively. Serum samples with MFI values greater than the cutoff were considered positive. Control beads were coupled with 1X PBS instead of WNV E-protein using the aforementioned coupling protocol to check for nonspecific attachment of serum proteins to the microspheres. Control beads mean IgG MFI for HI and NHI serum samples at

1:20 dilution were 18 and 11, and the IgM MFI were 27 and 63, respectively, which suggests that the nonspecific attachment of the serum proteins to the microspheres was minimal.

Complement addition to HI serum

HI serum was diluted 1:20 in PBS-1% BSA and 4 U of reconstituted guinea pig complement (C') (Sigma-Aldrich) was added to 240 μ L of diluted serum. In a separate group, HI serum after addition of C' were again heat-inactivated at 56° C for 30 min to inactivate the complement. HI serum without C', HI serum with C' and re-heat-inactivated HI serum with C' were tested by WNV E-MIA for detection of anti-WNV IgM and IgG antibodies.

Plaque reduction neutralization test (PRNT)

Serum samples used for conducting MIA were also used to screen for anti-WNV neutralizing antibodies by PRNT. Both NHI and HI sera were diluted serially from 1:20 to 1:160 and PRNT was conducted by using lineage I WNV strain (NY99), as described previously [30,32].

Anti-mouse IgG treatment of serum for removal of interfering IgG

For IgG depletions, twelve microliters of serum was mixed with 12 μ L of rabbit anti-mouse IgG (H+L) or goat anti-mouse IgG (Fc fragment specific) (2.5 mg/ml) (Code # 315-001-003, and 115-001-008, Jackson ImmunoResearch Laboratories, West Grove, PA.) followed by addition of 24 μ L of phosphate-

buffered saline (PBS) and centrifugation for 4 min at 14,000 rpm to remove antibody-bound IgG [8,26]. These pretreated serum samples were used in WNV E-MIA at final dilution of 1:20 using PBS-BSA.

Statistical analysis

All data are reported as mean MFI \pm standard deviation of at least two independent experiments conducted in duplicate using GraphPad Prism 5.0 software.

3.4 Results and Discussion

HI of serum enhanced the detection of anti-WNV IgM and IgG antibodies in mice

First, we examined the effect of HI of serum on the WNV E-MIA using both NHI serum and HI serum at 1:20 dilution. Using HI serum anti-WNV IgM first appeared at day 3 peaked at day 8 and then gradually decreased at days 10 and 24 after infection (Figure 1A). Similarly, anti-WNV IgG first appeared at day 3 and then demonstrated gradual increase up to day 24 after infection (Figure 1B). HI of serum enhanced the detection of anti-WNV IgM and IgG antibodies as there was approximately 2 to 10 fold increase in IgM and IgG MFI in HI serum as compared to NHI serum. WNV E-MIA optimized in this study is much more precise than traditional ELISA methods used for detection of anti-WNV antibodies in mice wherein anti-WNV IgM was first detected at day 4 after

infection and anti-WNV IgG was first detected at day 6 after infection [6]. These WNV-specific ELISA methods were also conducted using HI serum [33]. In addition, two WNV studies using animal models [11,34] demonstrated persistence of IgM antibody secreting cells or IgM antibodies until day 14 and 18 after infection in mice [11] and hamster [34], respectively. Similarly, persistence of WNV IgM antibodies was demonstrated in patients with WNV encephalitis for more than a year [35,36]. Our results demonstrate that IgM antibodies persist in WNV-infected mice until day 24 after infection, albeit at very low level suggesting that HI of serum enhances WNV E-MIA for the detection of anti-WNV IgM and IgG antibodies in mice. This increase in the MFI may be primarily due to the elimination of complement interference present in the serum as a result of heat treatment at 56°C for 30 min. However, heat treatment of samples may also change the accessibility of the antibody epitopes in a complex serum sample, thus can improve the performance of MIA [37]. In addition, previous studies using mouse models have employed polyclonal secondary antibody, which detect total anti-WNV antibodies (IgM, IgG, IgA) [10,11]. Whereas, in this study we employed monoclonal antibodies to independently detect anti-WNV IgM and IgG antibodies.

MIA data can be represented using various methods [7,8,10,11,38]. Signal-to-noise ratios is a common method of presenting data, where negative control sera are used as the denominator [8], or where test sample reacted on a control antigen is used as a denominator [38]. Often higher dilutions than those reported

by us are advantageous and may give better results for detection of WNV antibodies in human [8,14,38]. However, using this method false-negatives could potentially exist because non-specific reactions with the negative control antigen could generate artificially low numbers in the data transformation scheme [38]. Since detection of WNV antibodies using MIA in mice used MFI for data representation [10,11], we used this method to represent our data for comparison with existing data on detection of anti-WNV antibodies in mice.

To confirm the presence of anti-WNV neutralizing antibodies in serum from WNV-infected mice after heat-inactivation and dilution, we conducted PRNT on the same serum samples used for MIA using both NHI and HI sera diluted serially from 1:20 to 1:160. As expected, high levels of anti-WNV neutralizing antibodies were detected at days 6, 8, 10 and 24 after infection. PRNT, even though it is specific, did not detect very low levels of antibodies produced at day 3 after infection (Figure S1 A and B). However, WNV E-MIA detected these very low levels of antibodies produced early during infection (Figure 1).

Serum dilution enhances detection of anti-WNV IgG, but not IgM antibody

Another important parameter that can affect the performance of Luminex-based serological assays is the dilution of the serum. Serum dilution, though it contributes to low background signal, can also lead to decrease in sensitivity of the Luminex assay [23] and false-negatives could potentially exist [38]. In the previous studies, WNV E-MIA was conducted at a serum dilution of 1:100 as it

provided low background binding and optimal assay results [8,10,11,39].

However, these higher serum dilutions might have also decreased the sensitivity as well. Therefore, to determine the optimum serum dilution for WNV E-MIA, NHI and HI sera were serially diluted from 1:20 to 1:160 and anti-WNV IgM and IgG antibodies were detected. IgM MFI decreased with increase in serum dilution of NHI serum at day 6 after infection. However, there was very little increase in IgM MFI for higher dilutions (1:40 and 1:80) of NHI serum at days 8 and 10 after infection. No IgM was detected at days 3 and 24 after infection at any dilution of NHI serum (Figure 2A). In contrast, IgM MFI of HI serum at various time-points after infection was higher at 1:20 dilution, which gradually decreased with increase in serum dilution. HI serum at 1:20 dilution demonstrated improved sensitivity for IgM detection, particularly at days 3 and 24 after infection, where the IgM antibody levels are usually very low (Figure 2B). IgG MFI for NHI serum increased with increase in serum dilution at days 8, 10 and 24, while it was decreased at day 6 after infection (Figure 2C). Similar to IgM, IgG MFI for HI serum was highest for 1:20 dilution and then gradually decreased at 1:40, 1:80 and 1:160 dilutions (Figure 2D). Interestingly, IgG MFI obtained for higher dilutions (1:80 and 1:160) of the NHI serum at days 8, 10 and 24 are almost similar to that obtained for 1:20 and 1:40 dilutions of HI serum. Thus dilution of NHI serum enhanced the detection of anti-WNV IgG antibodies, however, the same effect was not observed for IgM antibodies. This observed change in MFI may be due to the prozone effect or high-dose hook effect as a result of high antibody titers in serum or the complement interference in the NHI serum.

Prozone effect and complement interference in the immunoassays can be eliminated by sufficient dilution of the serum [23,40], but this can also lead to decrease in sensitivity of the assay, as observed for IgM antibodies (Figure 2A). Therefore, we determined 1:20 to be the optimal dilution of HI serum for WNV E-MIA that can detect low-titer IgM and IgG antibodies during early and late time-points after infection.

Complement interferes in WNV E-MIA

To validate the role of complement interference in WNV E-MIA, we added exogenous guinea pig complement to the 1:20 dilution of the HI serum and then conducted WNV E-MIA. Addition of complement resulted in decrease in both IgM and IgG MFI at days 3, 6, 8, 10 and 24 after infection (Figure 3A and 3B). Surprisingly, IgM and IgG MFI obtained after complement addition to HI serum were almost similar to those obtained for NHI serum from WNV-infected mice (Figure 1). Further, to test if re-heat inactivation of HI serum with complement will restore the MFI values we re-heat-inactivated HI serum with complement and conducted WNV E-MIA. Interestingly, IgM MFI after heat-inactivation of HI serum with complement at days 8, 10 and 24 was increased as compared to non heat-inactivated HI serum with complement, but it did not restore completely, and remained unchanged for days 3 and 6 after infection (Figure 3A). Similarly, IgG MFI after heat-inactivation of HI serum with complement was increased at days 8, 10 and 24 after infection as compared to non heat-inactivated HI serum with complement (Figure 3B). However, in contrast to IgM, IgG MFI was restored after

heat-inactivation of HI serum with complement. The possible reason for this difference may be due to aggregation of IgM antibodies as a result of prolonged heat treatment at 56° for 1 hr. Mouse IgM antibodies are more sensitive to heat treatment whereas IgG1 and IgG2a antibodies are relatively heat-resistant [41]. Collectively, these results suggest that complement interferes in WNV E-MIA, thus causing the false-negative or false-low positive results. In addition, these results confirm the role of complement in causing the prozone effect in high antibody titer serum samples as fold increase in IgM and IgG MFI was higher for HI serum at days 8, 10 and 24 compared to NHI serum (Figure 1A and 1B). Similarly, Schnaidt et al [21] demonstrated that complement component C1 can cause prozone effect in the case of high antibody titers in Luminex based assay for HLA antibody detection. Complement can interfere in Luminex assays by binding to two or more closely spaced antibodies immobilized on a solid surface, which is only possible in the case of high antibody titers, but not with low antibody titer serum samples [21]. This complement binding blocks the Fc portion of antigen-specific antibodies and competitively prevents the binding of secondary antibodies, eventually giving false-negative results [21,23,42].

Anti-mouse IgG treatment of serum did not improve detection of anti-WNV IgM antibodies

To improve the sensitivity of WNV E-MIA for detection of anti-WNV IgM antibodies, we studied the effect of IgG depletion from mouse serum. IgG from the NHI and HI serum samples were removed using anti-mouse IgG (H+L) or Fcγ

fragment specific antibodies. Even after anti-mouse IgG treatment, IgG antibodies from the serum samples were not removed; instead IgG-depleted serum samples demonstrated increase in IgG MFI. (Figure S2 B, D and F). It is difficult to speculate the exact cause of this increased IgG MFI after anti-mouse IgG treatment of serum. One hypothesis for this increase in IgG MFI may be due to the formation of immunoglobulin aggregates as a result of anti-mouse IgG treatment [26]. Anti-mouse IgG (H+L) treatment of the NHI serum resulted in increase the IgM MFI at days 8, 10 and 24 after infection (Figure S2 A), but it was still similar to IgM MFI obtained for 1:20 dilution of HI serum. Anti-mouse IgG (Fc γ fragment specific) treatment of the NHI serum demonstrated increase in IgM MFI at day 8 after infection, but it was very low compared to IgM MFI obtained for HI serum at similar 1:20 dilution (Figure S2 E). HI serum after anti-mouse IgG (H+L) treatment did not show any increase in IgM MFI in WNV E-MIA in mice (Figure S2 C). In WNV-MIA developed by Wong *et al.*, (2004), depletion of IgG from human serum prior to testing did not improve the sensitivity for IgM detection. Similarly, in our study, IgG depletion from serum did not significantly improve the sensitivity of WNV E-MIA for detection of anti-WNV IgM antibodies in mice.

3.5 Conclusions and Significance

This study for the first time demonstrates that HI of the serum contributes to enhanced detection of anti-WNV IgM and IgG antibodies using MIA. The WNV E-MIA optimized in our laboratory is robust, sensitive and high throughput assay for

detection of anti-WNV IgM and IgG antibodies in mice, particularly at early and late time-points after WNV infection in mice. These findings led us to include a HI step of 56°C for 30 min and 1:20 as optimum serum dilution, as part of our sample preparation procedures for conducting WNV E-MIA in mice. These data strongly suggest that HI of serum and optimized serum dilution should be considered as one of the important parameter during development and optimization of other Luminex-based MIA. In conclusion, the significance of this study is that the optimized WNV E-MIA can be used to study WNV-specific IgM and IgG antibody response in various mice models [43,44] (Figure S3) as well as to detect the WNV-specific IgG antibodies in mouse hybridomas (Table 1)

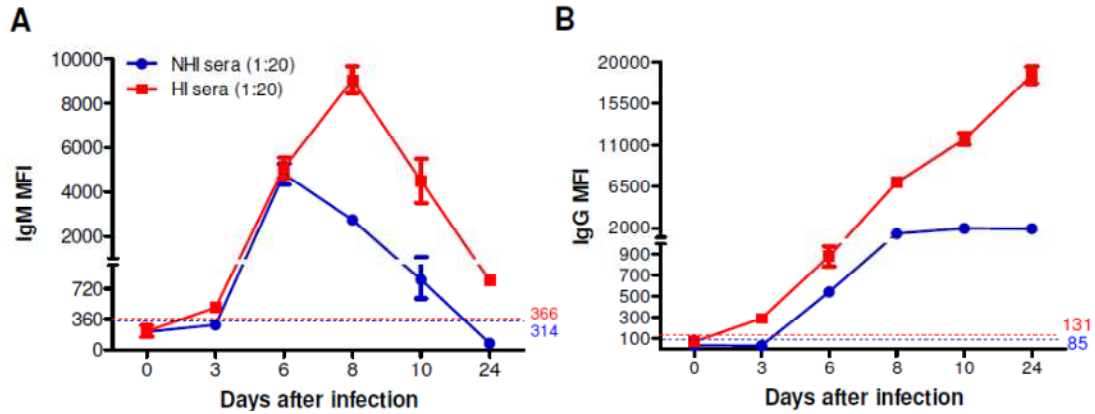


Figure 1. Effect of serum heat-inactivation on the detection of anti-WNV IgM and IgG antibodies in mice. Adult C57BL/6 mice were inoculated subcutaneously with 100 PFU of WNV. Mice (n=10 per time-point) were bled at 0, 3, 6, 8, 10 and 24 days after infection and serum was separated, and same time point serum was pooled. Serum samples were HI at 56°C for 30 min. NHI and HI sera were diluted 1:20 in PBS-1% BSA and were tested by WNV E-MIA for the presence of anti-WNV (A) IgM and (B) IgG antibodies. Results are reported as MFI per 100 microspheres. Data are expressed as MFI \pm SD and is representative of three independent experiments conducted in duplicate. Dotted line indicates the cutoff value. HI serum is depicted by red line and NHI serum by blue line.

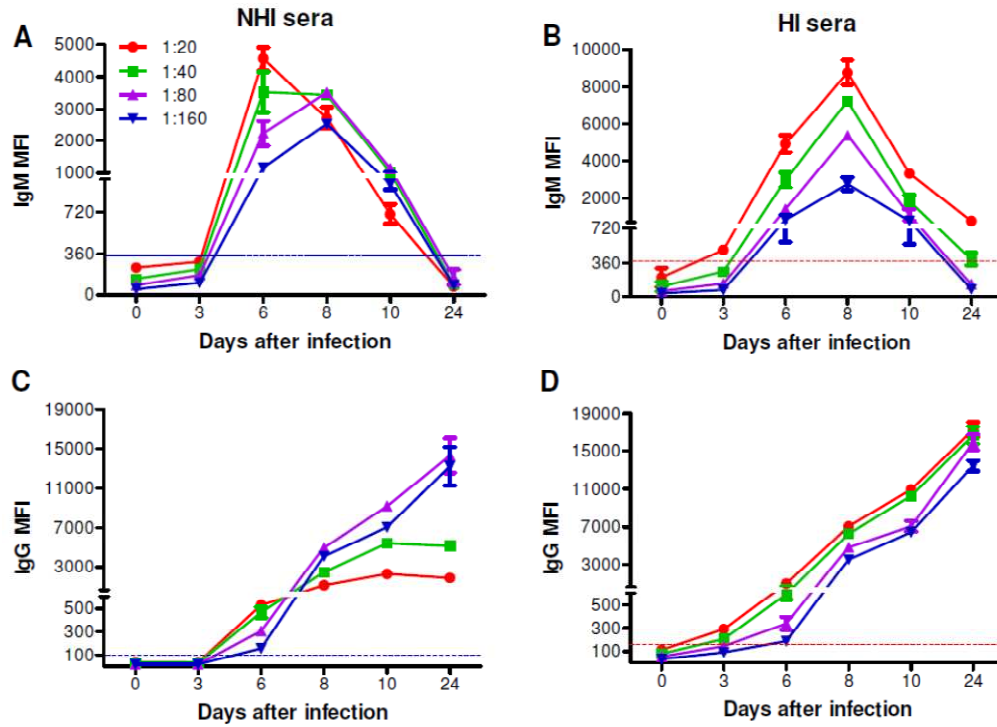


Figure 2. Effect of serum dilution on the detection of anti-WNV IgM and IgG antibodies in mice. NHI and HI sera at indicated time-points after infection were diluted serially from 1:20 to 1:160 in PBS-1% BSA and were tested by WNV E-MIA for the presence of anti-WNV (**A and B**) IgM and (**C and D**) IgG antibodies. Data are expressed as MFI \pm SD and is representative of two independent experiments conducted in duplicate. Dotted line indicates the cutoff value. HI serum is depicted by red line and NHI serum by blue line.

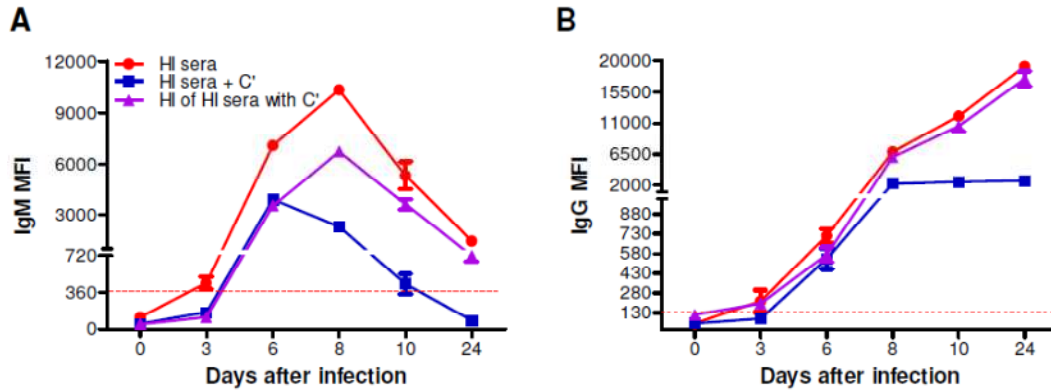


Figure 3. Effect of exogenous addition of complement to heat-inactivated mice serum in WNV E-MIA. HI serum at indicated time-points after infection were diluted 1:20 in PBS-1% BSA and 4 U of reconstituted guinea pig complement (C') were added to 240 μ L-diluted serum. Also, HI serum samples after addition of C' were again heat-inactivated at 56°C for 30 min to inactivate the complement. HI serum without C', with C' and heat inactivation of HI serum with C' were tested by WNV E-MIA for detection of anti-WNV (A) IgM and (B) IgG antibodies. Data are expressed as MFI \pm SD and is representative of two independent experiments conducted in duplicate. Dotted line indicates the cutoff value. HI serum is depicted by red line.

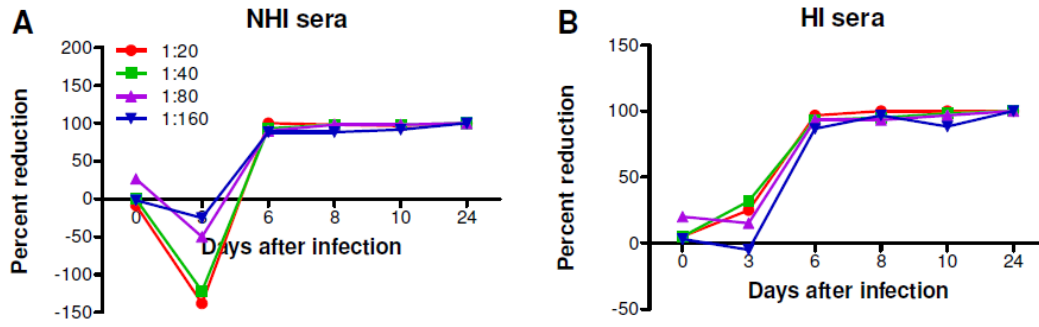


Figure S1. Plaque reduction neutralization test (PRNT) for detection of anti-WNV neutralizing antibodies in mice. NHI and HI sera from WNV-infected mice collected at 0, 3, 6, 8, 10 and 24 days after infection were serially diluted from 1:20 to 1:160 and PRNT assay was conducted. Percent reduction in number of plaques obtained per time point was calculated for both **(A)** NHI and **(B)** HI sera. Data are expressed as average percent reduction in number of plaques per time point conducted in duplicate.

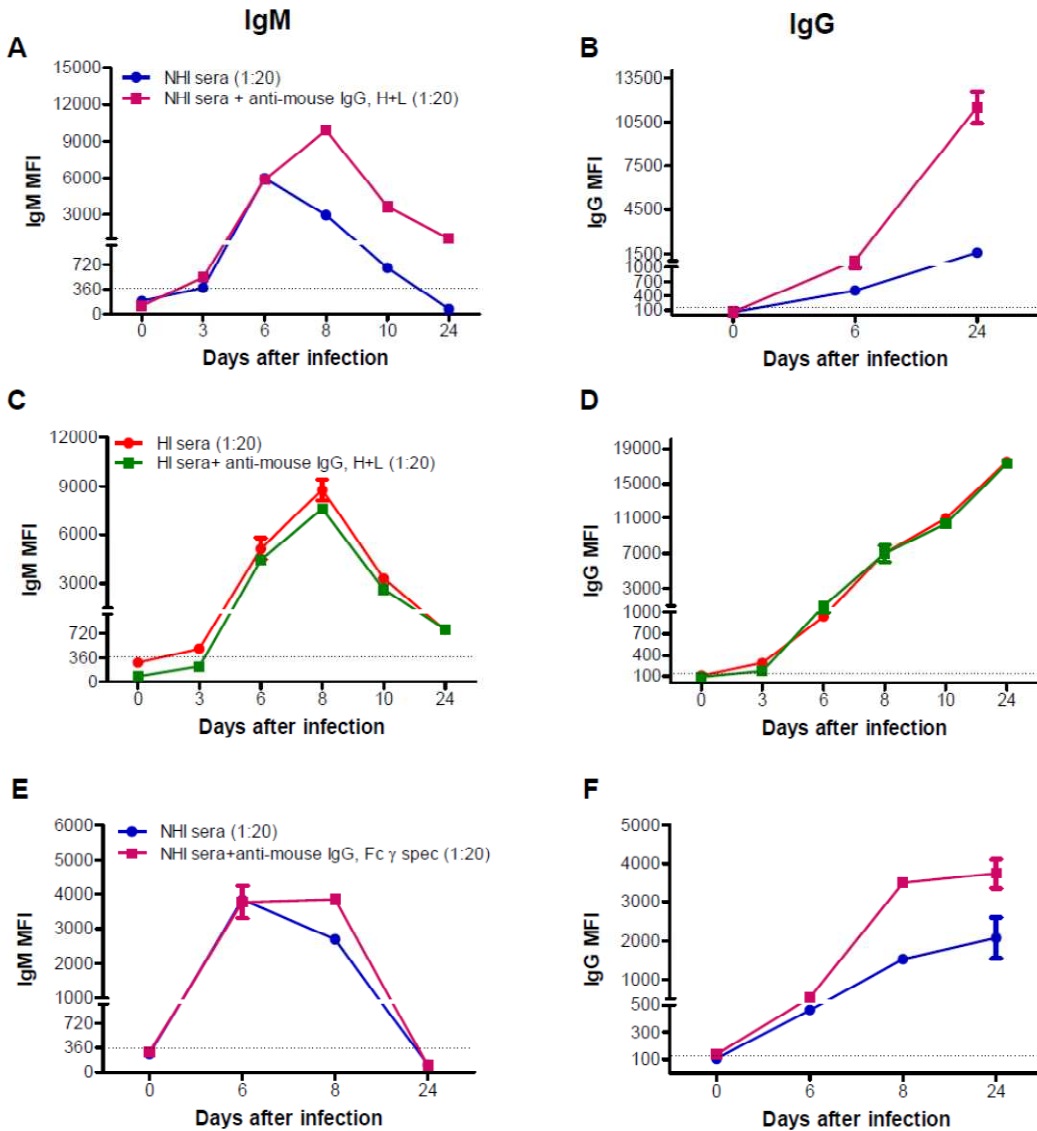


Figure S2: Anti-mouse IgG treatment of the serum does not improve the sensitivity of WNV E-MIA for detection of anti-WNV IgM antibodies in mice. NHI serum from WNV-infected mice collected at various time-points after infection were treated with rabbit anti-mouse IgG (H+L) (**A** and **B**) and goat anti-mouse IgG (Fc γ fragment specific) (**E** and **F**) to deplete the interfering mouse IgG and anti-WNV IgM and -IgG antibodies were detected by WNV E-MIA. Similarly, HI serum from WNV-infected mice collected at various time-points after infection were treated with rabbit anti-mouse IgG (H+L) and WNV E-MIA was used for detection of anti-WNV IgM (**C**) and -IgG antibodies (**D**). Data are expressed as MFI \pm SD and is representative of one or two independent experiments conducted in duplicate.

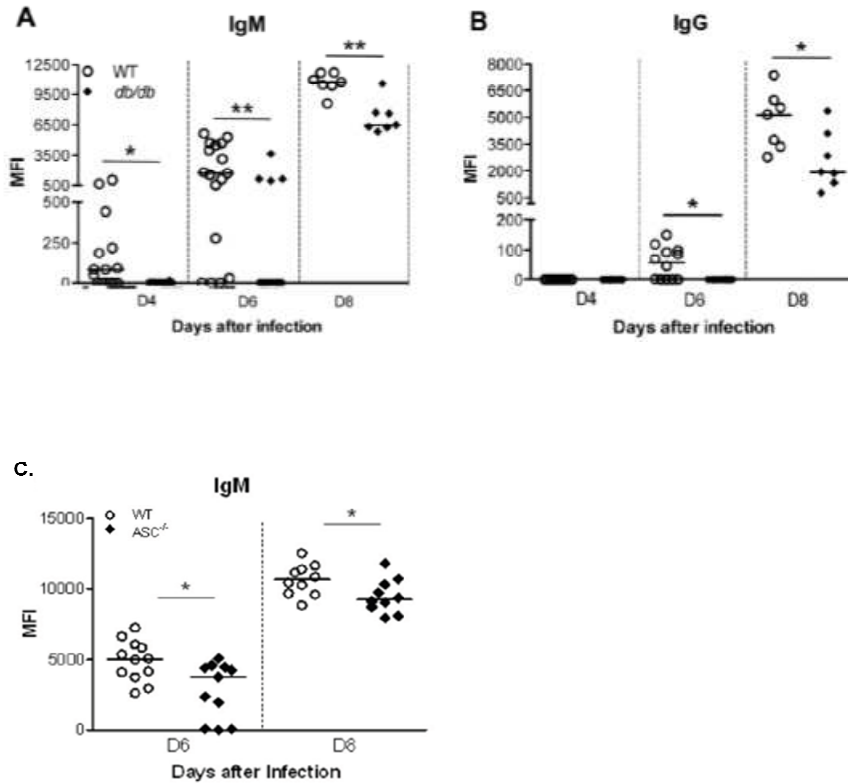


Figure S3: Application of optimized WNV E-MIA to study the WNV antibody response in various mice models. WNV E-MIA was conducted for detection of (A) anti-WNV IgM and (B) – IgG antibodies at indicated time-points in wild type (WT) and diabetic mice (db/db) model [43] as well as for detection of anti-WNV IgM antibodies (C) in WT and ASC^{-/-} mice [44] in two WNV immunopathogenesis studies in our laboratory. Data are expressed as MFI ± SD.

Table 1: Application of WNV E-MIA for detection of WNV-specific IgG antibodies in hybridomas

#	Samples (1:20 dilution)	Average IgG MFI					WNV IgG Ab Result
		Control		Antigen			
		PBS	BSA	DENV-2	DENV NS1 Ag	WNV E-protein	
1	Negative control human sera	42	166	126	176	179	Negative
2	DENV and WNV Ab positive human sera	68	119	4809	17065	11017	Positive
3	DENV Ab positive human sera	43	77	4990	17770	10620	Cross reactive DENV Abs
4	Negative control mice sera (HI)	15	23	25	27	46	Negative
5	Negative control mice sera (HI)	11	18	21	23	36	Negative
6	Positive control mice sera (HI)	35	71	1719	2374	18646	Positive
7	IMDM media control	9	10	14	18	14	Negative
8	DMEM media control	9	10	14	17	15	Negative
9	IMDM WNV E24 mouse hybridomas	14	12	15	19	13921	Positive
10	DMEM WNV E24 mouse hybridomas	11	10	16	18	13992	Positive
11	DMEM WNV E24+ Feeder cells mouse hybridomas	10	11	15	18	13669	Positive

WNV E-protein is truncated E protein (L2 diagnostics). DENV-2 antigen is highly purified preparation of Dengue 2 virus particles (Microbix Biosystems, Inc.)

DENV NS1 Antigen, recombinant DENV-2 NS1 protein (Hawaii Biotechnology Group, Inc); BSA, Bovine Serum Albumin; PBS, Phosphate Buffered Saline;

HI, Heat-inactivated; Positive control, Day 24 sera from WNV-infected mice; IMDM, Iscove's Modified Dulbecco's Medium; DMEM, Dulbecco's Modified Eagle's Medium.

Positive readings are highlighted in bold.

3.6 References

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Chapter 4

Validation and optimization of the in-house Luminex based DENV MIA for detection of dengue-IgM and IgG antibodies in human serum

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4.1 Abstract

In this study, newly developed in-house DENV Microsphere Immunoassay (MIA) was further validated for detection of anti-DENV IgG and IgM antibodies using different serum panels from Hawaii, Vietnam and Niue. DENV IgG MIA is 100% sensitive and 33-100% specific for detection of anti-DENV IgG antibodies as compared to plaque reduction neutralization test (PRNT). DENV IgM MIA is 71-100% sensitive and 11-98% specific for detection of anti-DENV IgM antibodies as compared to in-house DENV IgM Capture ELISA (MAC-ELISA) and a U.S. FDA approved InBios DENV IgM Capture ELISA. InBios DENV IgM Capture ELISA can be effectively used for rapid diagnosis of acute dengue infections as it demonstrated specificity and sensitivity of 94 and 92% for detection of anti-DENV IgM antibodies respectively from 79 human serum samples as compared to MAC-ELISA. The low specificity of DENV MIA for samples from Vietnam was not due to syphilis antibodies present in these serum samples. Use of alternative blocking agents such as polymer based blockers PVA and PVP, 5-10% animal serum (fetal bovine serum, mouse serum and human serum) in serum diluents did not reduce the IgM false-positives in DENV MIA. However, higher serum dilution, pretreatment of serum samples with high concentrations of BSA or BSA-coated beads were found to reduce the non-specific binding or high false-positive results to some extent in DENV IgM MIA, but it almost completely eliminated the low false-positives.

4.2 Introduction

Dengue is an acute viral disease caused by any of the four dengue serotypes, DENV-1,-2,-3 and -4 [1, 2]. Although most of the reported dengue cases in the continental United States are acquired by travelers or immigrants [3], it has recently re-emerged in southern Florida in 2009-2011 and also in Hawaii in 2011 [4]. To date, there is no vaccine or specific antiviral treatment for dengue infections in humans [5]. In many settings, dengue is diagnosed clinically; however the features of early infection are non-specific and mimic those of other febrile illnesses. An early dengue diagnosis would assist in patient triage and will have an increasingly important role as therapeutic drugs (antivirals), become available for dengue [6, 7]. IgM detection by ELISA in samples collected after day 4 of illness is routinely used for dengue diagnosis [8]. IgM antibody titers are usually lower in secondary dengue infection, as compared to primary dengue infection [9]. IgG seroconversion or a fourfold increase in paired serum samples is also among the criteria for dengue diagnosis [10]. One of the current research priorities is the evaluation of newly developed diagnostic assays and commercial kits for early and accurate dengue diagnosis. An ideal DENV serologic test should be sensitive to detect these low titer IgM antibodies and also should specifically detect dengue infection specifically in areas where multiple flaviviruses and other pathogens cocirculate [11].

Luminex-based microsphere immunoassays (MIA) are being developed for rapid serological diagnosis of several diseases [12-14]. In our laboratory, Luminex-based DENV MIA was developed for detection of DENV IgM and IgG

antibodies from human serum samples. In this study, this assay was further validated using different serum panels from Hawaii, Vietnam and Niue where dengue outbreaks have occurred in the past. In addition, DENV IgM MIA was further optimized for improved specificity and sensitivity for detection of anti-DENV IgM antibodies. Some of the key factors considered during DENV IgM MIA optimization to reduce the non-specific background signal, and 'false positive' results were sample diluents, serum concentration and type or concentration of reporter antibody. Human serum or plasma samples contain high-affinity, specific anti-animal antibodies or heterophile antibodies that can generate false-positive and negative signals in immunoassays [15]. Recent studies have shown that some human sera can directly bind to the carboxylated microspheres, causing false-positives and a very high level of non-specific background [16]. Sample diluent can minimize the disturbing influences such as heterophilic antibodies and unspecific binding, thus is an important factor in multiplex assay optimization [17]. Therefore, optimization of sample diluents by addition of alternative blocking agents can prevent the false-positive results. In addition, serum dilution if optimized also can minimize the nonspecific binding observed in the Luminex multiplexed immunoassay system. Another factor that can influence the non-specific binding is the type (monoclonal or polyclonal) and the concentration of the anti-human secondary antibody used for detection and thus should be optimized. In this study, all the above mentioned parameters were optimized to improve the specificity of DENV IgM MIA.

4.3 Materials and Methods

Serum samples

This study was approved by the University of Hawaii Committee on human subjects (CHS # 16857 and 16873). These included clinical samples from small DENV outbreak that occurred in Hawaii in March 2011, one clinical sample collected in 2012 from Hawaii, samples collected in 2004-2010 from persons infected in Hawaii 2001 outbreak, Vietnam plasma samples collected during the period of July-August 1997-1998 where a widespread epidemic of dengue hemorrhagic fever (DHF) occurred in southern Vietnam in 1998 [18]. Few samples from Niue were used only for validation of DENV IgM MIA because of limited sample volume. All assays were conducted in a blinded fashion using numerically coded serum or plasma samples.

Antigens

Inactivated Dengue 2 antigen (Microbix Biosystems Inc, Ontario, Canada, catalog no. EL-22-02) was covalently coupled to the magnetic microspheres. This dengue type 2 antigen (strain 16681) was propagated in Vero cells, concentrated from tissue culture supernatants by precipitation and ultracentrifugation. The antigen was then purified by sucrose density gradient centrifugation, inactivated by formaldehyde, neutralized by sodium bisulfite and lastly verified the inactivation by cell culture technique. Final antigen comprised of intact virions plus free viral proteins and nonstructural proteins. NS1 and E proteins were present in good quantity (Microbix Biosystems Inc, Ontario,

Canada) Immune affinity purified recombinant DENV-2 NS1 antigen was a kind gift from Hawaii Biotech Inc. (U.S.A).

Control beads to detect non-specific binding

Control beads coupled with bovine serum albumin (BSA) (Sigma A2058) and PBS (BIORAD) were included in the experiment to determine the non-specific binding of serum proteins to the microspheres [19, 20]. For control PBS beads, the coupling procedure was followed, in the absence of DENV antigens (Table 1).

Coupling of microspheres with antigens

Magnetic carboxylated microspheres (MagPlex™-C) were obtained from Luminex Corporation (Austin, TX, USA). A two-step carbodiimide process recommended by Luminex Corporation (Austin, TX) was used to link 10 μg of dengue antigens (Table 1) to the surface of 1.25×10^6 microspheres as described previously [21]. The antigen-conjugated microspheres were stored in 250 μL of PBN buffer (PBS with 1% BSA Fraction V, OmniPur, and 0.05% Ultra sodium azide, Sigma Aldrich) at 4°C.

DENV MIA

Serum samples were diluted 1:20 in PBS-1% BSA. For DENV IgM MIA, 12 μL of sample was treated with 80 μl of GullSORB (Meridian Bioscience, Inc) and then diluted in PBS-1%BSA. A total of 50 μL of PBS-1% BSA containing

approximately 1,250 of each DENV-2 antigen, DENV NS1 antigen, PBS and BSA coupled microspheres were added to each well of a flat-bottom black 96-well plate (VWR, PA). Fifty μL of diluted serum was added to the beads and incubated for 30 min in the dark. The plates were then washed twice with 200 μL of PBS-1% BSA and 50 μL of diluted red-phycoerythrin (R-PE) conjugated secondary antibody (2 $\mu\text{g}/\text{mL}$) was added to test wells and incubated for 45 min in the dark. The plates were washed twice with 200 μL of PBS-1% BSA. Microspheres were then resuspended in 100 μL of PBS-1% BSA per well and incubated for 5 min before analysis on the Luminex 100 machine (Qiagen, Valencia, CA). The median fluorescence intensity (MFI) was quantitated for 100 microspheres and recorded for each well. The secondary antibodies used were R-PE conjugated F(ab')₂ fragment goat anti-human IgG, Fc γ fragment specific and R-PE conjugated F(ab')₂ fragment donkey anti-human IgM, Fc5 μ fragment specific (Jackson ImmunoResearch, West Grove, PA). All assays were done in duplicate and all incubations were conducted on a plate shaker at 700 rpm and the wash steps were conducted using 96-well magnetic plate separator (Millipore Corp., Billerica, MA). Using PBS and BSA coupled beads, non-specific binding was monitored.

Cutoff determinations

Cutoff values were established based on analysis of positive and negative controls included during DENV IgG and IgM MIA of 44 samples collected in Hawaii in March 2011 (Table 2).

Reference dengue assays for characterization

1. DENV Plaque reduction neutralization test (PRNT)

Neutralizing antibodies to DENV1-4 were detected by using PRNT tests according to the protocol recommended by the Centers for Disease Control and Prevention (CDC) [22]. DENV-1 (1943), DENV-2 (DAKARA), DENV-3 (H87), and DENV-4 (H241) grown in Vero cells were employed for conducting PRNT. PRNT end point dilution was conducted on 67 serum samples whereas for another 35 serum samples PRNT was conducted only at 1:20 and 1:40 serum dilutions using 90% plaque reduction for all four DENV serotypes.

2. In-house Dengue IgM capture ELISA (MAC-ELISA)

In-house MAC-ELISA for detection of anti-DENV IgM antibodies was conducted as described previously [23, 24]. Briefly, inner 60 wells of Immulon II plates (Dynatech, VA) were coated with goat anti-human IgM antibodies (KPL, MD) diluted 1:2,000 in carbonate-bicarbonate buffer (pH-9.6) and the plate was incubated overnight at 4°C. Plates were aspirated and blocked with 200 μ L/well of 1X phosphate-buffered saline (PBS), 0.05% tween 20 and 5% milk for 30 min at room temperature. Further, plates were then washed five times with PBS containing 0.05% Tween 20 using an automated plate washer. Fifty μ L of 1:40 diluted serum samples were added in triplicate for the virus antigen wells and for the normal antigen wells and the plates were incubated for 1 hr at 37°C. Each plate included one positive and one negative control human sera. Plates were washed five times as described above. DENV viral antigens used were sucrose-

acetone extracts from DENV infected suckling mouse brain, obtained from the Division of Vector-Borne Diseases (DVBD), CDC. One hundred μL of 1:80, 1:160, 1:80 and 1:80 dilutions of DENV-1, -2, -3 and -4 antigens in PBS, respectively, were added to each virus well and 50 μL of 1:20 normal antigen was added to each control well. Plates were incubated overnight at 4°C, and then washed as described above. Fifty μL of flavivirus group-reactive monoclonal antibody 6B6C-1 conjugated to HRP (Hennessy Research, KS) diluted 1:2,500 in blocking buffer was added to each well. Plates were then incubated for 1 hr at 37°C, washed five times as described above, rotated and the plate were washed for five additional times. After washing, the wells were incubated with TMB substrate at room temperature for 10 min. The reaction was then stopped by addition of 50 μL of TMB stop solution (KPL, MD) and absorbance values were read at 450 nm using a Biotek fluorescence plate reader (Biotek, VT). Mean absorbance values for triplicate wells of each test serum (P) were divided by the mean values of the corresponding negative control serum (N). A P/N ratio of ≥ 3.0 was considered positive whereas P/N ratio of ≥ 2 and < 3 was considered presumptive positive and P/N ratio of < 2 was considered negative.

3. InBios DENV IgM Capture ELISA

InBios DENV IgM Capture ELISA was conducted according to the manufacturer's instructions. Briefly, 1:100 diluted serum samples were incubated in microtiter wells coated with anti-human IgM antibodies for 1 hr at 37°C followed by separate incubation with dengue-derived recombinant antigens (DENRA) and

normal cell antigen (NCA). After incubation and washing, the wells were treated with a DENV-specific monoclonal antibody labeled with the enzyme horseradish peroxidase (HRP). After a second incubation and washing step, the wells were incubated with tetramethylbenzidine (TMB) substrate. After addition of stopping solution, absorbance was read at 450 nanometers. The ratio of the DENRA and the control antigen wells (NCA), designated as immune status ratio (ISR) was used to determine the presence of DENV antibodies in the serum sample. All serum samples with ISR below 1.65 were considered negative for anti-DENV IgM antibodies, whereas samples with ISR above 2.84 were considered positive for anti-DENV IgM antibodies. Serum samples with ISR between 1.65 and 2.84 were considered equivocal. Equivocal serum samples were repeated in duplicate. If the serum was available, serum samples that remained equivocal after repeat testing were tested using the PRNT.

Syphilis IgG EIA

Syphilis IgG EIA was conducted using CAPTIA Syphilis IgG kit (Trinity biotech) on Vietnam samples that showed IgM false-positive results in DENV MIA to find out whether the syphilis antibodies are interfering in DENV MIA as they had been shown to interfere in WNV E-MIA for detection of anti-WNV antibodies [21].

DENV IgM MIA optimization

1. Use of alternative blocking agents in sample diluents or assay buffers

To prevent false-positive results from either cross reactive antibodies or from non-specific binding of assay reagents to BSA, several alternative blocking agents were added in sample diluents or assay buffers in DENV MIA such as polymer-based non-protein blockers for example, polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP) [25, 26] and 10% animal sera such as fetal bovine serum, mouse serum and normal human serum. PVA (average MW: 85,000 to 124,000, 98-99% hydrolyzed), PVA- P8136 (average MW: 30,000 to 70,000) and PVP-40 (average MW: 40,000) were obtained from Sigma Chemical Co. (St. Louis, Mo.) and PVP K90 (average MW: 360,000) was obtained from ICN Biomedicals Inc. Normal human serum AB was obtained from Gemini Bio-products, CA. Animal serum were heat-inactivated at 56°C in a water bath for 1 hour before adding it to the serum diluents.

2. Effect of serum dilution in DENV IgM MIA

Assay linearity was determined by serially diluting 8 selected serum or plasma samples using PBS-1%BSA. Out of 8 samples, 1 sample was DENV IgM MIA negative, 1 sample was DENV IgM MIA low false-positive, 2 were DENV IgM MIA high false-positive, 2 were DENV IgM low true-positive and 2 were DENV IgM high true-positive. Samples were treated with GullSORB diluted in a series of five 2-fold dilutions from 1:20 to 1:640 and DENV IgM MIA was conducted.

3. Use of high concentration of BSA or BSA-coated beads in sample diluents

Serum samples were treated with GullSORB, diluted 1:100 using higher concentration of BSA (5-20%) or BSA-coated beads in serum diluent and were kept on rotator overnight at 4°C or 1 hr at room temperature. After incubation, samples were centrifuged at 10,000g at 4°C for 20 min and DENV IgM MIA was conducted.

4. Optimization of secondary antibody used in DENV IgM MIA

Serum samples were treated with GullSORB, diluted 1:100 in PBS-1% BSA and DENV IgM MIA was conducted using different concentrations of donkey anti-human IgM PE from 0.5 to 4 $\mu\text{g}/\text{mL}$. In addition, the performance of DENV IgM MIA for detection of anti-DENV IgM antibodies was compared by using two different anti-human IgM PE, polyclonal donkey anti-human IgM PE (Jackson ImmunoResearch, West Grove, PA) and monoclonal mouse anti-human IgM PE (eBioscience, Inc., CA).

5. Use of alternative BSA-free storage buffer for coupled beads

Stabliguard (Surmodics Inc.), a BSA free blocker was used as a washing and storage buffer for DENV-antigen coupled beads as it has been shown to eliminate the false-positive results by avoiding the interference with anti-BSA IgM antibodies from serum samples.[27].

6. Use of heterophilic antibody blockers (HBRs)

HBR-ultra, HBR-24, HBR-25 and HBR-26 were obtained from Scantibodies Laboratories Inc., CA. These blocking agents contain immunoglobulins of murine origin with specific binders that neutralize by active attachment to the heterophilic antibody [28]. The HBR blockers were used according to the manufacturer's directions by adding them to the buffer used for the dilution of detection antibodies at a concentration of rate of 40 µg of HBR per sample.

Statistical analysis

Microsoft Excel software was used for statistical analysis. All data are reported as mean MFI ± standard deviation using GraphPad Prism 5.0 software.

Sensitivity and specificity of DENV MIA for diagnosis of DENV infection was analyzed by using 2×2 tables [29]. For evaluation of InBios DENV Detect IgM Capture ELISA, in addition to determination of sensitivity and specificity, percent agreement, 95% confidence intervals (95% CI) and kappa coefficients were also determined as measures of agreement. Levels of agreement as defined by kappa values were categorized as near perfect (0.81 to 1.0), substantial (0.61 to 0.8), moderate (0.41 to 0.6), fair (0.21 to 0.4), slight (0 to 0.2), or poor (<0) [30]. Equivocal results by the InBios DENV IgM Capture ELISA were considered negative for calculating percent sensitivity and positive for calculating percent specificity. Data analysis for evaluation of InBios assay was conducted using SAS software.

4.4 Results and Discussion

Validation of DENV MIA using different serum panels

DENV IgG MIA was found to be 100% sensitive and 33-100% specific for detection of anti-DENV IgG antibodies using different serum panels from Hawaii and Vietnam as compared to gold standard DENV PRNT assay (Table 3). Whereas, DENV IgM MIA was found to be 71-100% sensitive and 11-98% specific for detection of anti-DENV IgM antibodies using different serum panels from Hawaii, Vietnam and Niue as compared to in-house DENV IgM Capture ELISA (Table 4). Similarly using InBios DENV IgM Capture ELISA as gold standard, DENV IgM MIA was found to be 67-100% sensitive and 15-93% specific for detection of anti-DENV IgM antibodies (Table 5). These results demonstrated that sensitivity and specificity of DENV MIA for detection of anti-DENV antibodies may differ according to the origin of samples. Human sera may contain antibodies that directly bind to beads [31, 32]. The proportion of such “bead binders” differs in different serum panels and frequently exceeds 5% [16]. Up to 80% of human sera contain heterophile antibodies [32, 33] and up to 59% of bead binders were found in African serum collections [16]. These endogenous interfering antibodies that lead to non-specific binding may be of any immunoglobulins class, namely, IgG, IgA, which could be monomeric, dimeric or tetrameric or the pentameric IgM [34]. The interference we observed in DENV IgM MIA may be caused by the direct binding of IgM antibodies to surface-modified polystyrene microparticles, as IgG antibodies are removed during DENV IgM MIA. Also natural antibodies (nAbs) found in the serum are predominantly of

the IgM isotype, generated without the requirement for exogenous antigenic stimulation. [35].

Syphilis antibodies do not interfere in DENV MIA

Sera from patients with syphilis demonstrated a high frequency of false-positive results in Luminex-based polyvalent rWNV-E MIA for detection of anti-flavivirus antibodies [21]. Therefore, the lowest specificity (11%) of DENV IgM MIA for Vietnam plasma samples may be due to the presence of cross-reactive syphilis antibodies in these serum samples. However, comparison of syphilis IgG EIA and DENV IgM MIA data demonstrated that serum samples that did not contain syphilis antibodies also showed IgM false-positive results in DENV MIA (Table 6). These results confirm that syphilis antibodies present in serum samples from Vietnam are not the cause of IgM false-positive results in DENV IgM MIA and it may be due to non-specific binding of secondary detection antibodies.

Evaluation of InBios DENV IgM capture ELISA for the diagnosis of acute or recent DENV infection

InBios DENV Detect IgM Capture ELISA (InBios International, Inc., Seattle, WA) is the U.S. Food and Drug Administration (FDA) approved assay for qualitative detection of anti-DENV IgM antibodies [4]. In this study, Inbios DENV IgM Capture ELISA was evaluated in comparison with the in-house DENV MAC-ELISA using 79 clinical serum samples collected from Hawaii, Vietnam, Niue,

Singapore and American Samoa, where dengue outbreaks have occurred in the past.

The major performance characteristics used for evaluation of newly developed diagnostic tests as compared to “gold standard” or reference standard are sensitivity and specificity [11, 36]. However, if the reference standard is not available, then positive percent agreement (PPA) and negative percent agreement (NPA) are calculated [29]. In this study, for evaluation of sensitivity and specificity of the Inbios DENV IgM Capture ELISA, in-house DENV MAC-ELISA was used as a “gold standard” and data analysis was conducted using three criteria (Table 9). For the first criteria, equivocal and presumptive positive samples were considered as negative [37-39] whereas for the second criteria, the five equivocal and presumptive positive samples were excluded from data analysis, [36, 40] and for third criteria, MAC-ELISA presumptive-positives were considered as negative [39] and PPA and NPA were calculated as per InBios kit insert by considering the “worst-case scenario.” That is, equivocal samples were considered false-negative for the PPA and equivocal samples were considered false-positive for the NPA (Table 9). Based on 79 serum samples using first criteria, the sensitivity and specificity of InBios assay for detection of anti-dengue IgM antibodies was found to be 92% and 96%, respectively. Using second criteria, based on remaining 74 out of the 79 serum samples, the sensitivity and specificity was found to be 100% and 96%, respectively. The PPA (sensitivity) and NPA (specificity) using third criteria were found to be 92 and 91% respectively (Table 9).

Out of 22 positive serum samples by both assays, PRNT end point dilution was conducted for only six samples using InBios assay and MAC-ELISA due to limited sample volume. PRNT results based on end point serum dilution coupled with patient clinical history indicated that five out of the six (83%) serum samples were from patients with recent primary dengue infection and one sample (17%) was from a patient with recent secondary dengue infection. PRNT end point dilution was also conducted on three discordant samples (Table 7) to identify the infecting DENV serotype and to confirm the primary or secondary DENV infection. Out of 3 discordant samples, sample 5 from Vietnam was from a patient infected with DENV-2 in the past, the second sample 6 from Hawai'i was collected in 2010 from a patient infected with DENV-1 in 2001, and for the third sample 7 from Vietnam, DENV serotype could not be determined because of possible multiple past DENV infections (Table 7). Out of the 50 dengue IgM negative serum samples by both assays, PRNT was conducted at 1:20 and 1:40 serum dilutions on 39 samples to confirm the presence or absence of dengue infection, out of which 18 samples (46%) were PRNT positive and thus indicated past dengue infection.

We observed a significant correlation in detecting anti-DENV IgM antibodies between the InBios IgM Capture ELISA and the in-house MAC-ELISA (Pearson's $r = 0.80$, $P < 0.0001$) (Figure 1). Considering both InBios equivocal and MAC-ELISA presumptive positives, the agreement, sensitivity and specificity of InBios IgM Capture ELISA was 94 (95% CI, 86-98%), 92 (95% CI, 73-99%) and 94% (95% CI, 84-99%) respectively (Table 10). In addition, the InBios assay

demonstrated near perfect agreement ($\kappa = 0.87$) [30] to in-house MAC-ELISA (Table 10). Out of 79 serum samples, five serum samples exhibited discordant results (Table 7). The NCA used in InBios DENV IgM Capture ELISA is a culture supernatant of the COS-1 cell line (InBios International, Inc., Seattle, WA) and is used to detect nonspecific reactivity of test serum samples [40]. Dengue antigen (DENRA) and NCA optical density (OD) values obtained for 79 serum samples are depicted in Table 8. Out of the five samples that exhibited discordant results, three MAC-ELISA negative samples were InBios equivocal or positive (Table 7). These three samples exhibited DENRA OD values in the range of 0.19 to 0.21 (Table 7) that is closer to the highest DENRA OD value (0.14) obtained for negative serum samples using the InBios assay (Table 8). These data support the true-negativity of these serum samples by MAC-ELISA. In addition, sample 1 depicted high background as indicated by NCA OD value of 0.11 and thus interpreted as equivocal even though the DENRA OD value was 0.27 (Table 7). Thus, inclusion of positive as well as negative antigens are critical for interpretation of the test results as they detect background nonspecific reactivity of serum samples [40].

Two serum samples from Hawaii and Vietnam, from patients with previous dengue infection as determined by PRNT, produced false-positive reactions using InBios DENV IgM Capture ELISA (Table 7). However, ISR values for these 2 samples ranged from 3.0-3.4 and were closer to the ISR cut-off of 2.84 for InBios positives. Out of these two serum samples, serum sample 6 from Hawaii (Table 7) collected in 2010 from a patient infected with DENV-1 in 2001 [41] was

previously studied in our laboratory for long-term T cell memory responses (Gurary et al., unpublished data). In humans, dengue-epitope-specific tetramer positive CD8⁺T cells can be detected *ex-vivo* up to a year after natural primary dengue infection and even longer after secondary dengue infection [42]. In sample 6, we were unable to detect dengue-epitope-specific tetramer positive CD8⁺ T cells *ex vivo*. In addition, even after stimulation with cognate dengue epitope, sample 6 demonstrated lower dengue-specific CD8⁺ T cell proliferative response when compared to recently dengue-infected patients (Gurary et al., unpublished data). These data combined with MAC-ELISA data confirms the absence of recent secondary dengue infection and thus anti-dengue IgM antibodies in sample 6.

Sensitivity for other commercially available anti-dengue virus IgM tests varies from 21 to 99%, whereas the specificities are 77 to 98% as compared with reference solid-phase IgM antibody-capture ELISAs (MAC-ELISA) used by the CDC [11]. Recently, in one study by Blacksell *et al.*, sensitivities and specificities of the two commercial IgM antibody ELISAs for detection of acute dengue infection ranged from 85 to 89% and 88 to 100% respectively [43]. In addition, InBios DENV IgM Capture ELISA is advantageous as compared to MAC-ELISA since the results can be obtained in one day, whereas the latter requires 2-3 days.

Use of alternative blocking agents in sample diluent did not improve the specificity of DENV IgM MIA

In order to reduce or prevent the IgM false positive results in DENV IgM MIA, we analyzed and compared the use of several alternative blocking agents in sample diluents as well as assay buffers. Polymer based blockers, PVA and PVP have been shown to minimize background signals, by competing with nonspecific adsorption of proteins to the solid phase and thus have been added to the sample diluents in Luminex-based assays to reduce the non-specific binding [16, 26, 44, 45]. However, the addition of 0.5% (wt/vol) PVA and 0.8% (wt/vol) PVP to the sample diluent in DENV IgM MIA did not reduce the number of IgM false-positive results. Similarly, in other MIA, no effect of addition of 0.5% PVA and 0.8% PVP to the sample buffer was found [46, 47]. The addition of PVA and PVP to the secondary antibody dilution buffer in DENV MIA resulted in increased IgM and IgG MFI for positive controls; however it also increased the nonspecific background observed with false-positive samples. This may be as a result of PVP, which has been shown to increase the secondary antibody adsorption [45].

Normal animal sera such as fetal bovine serum (FBS) have also been added at concentration of 5-10% to the serum diluents to block or saturate the generalized binding interactions [48]. However, in this study, addition of 5-10% (vol/vol) of fetal bovine serum (FBS), mouse serum and normal human serum (NHS) in PBS buffer did not decrease the high background observed in DENV IgM MIA.

Minimizing serum concentration reduced non-specific protein binding to microspheres in DENV MIA

Highly concentrated serum or plasma sample can contribute to high background or non-specific reactivity (Luminex Corp.). Therefore, serum dilution should be optimized to minimize the non-specific binding that can occur with some serum samples. In addition, doubling dilutions test (i.e., serial dilution of a sample with assay diluent) have been used for detecting interference from endogenous antibodies [34]. Linear curves obtained upon two fold serial dilution of serum samples indicate true seropositivity whereas the nonlinear response or flat or undulating titration curves indicate false-positive results [34, 49]. To find out the effect of serum dilution on non-specific binding occurring in DENV IgM MIA, serum samples were treated with GullSORB, diluted serially from 1:20 to 1:640 using PBS-1% BSA. Two DENV IgM antibody positive samples demonstrated linear response with serum dilution. However, IgM MFI for 2 DENV IgM false-positive samples was drastically decreased at higher serum dilution. A serum dilution of 1:160 eliminated the low false-positives in DENV IgM MIA and also was able to discriminate the low true IgM positives and IgM negative samples (Figure 2).

Samples with increased non-specific binding show high MFI with BSA coated beads

Serum incubated with control (PBS) beads resulted in median MFI values for IgG, and IgM of 77 (range, 23.5–251), and 97.5 (range, 15–471), at 1:20 dilution respectively, whereas serum incubated with control (BSA) beads resulted in median MFI values for IgG, and IgM of 162.8 (range, 21–544), and 546 (range,

63–2749.5), at 1:20 dilution, respectively. This indicates that there was quite a variable level of non-specific binding. IgM false-positive samples produced high MFI readings with the control BSA beads (Figure 2).

As BSA is a serum protein; it has been shown to cause the non-specific ELISA signals in certain circumstances [25]. BSA antibodies and their effects on serological tests have been reported in healthy human sera, particularly from infants and are probably induced by dietary proteins [27, 50]. Use of BSA and casein to block reactive sites of the microtiter plate or polystyrene microspheres can contribute to elevated values, false-positive results, and high background readings as a result of heterophile and human anti-animal antibodies binding directly to the blocking protein [32]. In this case, the patient's heterophile antibody reacts with the BSA in the reaction well and is then detected by the labeled anti-human detection antibody, thus giving a false-positive reaction. However, a false-positive result was not seen when BSA was used in the specimen diluent, resulting in the heterophile antibodies being preabsorbed [51]. Herein in DENV IgM MIA, presence of 1% BSA in sample diluents did not decrease the observed false-positive results, thus indicating that these interfering antibodies are not specifically against BSA, but they are binding non-specifically to BSA beads and DENV antigen coupled beads. Similarly in another ELISA for detection of serum antibodies to the lipopolysaccharide (LPS) of *Escherichia coli* O157, serum samples with antibodies binding to BSA caused false-positive results [52]. However, these antibodies were not specific for BSA and the ELISA

values were reduced to a background level when plates were blocked with normal rabbit serum [52].

The absorption of human sera with high concentration of BSA or BSA-coated beads decreased the non-specific binding in DENV IgM MIA

In Luminex-based assay for determination of the specificity of HLA antibodies produced by left ventricular assist devices (LVAD) recipients, antibodies directed against BSA were found to be the cause of false-positive results. Absorption of these sera with BSA-coated beads completely abrogated these non-specific reactivity [53]. Similarly in DENV IgM MIA, sample pretreatment with 5-20% BSA and/or BSA-coated beads decreased the false-positive IgM MFI without reducing the specific responses. Overnight incubation of samples with BSA coated beads almost completely removed the interfering antibodies in DENV IgM MIA (Figure 3). However, sample pretreatment with BSA coupled beads for 1 hr did not completely reduce the nonspecific binding observed with high IgM false-positive samples (Figure 4)

Optimization of secondary antibody used in DENV IgM MIA

The concentration of IgM secondary antibody currently used for detection in DENV IgM MIA is 2 $\mu\text{g}/\text{mL}$. We studied the effect of different concentrations of IgM secondary antibody (0.5 to 4 $\mu\text{g}/\text{ml}$) on non-specific binding in DENV IgM MIA. As the concentration of anti-human IgM PE was increased, there was a proportionate increase in both true positive IgM MFI as well as false-positive IgM

MFI (Figure 5). Interestingly, the use of monoclonal mouse anti-human IgM PE for detection of anti-DENV IgM antibodies resulted in low BSA IgM MFI for false-positive samples as compared to polyclonal donkey anti-human IgM PE in DENV IgM MIA. However, the specific response or true positive IgM MFI was reduced as compared to polyclonal secondary antibody (Figure 6).

Use of Stabliguard, a BSA-free storage buffer for coupled beads did not reduce the IgM false-positives in DENV MIA

In Luminex-based assay for detection of pneumococcal antibodies, use of BSA free blocker, Stabliguard (Surmodics Inc., MN) as a washing and storage buffer for antigen coupled beads eliminated the false-positive results [27]. However, the use of Stabliguard for washing and storage of DENV antigen coupled beads did not reduce the high background observed with some serum samples in DENV IgM MIA.

Use of heterophilic antibody blockers (HBRs) did not decrease the false-positives in DENV MIA

Certain proteins present in serum samples, such as heterophile antibodies can also lead to increased non-specific signal or false-positive results in MIA (Luminex Corp.). Heterophile antibody interference in Luminex-based immunoassays for cytokines has been reported [32]. 'Heterophilic antibodies' are antibodies which bind other antigens than the specific antigen. They can be of the IgG, IgM, IgA or IgE type. Commercially available heterophile blocking agents

are effective in removing the heterophile antibodies from assay systems [28]. However, use of Scantibodies HBR's in secondary antibody diluents as well as serum diluents in DENV IgM MIA did not decrease the false-positive IgM MFI observed with some serum samples. Similarly in other studies, preabsorption of serum with heterophile blocking tubes did not decrease the non-specific binding [27].

Storage buffer of antigens can affect the efficiency of conjugation in MIA

Luminex recommends that if interfering substances are present in storage buffer of antigens or proteins, buffer exchange into an alternative buffer of the same pH should be conducted before coupling of proteins to the microspheres. BSA is used as an inert carrier or stabilizer to preserve the biological functions and allow prolonged storage of antigens/proteins. However, the presence of BSA in antigen preparations can lead to non-specific binding. For example, in detection of human NS1 antibodies to Japanese encephalitis virus using ELISA, sera containing antibodies to BSA reacted with BSA antigen remaining in immunoaffinity-purified NS1 antigen which was used to coat the microplates and thus caused false-positive results [54]. In this study, they were able to minimize the effect of BSA antibodies by reducing the BSA level to $\leq 1\%$ in the NS1 antigen preparation [54]. Therefore, the use of BSA as carrier proteins in immunoassays should be avoided.

Similarly in Luminex based multiplex Immunoassay for quantitative detection of IgG serum antibodies against measles, the excess of Vero cell

material present in commercially obtained measles virus preparation resulted in nonoptimal conjugation, and it also caused the nonspecific signal in the MIA for some serum samples. By preincubation of those samples before analysis with Vero cell material used for culturing the virus, they were able to inhibit the nonspecific signal [47]. In another Luminex-based multiplex assay for detection of antibodies against Human Papillomavirus (HPV), HPV antigens were bacterially expressed as glutathione S-Transferase (GST) fusion proteins. Antibodies against residual bacterial proteins and GST present in antigen preparations contributed to high non-specific background observed with some serum samples [16].

Dengue 2 antigen used in DENV MIA is stored in medium 199. Medium 199 is a medium that is used in cell biology for culturing cells. It contains amino acids and other additives. These other additives may interfere with conjugation of antigen to the microspheres and also can cause non-specific binding in MIA.

4.5 Conclusions

Validation of DENV MIA using different serum panels demonstrated that sensitivity and specificity of immunoassays may differ according to the origin of samples. The presence of interfering heterophilic antibodies, other endogenous antibodies and plasma proteins present in serum samples may lead to the reduced specificity of immunoassays. The use of reagent blank beads or PBS beads and BSA coupled beads can detect high-level background signals caused by these polyreactive antibodies or non-specific binding antibodies. Higher serum

dilution in DENV IgM MIA decreased the non-specific binding and thus eliminated the low false-positives. In addition, pretreatment of serum samples with high concentration of BSA or BSA-coated beads reduced the assay interference to some extent in DENV IgM MIA. Recently U.S. FDA approved InBios DENV Detect IgM Capture ELISA is used as a reference DENV diagnostic assay in this study. This assay can be used by public health laboratories for rapid and accurate detection of dengue infections during dengue epidemics, as it demonstrated good sensitivity and specificity for detection of dengue IgM antibodies. Results of this study conclude that sample pretreatment or the use of alternative blocking agents will vary for immunoassays. Therefore, it is important to optimize the blocking conditions for newly developed immunoassays to avoid the false-positive results.

Table 1: Concentration of antigens used for coupling of microspheres

Bead Region	Antigen	Concentration	Volume added to 100 μ L beads Suspended in PBS buffer
33	DENV-2 Ag	0.74 mg/mL	13.5 μ L
15	BSA	100 μ g/mL	100 μ L
007	NS1 Ag	1.84 mg/mL	5.4 μ L
42	1X PBS	-	13.5 μ L

Table 2: Cut off determinations for DENV IgG and IgM MIA

IgG MIA			IgM MIA		
Result	Cut offs ^a	Criteria used for cut off calculation	Result	Cut offs	Criteria used for cut off calculation
Negative	≤ 321	Mean +3SD for 7 -Ve controls	Negative	≤ 307	Mean +3SD for 14 -Ve controls
Positive	≥ 620	Mean +3SD of 3 PRNT -Ve and DENV IgG MIA low +Ve samples	Positive	≥ 414	Mean +3SD of 4 MAC-ELISA -Ve and DENV IgM MIA low +Ve samples
Equivocal	> 321 and < 620	-	Equivocal	> 307 and < 414	-

^a Cutoff values were established based on analysis of +Ve and -Ve controls included during DENV IgG and IgM MIA of 44 samples collected in Hawaii in March 2011.

-Ve, negative control; +Ve, positive control; SD, standard deviation

Table 3: Comparison of DENV IgG MIA and PRNT for detection of anti-dengue IgG antibodies

Cohort/Place	Total number of Samples	DENV IgG MIA	PRNT ₉₀		Criteria 1 ^a		Criteria 2 ^b		Criteria 3 ^c	
			Negative	Positive	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Hawaii (2011- 2012)	45	Negative	34	0	100 (10/10)%	97 (34/35)%	100 (10/10)%	97 (34/35)%	100 (10/10)%	97 (34/35)%
		Equivocal	0	0						
		Positive	1 ^d	10						
		Total	35	10						
Hawaii (2004-2010)	21	Negative	3	0	100 (17/17)%	100 (4/4)%	100 (17/17)%	100 (3/3)%	100 (17/17)%	75 (3/4)%
		Equivocal	1	0						
		Positive	0	17						
		Total	4	17						
Vietnam (1998)	35	Negative	2	0	100 (29/29)%	83 (5/6)%	100 (29/29)%	67 (2/3)%	100 (29/29)%	33 (2/6)%
		Equivocal	3	0						
		Positive	1	29						
		Total	6	29						

PRNT, Plaque Reduction Neutralization Test; Sensitivity- Tp/Tp+Fn; Specificity- Tn/Tn+Fp.

^a Equivocal samples were considered as negative. ^b Equivocal samples were excluded.

^c Equivocal samples were considered false-negative for the sensitivity and equivocal samples were considered false-positive for the specificity.

^d This sample had 80% reduction for DENV-4 PRNT and was positive for IgG based on CDC data.

Table 4: Comparison of DENV IgM MIA to In-House DENV IgM Capture ELISA for detection of anti-dengue IgM antibodies

Cohort/Place	Total number of Samples	DENV IgM MIA	In-House DENV IgM Capture ELISA			Criteria 1 ^a		Criteria 2 ^b		Criteria 3 ^c	
			Negative	Presumptive Pos	Positive	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Hawaii (2011- 2012)	45	Negative	37	0	0						
		Equivocal	4	0	0						
		Positive	1	0	3	100 (3/3)%	98 (41/42)%	100 (3/3)%	97 (37/38)%	100 (3/3)%	88 (37/42)%
		Total	42	0	3						
Vietnam (1998)	36	Negative	4	0	0						
		Equivocal	3	0	0						
		Positive	29	0	0	- ^d	19 (7/36)%	- ^d	12 (4/33)%	- ^d	11 (4/36)%
		Total	36	0	0						
Niue	23	Negative	2	0	0						
		Equivocal	2	0	5						
		Positive	2	0	12	100 (12/12)%	82 (9/11)%	100 (12/12)%	50 (2/4)%	71 (12/17) %	33 (2/6)%
		Total	6	0	17						

Sensitivity- $Tp / (Tp + Fn)$; Specificity- $Tn / (Tn + Fp)$. MAC-ELISA Pos, $P/N \geq 3$, MAC-ELISA Presumptive Pos, $P/N \geq 2$ and < 3 , MAC-ELISA Neg, $P/N < 2$.

^a Equivocal samples were considered as negative. ^b Equivocal samples were excluded.

^c Equivocal samples were considered false-negative for the sensitivity and equivocal samples were considered false-positive for the specificity.

^d Sensitivity can not be determined as there are no true IgM positive samples from Vietnam cohort.

Table 5: Comparison of DENV IgM MIA to InBios DENV IgM capture ELISA to confirm IgM false-positive results

Cohort/Place	Total number of Samples	DENV IgM MIA	InBios DENV IgM Capture ELISA			Criteria 1 ^a		Criteria 2 ^b		Criteria 3 ^c	
			Negative	Equivocal	Positive	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Hawaii (2011- 2012)	17	Negative	9	0	0	100 (3/3)%	93 (13/14)%	100 (3/3)%	90 (9/10)%	100 (3/3)%	64 (9/14)%
		Equivocal	4	0	0						
		Positive	1	0	3						
		Total	14	0	3						
Vietnam (1998)	27	Negative	4	0	0	- ^d	27 (7/26)%	- ^d	17 (4/23)%	- ^d	15 (4/26)%
		Equivocal	3	0	0						
		Positive	19	1	0						
		Total	26	1	0						
Niue	19	Negative	2	0	0	100 (8/8)%	80 (8/10)%	100 (8/8)%	50 (2/4)%	67 (8/12) %	33 (2/6)%
		Equivocal	2	0	4						
		Positive	2	1	8						
		Total	6	1	12						

Sensitivity- $Tp / (Tp + Fn)$; Specificity- $Tn / (Tn + Fp)$. InBios IgM Pos, $ISR > 2.84$; InBios IgM equivocal, $ISR 1.65-2.84$; InBios IgM Neg < 1.65 .

^a Equivocals were considered as negative. ^b Equivocals were considered as positive.

^c Equivocal samples were considered false-negative for the sensitivity and equivocal samples were considered false-positive for the specificity.

^d Sensitivity can not be determined as there are no true IgM positive samples from Vietnam cohort.

Table 6: Detection of Syphilis antibodies in Vietnam samples tested using DENV IgM MIA

Syphilis IgG EIA	DENV IgM MIA			Total number of Samples
	Positive	Equivocal	Negative	
Positive	16 ^a	1	3	20
Equivocal	0	1	0	1
Negative	20 ^b	5	3	28
Total	36	7	6	49

^a In-house DENV IgM Capture ELISA was done for 13 samples and were negative.

^b In-house DENV IgM Capture ELISA was done for 15 samples and were negative.

Table 7: Results of MAC-ELISA and InBios DENV IgM Capture ELISA equivocal and discordant samples

Sample	MAC-ELISA		InBios DENV Detect IgM Capture ELISA				PRNT
	P/N	Results	DENRA (OD)	NCA (OD)	ISR	Results	Results
1	7.6	Pos	0.27	0.11	2.4	Equivocal	QNS
2	4.3	Pos	0.22	0.08	2.6	Equivocal	QNS
3	2.9	Presumptive Pos	0.13	0.06	2.2	Equivocal	QNS
4	2.8	Presumptive Pos	0.16	0.07	2.4	Equivocal	QNS
5	1.2	Neg	0.21	0.11	1.9	Equivocal	DENV-2 Pos
6	1.7	Neg	0.21	0.06	3.4	Pos	DENV-1 Pos
7	1.2	Neg	0.19	0.06	3.0	Pos	DENV Pos

DENRA, Dengue-derived recombinant antigens; NCA, Normal cell antigen; ISR, Immune status ratio; P/N, Positive to negative ratio; QNS - Quantity not sufficient.

InBios IgM Pos, ISR > 2.84; InBios IgM equivocal, ISR 1.65-2.84; InBios IgM Neg < 1.65

MAC-ELISA Pos, P/N ≥ 3, MAC-ELISA Presumptive Pos, P/N ≥ 2 and < 3, MAC-ELISA Neg, P/N < 2

Table 8: Results obtained for 79 samples tested by using InBios DENV Detect IgM Capture ELISA

InBios DENV IgM Capture ELISA results	Number of Samples	ISR (Ratio)	DENRA (OD)	NCA (OD)
Positive	24	12.1 (3.0 - 33.0)	0.83 (0.19 - 2.11)	0.07 (0.06 - 0.14)
Equivocal	5	2.4 (1.9 - 2.6)	0.21 (0.13 - 0.27)	0.08 (0.06 - 0.11)
Negative	50	1.0 (0.9 - 1.3)	0.07 (0.06 to 0.14)	0.07 (0.06 - 0.13)

ISR, Immune status ratio; DENRA, Dengue-derived recombinant antigens; NCA, Normal cell antigen.

InBios IgM Pos, ISR > 2.84; InBios IgM equivocal, ISR 1.65-2.84; InBios IgM Neg < 1.65. Average (Range of Min to Max).

Table 9: Calculated specificity, sensitivity, positive percent agreement, negative percent agreement, positive and negative predictive values for InBios DENV detect IgM capture ELISA in comparison to the In-house MAC-ELISA

	Criteria 1^a	In-house MAC-ELISA		Criteria 2^b	In-house MAC-ELISA		Criteria 3^c	In-house MAC-ELISA	
	n = 79	Positive	Negative	n = 74	Positive	Negative	n = 79	Positive	Negative
InBios DENV detect	Positive	22	2	Positive	22	2	Positive	22	2
IgM capture ELISA	Equivocal	0	0	Equivocal	0	0	Equivocal	2	3
	Negative	2	53	Negative	0	50	Negative	0	50
	Total	24	55	Total	22	52	Total	24	55
Sensitivity		92%			100%			-	
Specificity		96%			96%			-	
Positive percent agreement		-			-			92%	
Negative percent agreement		-			-			91%	
Positive predictive value		92%			92%			-	
Negative predictive value		96%			100%			-	

% Sensitivity, (TP/TP+FN) x 100; % Specificity, (TN/TN+FP)x 100; Positive percent agreement (PPA): 100% x TP/Total samples;

Negative percent agreement (NPA):100% x TN/ Total samples; Positive predictive value, (TP/TP+FP) x 100; Negative predictive value, (TN/TN+FN) x 100

^a Equivocal and presumptive positive samples were considered as negative. ^bEquivocal and presumptive positive samples were excluded.

^c PPA and NPA are calculated as per InBios kit insert by considering the "worst-case scenario." That is, equivocal samples were considered false-negative for the PPA and equivocal samples were considered false-positive for the NPA.

Table 10: Calculated specificity, sensitivity, percent agreement and kappa value for InBios DENV detect IgM capture ELISA comparison to the In-house MAC-ELISA

	n = 79	In-house MAC- ELISA			% sensitivity (95% CI)	% specificity (95% CI)	% agreement (95% CI)	Kappa value
		Positive	Negative	Presumptive positive				
InBios DENV detect IgM capture ELISA	Positive	22	2	0				
	Negative	0	50	0	92	94	94	0.87
	Equivocal	2	1	2	(73, 99)	(84, 99)	(86, 98)	
	Total	24	53	2				

Equivocal samples were considered false-negative for the sensitivity and equivocal samples were considered false-positive for the specificity.

InBios IgM Pos, ISR > 2.84; InBios IgM equivocal, ISR 1.65-2.84; InBios IgM Neg < 1.65

MAC-ELISA Pos, P/N ≥ 3, MAC-ELISA Presumptive Pos, P/N ≥ 2 and < 3, MAC-ELISA Neg, P/N < 2

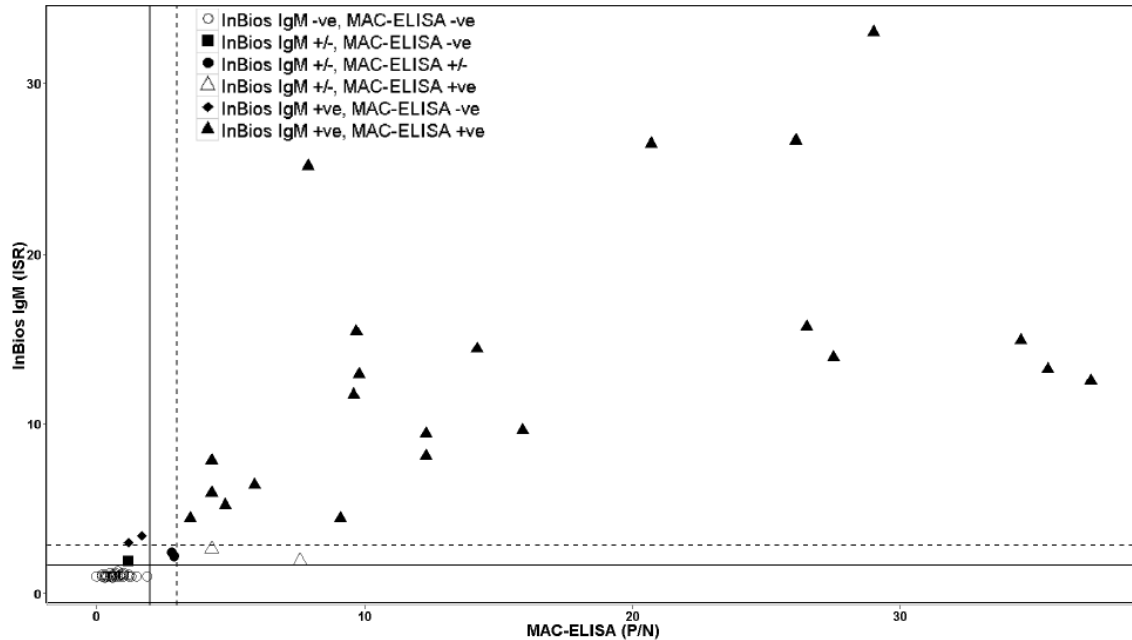


Figure 1. Correlation between the in-house DENV MAC-ELISA and InBios DENV IgM Capture ELISA. Scatter plot depicting in-house DENV MAC-ELISA versus InBios DENV IgM Capture ELISA determined using 79 human serum samples. Left of the vertical solid line are MAC-ELISA negatives, $P/N < 2$; right of the vertical dotted line are MAC-ELISA positives, $P/N \geq 3$; and all values in between the vertical solid and dotted lines are MAC-ELISA presumptive positive samples, $P/N \geq 2$ and < 3 . Below the horizontal solid line are InBios IgM ELISA negatives, $ISR < 1.65$; above the dotted horizontal line are InBios IgM ELISA positives, $ISR > 2.84$; all samples in between these two lines are InBios IgM Capture ELISA equivocal ($ISR = 1.65$ to 2.84).

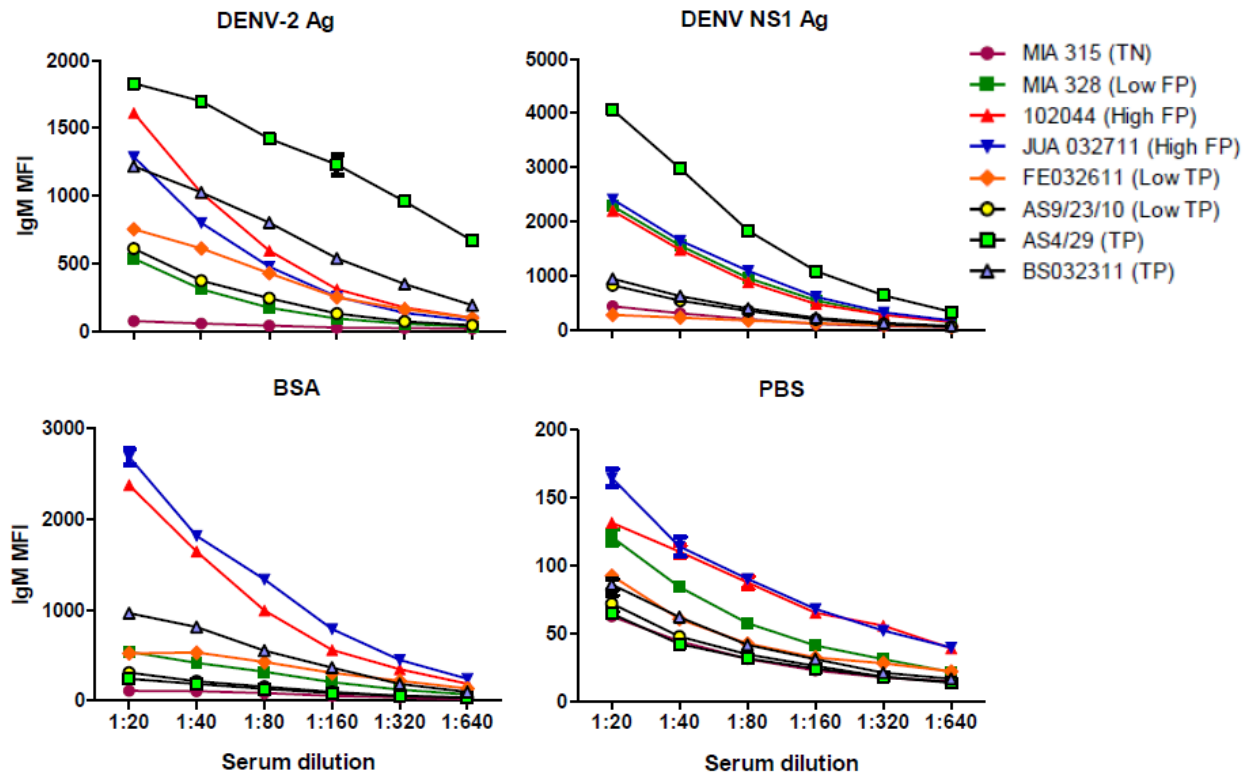


Figure 2. Effect of serum dilution on the non-specific binding observed in DENV IgM MIA.

Selected 8 Serum samples were treated with GullSORB and diluted serially from 1:20 to 1:640 using PBS-1% BSA and were tested by DENV IgM MIA for the presence of anti-DENV IgM antibodies. Data are expressed as MFI \pm SD.

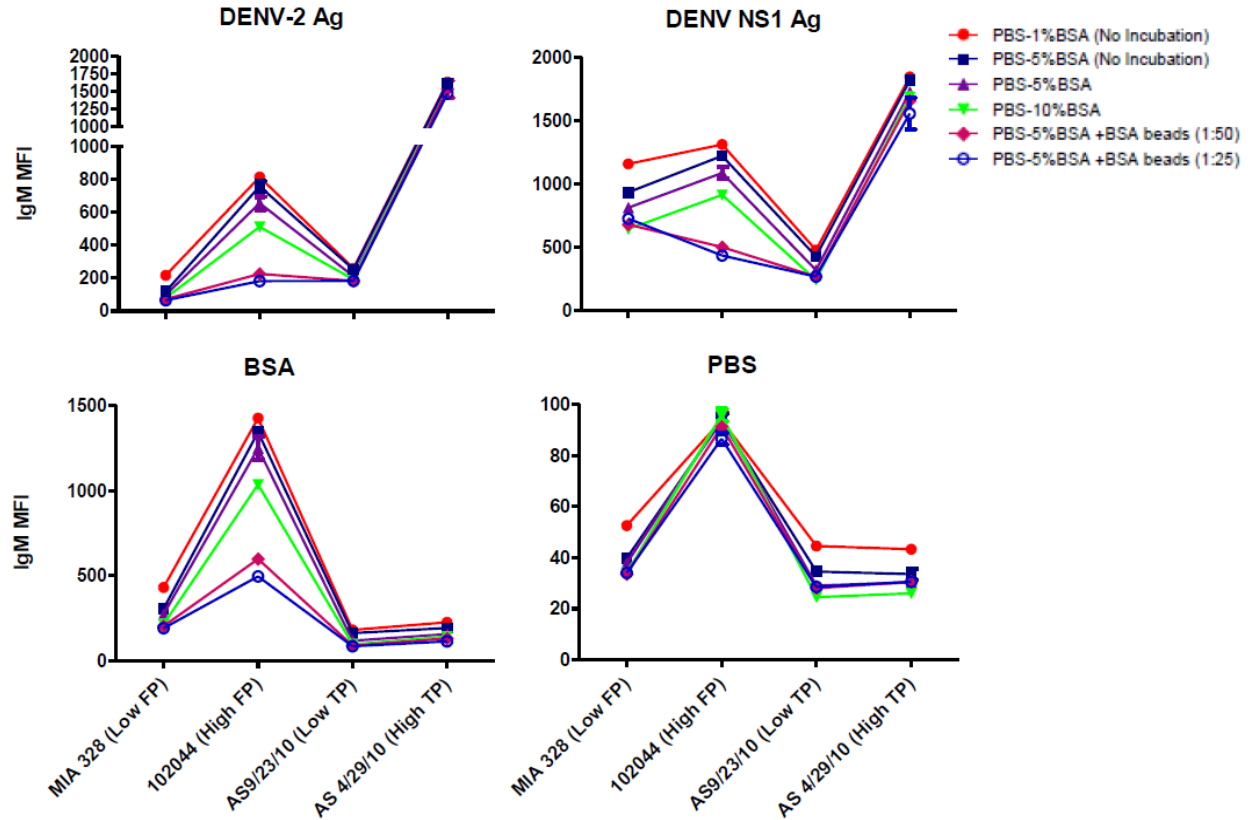


Figure 3. Effect of overnight sample pretreatment with high concentration of BSA or BSA-coated beads on the non-specific binding observed in DENV IgM MIA. Serum samples were treated with GullSORB; diluted 1:100 using higher concentration of BSA (5-10%) or BSA-coated beads in serum diluents and were kept on rotator overnight at 4°C. After incubation, samples were centrifuged at 10000g at 4°C for 20 min and we re tested by DENV IgM MIA for the presence of anti-DENV IgM antibodies. Data are expressed as MFI \pm SD.

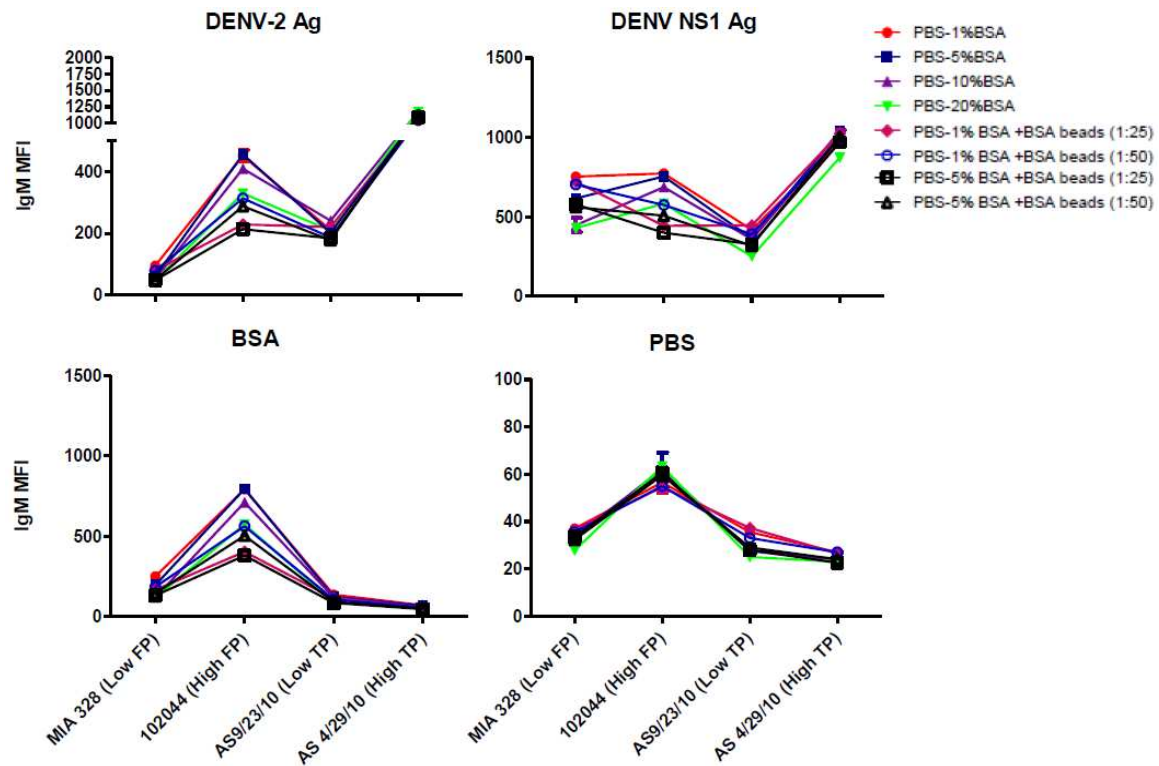


Figure 4. Effect of 1 hr sample pretreatment with high concentration of BSA or BSA-coated beads on the non-specific binding observed in DENV IgM MIA. Serum samples were treated with GullSORB; diluted 1:100 using higher concentration of BSA (5-20%) or BSA-coated beads in serum diluents and were kept on rotator for 1 hr at room temperature. After incubation, samples were centrifuged at 10000g at 4°C for 10 min and we re tested by DENV IgM MIA for the presence of anti-DENV IgM antibodies. Data are expressed as MFI \pm SD.

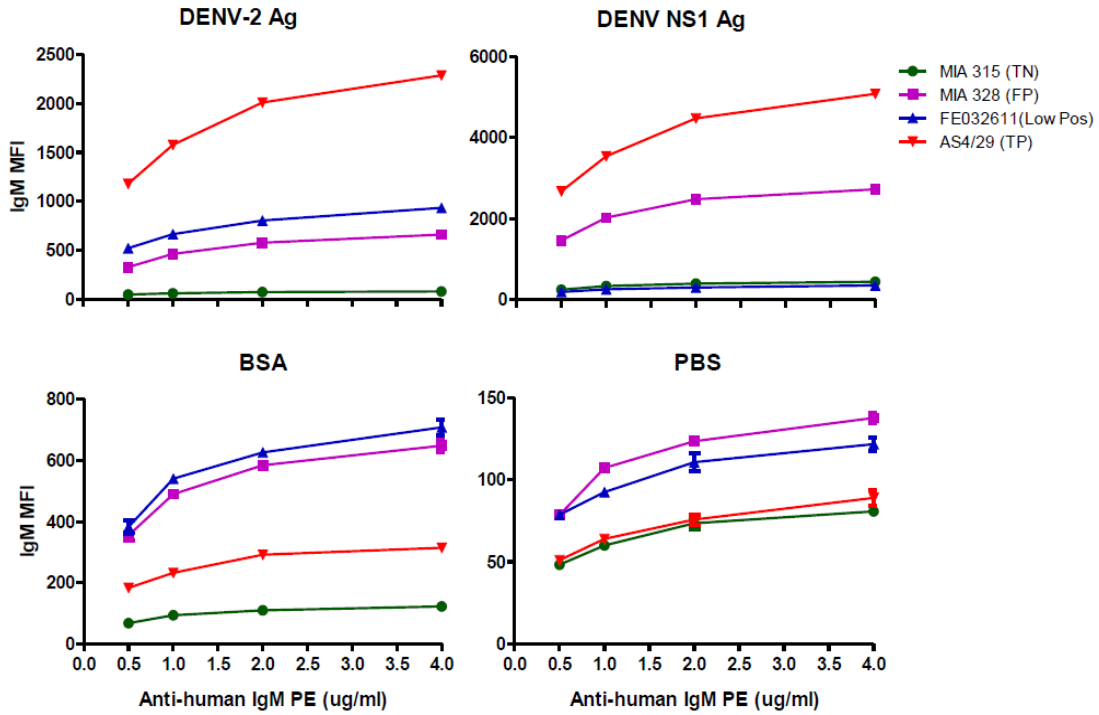


Figure 5. Effect of different concentrations of IgM secondary antibody on the DENV IgM MIA results. Serum samples were treated with GullSORB; diluted 1:100 using PBS-1% BSA and DENV IgM MIA was conducted using different concentration of donkey anti-human IgM PE from 0.5 to 4 μ g/ml. Data are expressed as MFI \pm SD.

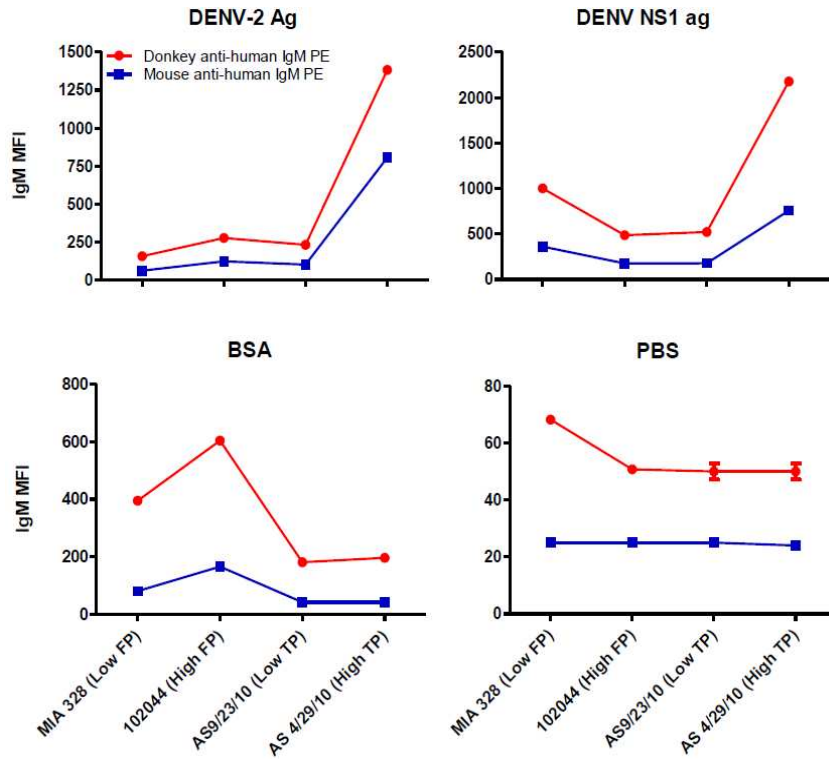


Figure 6. Comparison of DENV IgM MIA results using two different anti-human IgM PE.

Serum samples were treated with GullSORB; diluted 1:100 using PBS-1% BSA and were tested by DENV IgM MIA for the presence of anti-DENV IgM antibodies using donkey anti-human IgM PE (polyclonal) and mouse anti-human IgM PE (monoclonal). Data are expressed as MFI \pm SD.

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Chapter 5

Validation of the in-house polymerase chain reaction- microsphere bead assay (PCR-MBA) for detection of dengue virus serotypes and West Nile virus

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5.1 Abstract

In this study, the in-house newly developed Luminex-based Polymerase Chain Reaction-Microsphere Bead Assay (PCR-MBA) was further validated for differential detection of four DENV serotype-specific RNA using CDC DENV panel samples and WNV-specific RNA. The specificity of PCR-MBA for detection of four DENV serotypes from CDC DENV panel samples was found to be 100% as compared to 'gold standard' CDC DENV 1-4 real time RT-PCR assay. However, the sensitivity of PCR-MBA varied from 50-100% for DENV-1, 29-100% for DENV-2, 100% for DENV-3 and 80-100% for DENV-4. The sensitivity may be compromised due to the possible genotypic differences among DENV strains and the resulting mismatch in sequences between the amplicon and probe. PCR-MBA for detection of WNV is specific and more sensitive as compared to WNV-specific qRT-PCR. For further validation of PCR-MBA for detection of low levels of WNV viremia, panel of human WNV viremic serum specimens will be required. Moreover, PCR-MBA can be further verified for simultaneous detection of additional arboviruses including CHIKV, JEV, SLEV, WNV and YFV.

5.2 Introduction

Arthropod-borne viruses (arboviruses) are some of the most important emerging and resurging pathogens of global significance which include medically important flavivirus such as Dengue virus (DENV), West Nile virus (WNV), Yellow fever virus (YFV), Japanese encephalitis virus (JEV), St. Louis encephalitis virus

(SLEV) as well as Chikungunya virus (CHIKV) which belongs to the Alphavirus genus of the Togaviridae family [1]. Clinical diagnosis of arboviral infections is difficult because the symptoms are quite similar among different arboviruses as well as to other diseases such as enteric fever, leptospirosis, viral hepatitis, and malaria. [2]. Dengue is an acute viral disease caused by any of the four dengue virus serotypes (DENV-1, -2, -3 and -4) [3]. Dengue clinical manifestations range from asymptomatic infection to a severe disease characterized by hemorrhage and shock [4]. The emergence of WNV in the United States in 1999 resulted in dramatic increase in the severity of human infection, thus making WN viral encephalitis a public health concern [5]. Moreover, arboviruses such as DENV, WNV and CHIKV can also be acquired through blood transfusion from asymptomatic donors [6, 7]. Therefore, rapid differential diagnosis of arbovirus infections is crucial for effective patient management and implementation of control measures [1]. The current procedure for establishing a laboratory diagnosis of arbovirus infection is detection of viral nucleic acids and specific antibodies in clinical samples [8]. Virus isolation (mainly from mosquito cell lines such as *A. albopictus*), the identification by immunofluorescence assay using virus-specific monoclonal antibodies and the genomic detection by RT-PCR or real-time RT-PCR confirm the infection [9]. Serum samples collected in the first five days of fever are useful for virus detection [10]. During the febrile phase, dengue viremia has been shown to range from 10^2 to 10^7 PFU/ml depending on serotype and time after onset [11]. However, WNV-infected humans do not develop high-level viremia. Low levels of WNV (viremia) found in asymptomatic

blood transfusion or cell/tissue donors with an infection poses a health threat to recipients [12]. Various assays based on nucleic acid amplification technology (NAAT) have been developed for detection of DENV serotypes and WNV including RT-PCR [13, 14], TaqMan real-time RT-PCR [7, 15-18] and nucleic acid sequence-based amplification (NASBA) [19-21]. These assays are typically more sensitive than virus culture, but are not able to detect low levels of virus in clinical specimens obtained at day 3 to 5 of the illness, by when virus-specific antibodies are detectable [22]. In addition, there is chance of nonspecific amplification as multiple primer sets are used and thus can result in false-positives. Moreover, singleplex qRT-PCR is more prone to contamination and thus require high technical expertise [23]. Also qRT-PCR is not ideal for multiplexing due to the limited number of fluorophores. Therefore, development of rapid, specific and sensitive assays for differential detection of dengue serotypes as well as of low levels of WNV will be valuable for early diagnosis of infections and will also provide better understanding of virus dynamics in humans as well as animal models.

Luminex-based assays are being developed for rapid, high-throughput multiplexed nucleic acid detection for diagnosis of several viral and bacterial diseases [24-27]. In our laboratory, Luminex-based Polymerase Chain Reaction-Microsphere Bead Assay (PCR-MBA) was developed for differential detection of WNV, DENV serotypes with the future potential of multiplexing other medically important arboviruses. PCR-MBA employs a single set of pan-flavivirus primers, a single step reverse transcriptase-polymerase chain reaction (RT-PCR) step,

serotype-specific probes and the Luminex platform to detect and differentiate between DENV serotypes. In this study, this assay was further validated using CDC DENV panel samples for accurate detection of DENV serotypes as comparison to CDC DENV-1-4 Real-Time RT-PCR Assay which has been approved by the FDA for detection of DENV1-4 serotypes in serum or plasma samples from suspected, symptomatic cases for use in the United States. The key criteria that are critical in validation of any newly developed virus detection assay are sensitivity and specificity [28]. Sensitivity is a measure of the limit of detection of the assay that is how few viral targets are necessary to produce a positive result. Whereas the specificity is a measure of the ability of the assay to specifically amplify and detect a particular viral RNA while producing negative results with other nucleic targets [29]. It is also important that assay should be able to detect all the strain variants of viral target [28].

5.3 Materials and Methods

CDC DENV panel samples

DENV- 1 to 4 serotypes; 10 samples for each serotype ($n = 40$), proficiency panel specimens ($n = 12$), Limit of detection (LOD) panel samples; 8 samples for each serotype ($n = 32$) were obtained from the Dengue Branch, Centers for Disease Control and Prevention (CDC), San Juan, Puerto Rico. All assays were conducted in a blinded fashion using numerically coded serum or plasma samples.

RNA extraction

RNA was extracted from 70 or 140 μ L of human serum samples or virus-infected tissue culture supernatants of four different DENV serotype strains [DENV-1 (Hawaii), DENV-2 (NG'C"), DENV-3 (H 87) and DENV-4 (H 241) grown in Vero cells] which were employed as positive control, using the QIAamp viral RNA kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions.

CDC Quanta qRT PCR

Singleplex CDC DENV 1-4 Real time RT-PCR was conducted using Quanta biosciences™ qScript™ One Step qRT-PCR kit as described previously [16]. Briefly, 5 μ L of RNA was combined with 25 pmol of each primer and 4.5 pmol of the probe in a 25 μ L of Quanta biosciences™ qScript™ One Step qRT-PCR kit. Each reaction mixture contained a single DENV serotype primer pair and probe; therefore, in singleplex assays four separate reactions were carried out for each RNA sample. Reverse transcription of 30 min at 50°C was followed by 45 cycles of amplification in an Icyler iQ Real-Time Detection System to Quanta One-Step RT-PCR kit instructions for real-time RT-PCR conditions and using a 60°C annealing temperature.

WNV qRT PCR

WNV RNA was extracted from Lineage I WNV strain NY99 originally isolated from a crow brain in New York which was further propagated in Vero cells (5×10^8 PFU/mL). cDNA was prepared using iScript™ cDNA Synthesis Kit (Bio-Rad, CA) according to the manufacturer's protocol. To determine the limit of detection

(LOD) and specificity of PCR-MBA for detection of WNV, 10-fold serial dilutions of cDNA was prepared with concentrations ranging from 3×10^7 PFU/mL to 0.3 PFU/mL. WNV specific qRT-PCR was conducted using primers and probes directed against 3' non-coding region of WNV genome as described previously [15].

PCR-MBA

Primers and probes were directed against the RNA-dependent RNA polymerase (RdRp) domain located at the carboxy terminus of nonstructural protein 5 (NS5) which is the most conserved coding region in the *Flavivirus* genomes and against *E1* region for Chikungunya virus as described previously [17, 30, 31]. For Luminex assay, reverse primers were labeled with biotin at 5' end and arbovirus probes were modified with C12 amino site on 5' end. Briefly, a one-step RT-PCR reaction was conducted using an asymmetric PCR with 1:5 forward: reverse flavivirus or CHIKV primers. The PCR cycling conditions consisted of an initial 50°C for 30 min; 95°C for 15 min followed by 35 cycles of 94°C for 30s, 52°C for 30s, 72°C for 1 min with a final 10 min extension at 72°C. Positive and negative control RNA samples as well as a negative control (nuclease-free water) were included on each 96-well plate. Probes were coupled to carboxylated Luminex beads with EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) using Luminex protocol. Hybridization of coupled oligo beads to the PCR products was conducted at 45°C for 15 min using the oligonucleotide hybridization protocol from Luminex Corporation. Detection of the amplicon was

conducted by the Luminex 100 (Luminex Corporation, Austin, TX) after addition of streptavidin PE as the reporter molecule. Results were reported as median fluorescent intensity (MFI). Negative control (nuclease-free water) and positive controls were included with each run. MFI cut-offs were established based on data analysis of 57 healthy controls tested in duplicates and final MFI cutoff was calculated using mean MFI \pm 3 standard deviation.

Statistical analysis

Microsoft Excel and GraphPad Prism 5.0 software was used for statistical analysis. Sensitivity and specificity of PCR-MBA for detection of DENV serotypes and WNV was calculated using quantitative real time RT-PCR as the 'gold standard'.

5.4 Results and Discussion

Sensitivity of PCR-MBA for detection of DENV serotypes

PCR MBA demonstrated varied sensitivity of 50-100% for DENV-1, 29-100% for DENV-2, 100% for DENV-3 and 80-100% for DENV-4 for detection of DENV serotypes as compared to CDC DENV 1-4 real time RT-PCR assay (Table 1, 2 and 3). The lower sensitivity of PCR-MBA for detection of DENV-1 and- 2 may be due to the mismatch in sequences between the amplimer and probe as a result of continual evolution of viral RNA. DENV is a positive strand RNA virus and thus has high potential for mutation which results in genotypic differences. To detect all genotypes and strains within a DENV serotype by PCR-MBA, additional

probes that would bind to these new DENV genotype-specific sequences can be included in the assay. In addition, the sensitivity can also be affected by various factors including the copy number of the amplification target; primer selection; the method of amplification as well as the method used for detection [28]. Due to the strong affinity of biotin to streptavidin PE used for detection in PCR-MBA, the detection of biotinylated molecules is very sensitive. However, PCR-MBA can be further optimized for improved sensitivity for detection of dengue serotypes and WNV by changing the concentration of probe coupled, concentration of the input RNA, hybridization temperature and time. In addition, proper transport and storage of serum specimens is also required for viral detection by nucleic-acid based assays as otherwise it may lead to virus inactivation and thus false-negative results.

Specificity of PCR-MBA for detection of DENV serotypes

The specificity of PCR-MBA for detection and differentiation of four DENV serotypes from CDC DENV panel samples was found to be 100% as compared to CDC DENV 1-4 real time RT-PCR assay (Table 1 and 2). The specificity of the PCR-MBA can be affected by the ability to distinguish dengue viruses from other flaviviruses; the uniqueness of the target sequence, specifically for dengue genotyping; and false-positives resulting from contamination [28]. Low background MFI was observed for the arboviruses included in PCR-MBA and thus indicated negligible probe cross reactivity for the DENV serotypes.

PCR-MBA for detection of WNV

PCR-MBA for detection of WNV demonstrated a lower limit of detection of 3×10^1 PFU/ml which was equivalent to that of real-time PCR assay (Table 4). However, as the WNV cDNA concentration was decreased, there was corresponding increase in Ct value, but the MFI values did not show similar decrease, thus indicating improved detection of WNV by PCR-MBA, even at lower concentration of WNV cDNA. The quantitative relationship between real-time PCR Ct values and PCR-MBA MFI for detection of WNV is shown in Figure 1. There was good correlation observed between real-time qRT-PCR and PCR-MBA for detection of WNV ($R^2 = 0.92$). Moreover, PCR-MBA specifically detected WNV as the background MFI for other arboviruses were below the respective cut-off values (Table 5). PCR-MBA if optimized using cycling as well as hybridization conditions may further improve the sensitivity for detection of low levels of WNV (viremia) found in human serum samples after infection. For further validation of PCR-MBA for detection of WNV, panel of human viremic samples will be required.

5.5 Conclusions

PCR-MBA efficiently detected and differentiated the DENV serotypes from CDC DENV panel samples as well as WNV. The optimization of cycling and hybridization conditions may further improve the specificity and sensitivity of PCR-MBA for detection of DENV serotypes and WNV. The probes included in the PCR-MBA should be routinely checked against all available sequences periodically to ensure that the PCR-MBA will detect newly circulating DENV

genotypes. Multiplexed probes designed for other arboviruses did not result in false-positives for dengue positive samples and for WNV. PCR-MBA thus has the future potential of multiplexing other arboviruses. It will be helpful in regions where multiple flaviviruses are co-circulating. A large number of viremic samples from a panel of arboviruses (DENV-1, -2, -3, -4, CHIKV, JEV, SLEV, WNV and YFV) would be required to validate the entire panel of arboviruses included in the PCR-MBA.

Table 1: Comparison of San Juan dengue samples qRT-PCR* results to PCR-MBA

DENV-1 ^a			DENV-2 ^b			DENV-3 ^c			DENV-4 ^d		
Sample ID	PCR-MBA	qRT-PCR	Sample ID	PCR-MBA	qRT-PCR	Sample ID	PCR-MBA	qRT-PCR	Sample ID	PCR-MBA	qRT-PCR
	MFI	CT value		MFI	CT value		MFI	CT value		MFI	CT value
E	10980	16	i	262	28	B	14757	22	J	13598	20
D	10103	22	J	249	29	G	11752	26	A	13563	20
J	10066	22	B	121	36	A	7173	30	G	13461	19
G	9786	23	D	44	33	J	1234	31	B	13032	23
B	9014	24	F	38	37	E	634	35	H	12744	22
A	7043	25	H	38	33	i	572	33	D	12202	24
i	3643	27	A	24	36	H	289	34	i	3545	27
F	3380	27	G	22	34	C	107	37	E	1733	30
C	395	33	E	22	26	F	78	40	F	97	41
H	110	37	C	21	21	D	14	40	C	85	37
Cut-offs [#]	360			135			195			2311	

Positive readings are highlighted in bold. Ct, Cycle Threshold.

*CDC Quanta Real time RT-PCR protocol was used for detection of 4 DENV serotypes. Positive: Ct value < or = 35; Negative: Ct value > 35.

[#]MFI cut-offs were established based on 57 healthy controls and was part of E. Volper's thesis. MFI values above the respective cut-offs were considered positive.

^aSensitivity, 100%; specificity, 100%

^bSensitivity, 29%; specificity, 100%

^cSensitivity, 100%; specificity, 100%

^dSensitivity, 87%; specificity, 100%

Table 2: Evaluation of PCR-MBA using dengue proficiency panel samples from CDC

#	Sample ID	DENV serotype	PCR-MBA (MFI)				Result	qRT-PCR*	
			DENV-1 ^a	DENV-2 ^b	DENV-3 ^c	DENV-4 ^d		CT value	Result
1	A1	DENV-1	2564	74	55	68	Pos	20	Pos
2	T3	DENV-1	278	121	76	94	Neg	28	Pos
3	D5	DENV-1	107	81	58	80	Neg	37	Neg
4	F10	DENV-2	114	1538	74	71	Pos	25	Pos
5	R12	DENV-2	148	115	77	76	Neg	33	Pos
6	B17	DENV-3	117	142	12385	252	Pos	21	Pos
7	O19	DENV-3	125	103	2575	101	Pos	29	Pos
8	S21	DENV-3	145	117	75	93	Neg	37	Neg
9	X26	DENV-4	155	148	64	12526	Pos	24	Pos
10	I28	DENV-4	131	104	68	2487	Pos	31	Pos
11	K33	NEG Control	119	100	64	82	Neg	39	Neg
12	G34	NEG Control	106	95	70	84	Neg	36	Neg
Cut-offs [#]			360	135	195	2311		35	

Positive readings are highlighted in bold. Ct, Cycle Threshold.

*CDC Quanta Real time RT-PCR protocol was used for detection of 4 DENV serotypes. Positive: Ct value < or = 35; Negative: Ct value > 35.

[#] MFI cut-offs were established based on 57 healthy controls and was part of E. Volper's thesis.

[#] MFI values above the respective cut-offs were considered positive.

^aSensitivity, 50%; specificity, 100%

^bSensitivity, 50%; specificity, 100%

^cSensitivity, 100%; specificity, 100%

^dSensitivity, 100%; specificity, 100%

Table 3: Evaluation of PCR-MBA using dengue limit of detection (LOD) panel samples from CDC

Dilution	DENV-1 ^a		DENV-2 ^b		DENV-3 ^c		DENV-4 ^d	
	PCR-MBA	qRT-PCR*	PCR-MBA	qRT-PCR	PCR-MBA	qRT-PCR	PCR-MBA	qRT-PCR
	MFI	Ct value	MFI	Ct value	MFI	Ct value	MFI	Ct value
1	2944	20	7255	20	12318	21	12582	19
1/10	750	23	1808	23	10689	24	12380	22
1/100	379	26	663	27	4807	28	10253	26
1/1000	148	30	240	30	1046	31	2050	29
1/10000	111.75	33	143	33	254	34	1176	31
1/100000	139	35	92	36	85	37	492	37
1/1000000	233	NEG	60	NEG	129	NEG	120	NEG
1/10000000	258	NEG	106	NEG	89	NEG	81	NEG
Cut-offs [#]	360		135		195		2311	

Positive readings are highlighted in bold. Ct, Cycle Threshold.

*CDC Quanta Real time RT-PCR protocol was used for detection of 4 DENV serotypes. Positive: Ct < or = 35; Negative: Ct > 35.

[#]MFI cut-offs were established based on 57 healthy controls and was part of E. Volper's thesis.

[#]MFI values above the respective cut-offs were considered positive.

^aSensitivity, 50%

^bSensitivity, 100%

^cSensitivity, 100%

^dSensitivity, 80%

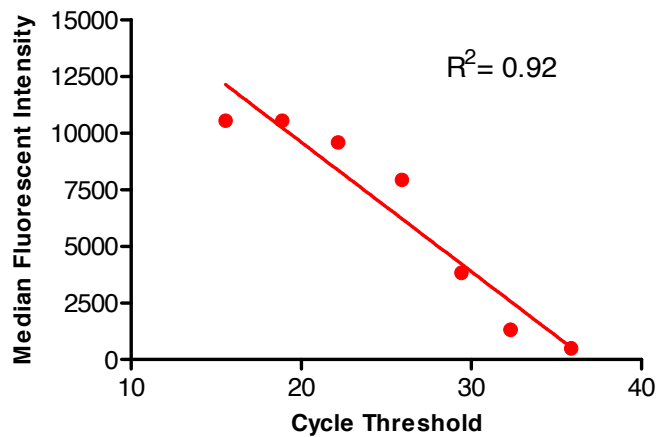


Figure 1. Correlation of WNV specific qRT-PCR and PCR-MBA for detection of WNV. WNV cDNA was diluted serially (10-fold dilutions) from 3×10^7 PFU/ml to 0.3 PFU/ml and WNV specific qRT-PCR and PCR-MBA was conducted. Data was analyzed to assess the sensitivity and specificity of PCR-MBA for detection of WNV.

Table 4: Sensitivity of PCR-MBA for detection of WNV

WNV Titer (PFU/mL)	WNV ¹ cDNA Dilutions	qRT-PCR		
		CT value	WNV titer (PFU/ml)	PCR-MBA MFI
3 x 10 ⁷	Undiluted	16	2.88E+07	10545
3 x 10 ⁶	1/10	19	2.75E+06	10546
3 x 10 ⁵	1/100	22	2.75E+05	9585
3 x 10 ⁴	1/1000	26	1.98E+04	7927
3 x 10 ³	1/10000	29	1.71E+03	3828
3 x 10 ²	1/100000	32	2.25E+02	1323
3 x 10 ¹	1/1000000	36	1.84E+01	500
3 x 10 ⁰	1/10000000	N/A	N/A	148
3 x 10 ⁻¹	1/100000000	N/A	N/A	150

CT, Cycle Threshold; qRT-PCR, Quantitative real-time reverse transcriptase PCR; MFI cut-off for WNV- 161.

PCR-MBA, Polymerase Chain reaction- Microsphere Bead Assay; MFI, Median Fluorescent Intensity.

¹ Lineage I WNV strain NY99 was originally isolated from a crow brain in New York and further propagated in Vero cells. Positive readings are highlighted in bold.

Table 5: Specificity of PCR-MBA for detection of WNV

Sample/Virus	Median Fluorescent Intensity								
	YFV	SLEV	CHIKV	DENV-1	DENV-2	DENV-3	DENV-4	JEV	WNV
Coupled beads	13	14	9	19	9	14	10	21	17
Coupled beads+ PCR water product	3	19	58	19	14	9	24	14	13
EV (DENV Neg control serum)	10	15	63	22	22	8	26	12	19
ASD11 (DENV-2 Positive serum)	33	30	48	27	3332	48	1138	38	22
DENV-1 Hawaii strain	22	24	64	8222	33	17	49	27	31
DENV-2 NG ¹ C ¹ strain	131	38	77	43	7998	177	529	74	35
DENV-3 H87 strain	32	32	72	63	91	11461	301	85	47
DENV-4 H241 strain	222	31	70	42	238	25	10520	58	45
Undiluted WNV ¹ cDNA	69	68	118	111	91	58	86	79	10545
1/10, WNV cDNA	69	71	125	111	89	63	77	75	10546
1/100, WNV cDNA	67	78	116	112	96	56	79	62	9585
1/1000, WNV cDNA	74	71	121	125	100	67	79	66	7927
1/10000, WNV cDNA	75	85	128	130	102	69	78	72	3828
1/100000, WNV cDNA	69	77	129	141	99	62	79	65	1323
1/1000000, WNV cDNA	76	91	138	149	107	74	91	78	500
1/10000000, WNV cDNA	78	98	153	170	123	80	99	89	148
1/100000000, WNV cDNA	88	92	137	170	121	74	84	91	150
Cut-offs ²	189	189	200	360	135	195	2311	128	161

Positive readings are highlighted in bold. WNV, West Nile virus; DENV, Dengue virus.

¹ Lineage I WNV strain NY99 was originally isolated from a crow brain in New York and further propagated in Vero cells

² MFI cut-offs were established based on 57 healthy controls and was part of E. Volper's thesis and values above cut-offs were considered positive

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Chapter 6

Summary, Conclusion and Future Directions

The WNV Envelope protein-Microsphere Immunoassay (WNV E-MIA) optimized in this study is robust, sensitive and high throughput for detection of anti-WNV IgM and IgG antibodies particularly at early and late time-points after WNV infection in mice. This optimized WNV E-MIA can be used to study WNV-specific IgM and IgG antibody response in various mice models and can detect the WNV-specific IgG antibodies in mouse hybridomas. Moreover, these data strongly suggest that heat inactivation (HI) of serum and optimized serum dilution should be considered as important parameters during development and optimization of other Luminex-based MIAs. Validation of DENV MIA using different serum panels demonstrated that sensitivity and specificity of immunoassays may differ according to the origin of samples. Therefore, it is important that newly developed assays should be validated using serum samples from different populations or geographic regions. Moreover, the results of this study conclude that the sample pretreatment or the use of alternative blocking agents in assay buffers will vary for immunoassays. Therefore, it is important to optimize the blocking conditions for newly developed immunoassays to improve the assay specificity. In addition, U.S. FDA approved InBios DENV Detect IgM Capture ELISA which is used as a reference standard for detection of anti-DENV IgM antibodies in this study can be used by public health laboratories for rapid and

accurate detection of dengue infections during dengue epidemics, as it demonstrated good sensitivity and specificity for detection of dengue IgM antibodies. PCR-MBA efficiently detected and differentiated DENV serotypes and WNV. However, the optimization of cycling and hybridization conditions may further improve the specificity and sensitivity of PCR-MBA for detection of DENV serotypes and WNV. For further validation of PCR-MBA for detection of low levels of WNV viremia, panel of human WNV viremic serum specimens will be required. PCR-MBA can be further verified for simultaneous detection of additional arboviruses including CHIKV, JEV, SLEV and YFV.