**DISSERTATION**

# CHARACTERIZATION OF EPITOPE-BLOCKING ELISA FOR DIFFERENTIAL DIAGNOSES OF SECONDARY FLAVIVIRUS INFECTIONS

**Submitted by**

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**WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY MARÍA ALBA LOROÑO PINO "CHARACTERIZATION OF EPITOPE-BLOCKING ELISA FOR DIFFERENTIAL DIAGNOSIS OF SECONDARY FLAVIVIRUS INFECTIONS" BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.**

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**ABSTRACT OF DISSERTATION**

**"CHARACTERIZATION OF EPITOPE-BLOCKING ELISA FOR DIFFERENTIAL DIAGNOSES OF SECONDARY FLAVIVIRUS INFECTIONS"**

West Nile virus (WNV) and Dengue virus (DENV) infections cause significant public health and animal health problems in countries around the world. Accurate laboratory results and diagnoses are essential elements of effective treatment of patients. On a broader scale, accurate diagnoses are critical for public health officials to select appropriate control and prevention measures. However, accurate diagnoses of WNV and DENV infections are currently complicated in areas where multiple flaviviruses circulate.

To address this complication, the dissertation project investigated the ability of WNV-specific monoclonal antibodies to compete actively in binding the epitopes on the NS1 peptides and to distinguish between antibodies induced by different flaviviruses. Developing a test distinguish between antibodies to different flaviviruses would significantly improve differential diagnostic capabilities, and reduce false positive WNV diagnoses for humans and horses potentially infected by other flaviviruses.

For the diagnosis of WNV infections in humans, an epitope-blocking enzyme-linked immunosorbent assay (b-ELISA) using the WNV-specific monoclonal antibody (MAb) 3.1112G and the flavivirus-specific MAb 6B6C-1 was evaluated. Sera from patients previously diagnosed with WNV infections

and with dengue infections were tested. The WNV- specific b-ELISA was efficacious in diagnosing WNV infections in humans with primary infections. The sensitivity and specificity of this test were 90.8% and 91.9%, respectively. However, in tropical regions where people have experienced multiple flavivirus infections, the use of the b-ELISA for WNV diagnosis is contraindicated, due to the 80% false positive rate using this protocol.

Arrays of synthetic peptides of the non-structural-1 (NS1) and the envelope (E) proteins of WNV were also evaluated as diagnostic reagents for peptide-based ELISA for WNV. All WNV peptides investigated accurately diagnosed WNV infections; however, the WNV NS1-1 peptide was found to be the best peptide to distinguish between recent dengue infections and sera classified as negative for flavivirus infections.

Finally, b-ELISA was evaluated for its ability to detect antibodies to WNV in 14 horses sequentially infected with WNV and SLEV, SLEV and WNV, or DENV and WNV. The sensitivity and specificity of b-ELISA for detecting antibodies to WNV were 90.9% and 91.7%, respectively in these test results. B-ELISA was specific for detecting antibodies to WNV.

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# CHAPTER 1

# LITERATURE REVIEW

## 1.1. INTRODUCTION.

The introduction of West Nile virus (WNV) into New York and its subsequent spread throughout the American Hemisphere is a textbook example of the epidemic potential of an emerging virus disease. However, the epidemiological consequences of the introduction and spread of the virus in the Americas has differed dramatically in temperate and tropical regions. In the US and Canada, the introduction of WNV was a public and veterinary health disaster, which resulted in significant morbidity and mortality in humans, equines, and birds. In contrast, WNV introduction into tropical regions of the Americas has not resulted in public and veterinary health problems. Indeed, there have been no diagnoses of human WNV infections in tropical America, even in areas and cities where the virus has been demonstrated to be circulating in birds and equines. The reasons for the disparate outcomes remain to be determined. The principal hypotheses for the lack of human cases in tropical America include: 1) the attenuation of WNV as it trafficked from North to Central America in migratory birds, and 2) the protective effect of pre-existing antibodies to other flaviviruses, especially dengue virus, in the human populations. Investigating the latter hypothesis in tropical American countries has been difficult because of the the extensive cross reactions among flaviviruses in serological diagnostic tests and the Original Antigenic Sin (OAS)

phenomenom associated with sequential flavivirus infections. Indeed, WNV infections in humans with previous dengue infections (the majority of the people in many tropical areas) could be being misdiagnosed as dengue infections because of OAS and/or serological cross reactions. Development of a rapid and inexpensive serologic test capable of the differential diagnosis of flavivirus infections, especially secondary flavivirus infections, which could be used in disease endemic countries would be a significant public health contribution. The overarching goal of this dissertation was to determine if the the highly specific b-ELISA test, which is based upon serologic recognition of a specific a West Nile Virus NS1 epitope, could be exploited for the differential diagnosis of flavivirus infections in humans. The diagnostic efficacy of the test was determined and the tests characterized using human specimens from flavivirus-endemic and naïve regions of the world and serum specimens from sequentially infected horses. The potential use of WNV synthetic peptides for differentional diagnosis in a peptide based ELISA was also evaluated.

Many excellent review articles concerning WNV have been published recently that are pertinent to this dissertation (11, 13, 71, 91, 142, 147). In this chapter, some of the major issues regarding WNV will be reviewed including: the characteristics of the introduction and spread of WNV into the Western Hemisphere, the molecular biology of WNV, the epidemiology of WNV, WNV pathogenesis in humans and other animals, the human and horse immune responses to infections with the virus, and the progress in diagnostic procedures for WNV (91, 113, 142, 207). Essential information on dengue virus will also be reviewed, including: a brief overview of the history of dengue virus in the New World, the serological responses of humans to dengue virus

infections, and the current methods used for diagnosing dengue infections. Then problems associated with the differential diagnosis of flavivirus infections and the critical need for new diagnostics will be reviewed. Finally, the goals and specific aims of this research program in terms of developing and characterizing a test for the differential diagnosis of West Nile and dengue virus infections in regions of the world where multiple flaviviruses circulate will be presented.

## 1.2. WEST NILE VIRUS.

West Nile Virus (WNV) is an arthropod-borne virus (arbovirus). The arboviruses are a group of over 530 viruses that replicate in and are transmitted by arthropods. Most arboviruses that affect humans are part of the families: *Flaviviridae, Togaviridae, Bunyaviridae, Reoviridae and Rhabdoviridae.* The family *Flaviviridae* contains 73 members, of which half have been associated with disease in humans and other animals, for example dengue fever, yellow fever, West Nile encephalitis, Japanese encephalitis, St. Louis encephalitis, and tick-borne encephalitis. Flaviviruses are separated into eight antigenic complexes based on their cross-neutralization reactions with polyclonal hyperimmune ascitic fluids against each member of the group (25). These complexes are: Japanese encephalitis (10 viruses), tick-borne encephalitis (12), Rio Bravo (6), dengue (4), Uganda S (4), Modoc (5), Tyuleniy (3) and Ntanya (5). The remaining viruses have not been assigned to any of the complexes (24). The research in this dissertation will mainly focus on West Nile virus (WNV), a member of the Japanese encephalitis complex, and the 4 members of the Dengue virus complex (DEN 1-4).

### 1.2.1. Emergence of West Nile Virus in North America.

West Nile virus (WNV) was first isolated in 1937 from a febrile woman in the West Nile province in Uganda (191). Sequence analysis of many isolates of WNV from different regions and different sample origins has identified two lineages: lineage 1 (containing 4 clades) includes pathogenic strains, including the viruses associated with human diseases from around the world; whereas lineage 2 (with 1 clade) includes only strains maintained in enzootic foci in Africa and Madagascar that do not cause severe human diseases (125).

WNV is commonly found in Africa, West Asia, India, the Middle East, Europe, Australia and now the United States (90). The virus infects mosquitoes, birds, humans, horses and many other animals (92, 121, 147). At least 60 species of mosquitoes have been implicated as vectors of WNV (71, 219), and the numbers are still increasing.

The first outbreak of West Nile virus (WNV) encephalitis in the United States of America occurred in July of 1999 (7). WNV has subsequently infected thousands of humans, tens of thousands of birds, and a broad range of other animal species. WNV spread throughout the Western Hemisphere with remarkable speed. After introduction into the northeast region of North America in 1999 (128), WNV moved rapidly from east to west and later south through the entire hemisphere (117), reaching Argentina in 2006 (153). From 1999 through 2007, there were 27,379 recorded human cases and 25,323 recorded horse cases of WNV infections reported in the U.S. (Table 1.1.1). Individuals from more than 290 species of birds and 30 species of mammals have been reported to be infected with the virus. Transmission of WNV in the US has been most frequent during the warm months of July through October. The public health

importance of WNV has motivated many groups of scientists in the government, academic, and private sectors to study the virus.

**Table 1.1.1 Human and horses cases of West Nile virus infections in United States, 1999-2007.**

| YEAR | HUMAN CASES [a] | HORSES CASES [b] |
|---|---|---|
| 1999 | 62 | 25 |
| 2000 | 21 | 60 |
| 2001 | 66 | 738 |
| 2002 | 4,156 | 15,257 |
| 2003 | 9,862 | 5,181 |
| 2004 | 2,539 | 1,406 |
| 2005 | 3,000 | 1,088 |
| 2006 | 4,269 | 1,086 |
| 2007 | 3,404 | 484 |
| Total | 27,379 | 25,325 |

[a] Data obtained from http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm
[b] Data obtained from http://www.aphis.usda.gov/vs/nahss/wnv/wnv_distribution_maps.htm

### 1.2.2. Bird deaths in the Bronx Zoo.

In July 1999, captive exotic birds and non-captive free-flying wild crows at the Bronx Zoo in New York started dying or getting sick with encephalitis. In response to the unexplained deaths, a veterinary pathologist at the zoo, Dr. Tracey MacNamara, started studying samples from dead and dying birds and sought help from other experts in determining the cause of the disease and death of the birds. Also, an outbreak of

human arboviral encephalitis was identified in August in neighboring counties in New York state (7). Sequences of a virus RNA similar to Kunjin-West Nile virus were amplified from four brain samples from fatal encephalitis patients using several degenerate primer sets for the NS3 gene (21). Scientists from several US agencies subsequently joined the search for solutions to solve the mystery of the bird deaths. Scientists at the US Army Medical Research Institute of Infectious Diseases ruled out Eastern equine encephalitis virus as the causal agent. Other tests suggested that the Saint Louis Encephalitis virus was the probable cause (53). Researchers at the US Department of Agriculture National Veterinary Services Laboratory isolated a virus from birds, but they could not identify it. Finally, researchers from the Division of Vector-Borne Infectious Disease at the Centers for Disease Control and Prevention unequivocally identified the virus as West Nile (128). This finding was subsequently corroborated by other agencies. During the same time frame, dozens of human encephalitis cases were detected in the same area, and immediate mosquito control measures were instituted in New York City. Studies were promptly initiated to identify vectors and vertebrate hosts of the virus. Many different species of mosquitoes were tested in laboratory studies to determine which specie(s) could be implicated in the transmission and spread of WNV (206). Wild birds and other animals in New York and New Jersey were investigated for WNV infections (147). Detailed information on these initial studies on the captive wildlife populations in the Bronx zoo, mosquito species, and the original WNV isolates has been published (128, 139, 148, 196, 206, 207).

After the initial studies, many other scientists started research programs to answer questions about WNV including: the effects of WNV in humans, wild life, and

domestic animals, virulence of the virus, vector competence, the molecular genetic characteristics of WNV, and the sensitivity, specificity, and selectivity of WNV identification methods. During the intervening years a remarkable amount of information has been published regarding the characteristics of WNV, its vectors, WNV diagnosis, and other factors involving transmission and control (67). Even so, many aspects involving the virus, vectors, hosts, and their interactions remains either unknown or only partially characterized. Some of the remaining questions include: What mechanisms condition the trafficking of the virus in the Western Hemisphere? What vectors and reservoirs are involved in the transmission of the virus in each country? Is WNV evolving under the selection pressures of infecting different vectors and hosts? How specific or selective are the current diagnostic tests in areas where several flaviviruses circulate? What is the effect of the high prevalence of dengue antibodies in dengue endemic populations on WNV infections? And how effective are current vector control strategies in preventing outbreaks or in diminishing the magnitude of potential epidemics?

### 1.2.3. Transmission, dissemination, and establishment of West Nile virus in USA.

The main actors involved in the effective transmission, rapid dissemination and final establishment of the WNV in the US were determined to be mosquitoes and birds. The principal mosquito vectors were *Culex pipiens* (ornithophilic) and *Cx. tarsalis* (general feeder) (8). Many species of birds were found to be extremely susceptible to WNV infections. The American crow (*Corvus brachyrhychos*), the blue jay (*Cyanocitta cristata),* and other members of the Corvidae family were found to be exquisitely susceptible to WNV infection, typically developed high titered viremias, and

experienced high fatality rates after infection (47, 116). The high temperatures during 1999 summer in New York probably facilitated and enhanced the intensity of transmission and dissemination of WNV. Higher temperatures favor the increase of mosquito vector populations, increase mosquito metabolism and blood feeding, and decrease the extrinsic incubation period of the virus. The presence and concentration of large numbers of two species of birds in NYC that were extremely susceptible to WNV infections, (the American crow and the blue jay), also promoted the rapid spread of WNV (118). In addition to the presence of susceptible vector hosts, intensity of virus transmission depends on: the number of mosquito species participating as vectors, the densities of populations of those mosquito vectors, the feeding behaviors of the infected vectors, and the local ecological determinants of exposure to mosquitoes. WNV transmission rates increase during the warmer months (92).

The rapid and widespread dissemination of the virus in the Western Hemisphere has been found to depend mainly on birds. Birds are highly competent reservoirs and amplifiers for mosquito infection with WNV. Birds spread the virus over broad ranges through their movements and migration, and they are preferred hosts for many mosquitoes.

### 1.2.4. The introduction of WNV into the Yucatán and México.

Just 6 months after confirmation that WNV was circulating in Northeastern US, a program of avian surveillance was established in the Yucatán State of México in March 2000. Because WNV has been isolated from birds migrating from Eurasia to Africa, there was great concern that the virus might traffic to México and Central America in a similar way. Bird capture sites were established in México (Yucatán and

Tamaulipas) in the US-México migratory flyways to monitor resident and migrating (both incoming and outgoing) birds for WNV. The main purpose of the program was to determine if and when WNV would be introduced into México in order to alert public health authorities. The study also was to determine (by capture and release serum sampling) which migratory birds were involved in WNV trafficking and which resident birds would be infected, becoming potential reservoirs for the virus in México. The first study was performed as collaboration between Colorado State University and the Universidad Autónoma de Yucatán. Financial support was provided by the National Institutes of Health (NIH) and the Centers for Disease Control and Prevention (CDC). Prior to initiation of the work, all necessary permissions were sought from and granted by the Secretaría del Medio Ambiente y Recursos Naturales de México (SEMARNAT) and the Ethical Committee from the Universidad Autónoma de Yucatán.

The Yucatán peninsula is the eastern-most part of México, jutting North into the Gulf of México. As such, it is the closest geographical location for birds migrating south across the Gulf of México into Central and South America. This makes it an ideal collection point along a key migration route for many US birds. Migrating birds from the Northeast, the Eastern coast, and the Southern US all use this route. The Yucatán is both a wintering ground and a resting place during the annual migration (www.npwrc.gov/resource/birds/migration.routes.htm) of many bird species. Three ecological reserves were selected as representative sampling locations: Biosphere Ría Celestún, Hobonil Ranch, and Cuxtal (Figure 1.1.1). The Biosphere Ría Celestún is a 60,000-ha estuary located in the northwest of Yucatán state. It is characterized by coastal vegetation, such as red and black mangroves. The Hobonil Ranch occupies

1,200-ha outside of Tzucacab in the central region of the State of Yucatán. Part of the ranch has been modified for horse and cattle breeding, and grass has been introduced in some areas. Finally, Cuxtal is a 10,800-ha ecological reserve located south of Mérida city. It is characterized by forest and secondary vegetation. Over a period of 36 months, birds were trapped at each site for 3 or 4 days a week, during the second week of every month. More than 8,600 birds were captured, belonging to 182 species (28 families, 14 orders), for identification, blood sampling, and release (Table 1.2.1). Using plaque reduction neutralization test (PRNT), five (5) birds out of 8,611 total birds were confirmed with WNV antibodies: a gray catbird, a brown-crested flycatcher, a rose-breasted grosbeak, a blue bunting, and an indigo bunting. The first WNV-positive birds were captured in December 2002 (59, 134). One migratory bird, a hooded warbler (*Wilsonia citrina*), captured in September 11, 2001, was classified serologically as a "presumptive WNV infection" (59).

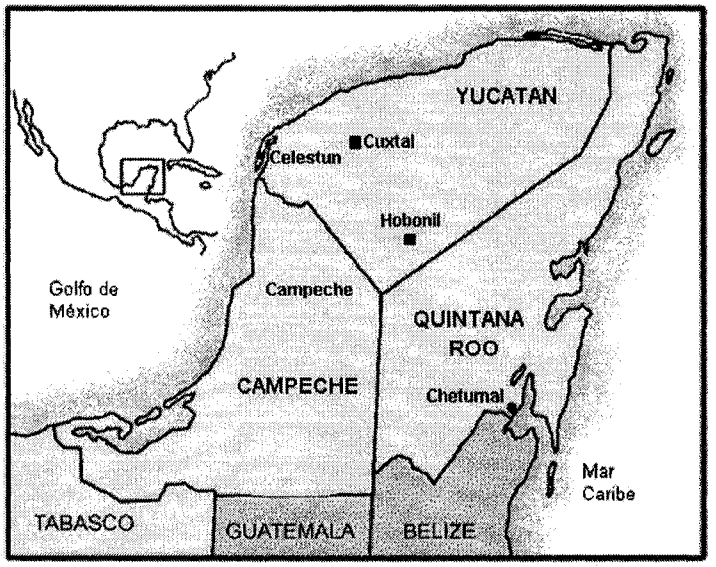



**Figure 1.1.1. Ecological reserves selected as representative sampling locations: Biosphere Ría Celestún, Hobonil Ranch, and Cuxtal.**

In conjunction with the migratory bird surveillance for WNV, blood samples from 252 horses were taken from 14 locations in Yucatán in July 2002. These samples were collected for determination of specific antibodies against WNV. Three samples (1.2%) were confirmed to have antibodies to WNV by blocking enzyme-linked immunosorbent assay (b-ELISA), hemagglutination inhibition (HI) and plaque reduction neutralization test (PRNT) (135). The first WNV confirmation in this study was from a horse from Tizimín, Yucatán. The overall seroprevalence rate was 1.2% (Table 1.2.1).

An additional surveillance study was conducted on 441 horses from 14 states of México. The first horse confirmed positive to WNV was diagnosed in a sample from Coahuila in July, 2002 (54). In March 2003, blood samples from 88 healthy and non-WNV-vaccinated horses from Nuevo León were collected and tested for flavivirus and WNV specific antibodies, using b-ELISA and PRNT. The purpose of this study was to determine the prevalence of WNV and SLE infections in horses from Nuevo León. Twenty horses (22.7%) were confirmed by PRNT to have had WNV infections and 1 horse (1.1%) had antibodies to SLE (143).

A number of other smaller surveillance studies have also been conducted in the Yucatán Peninsula to monitor the arrival of the WNV into the country (Table 1.2). During spring 2002, scientists from institutions from New York State performed a small survey of WNV activity in migratory and resident birds on the coast of Yucatán. Of the 172 birds captured, one (0.58%) had neutralizing antibodies to WNV and another one (0.58%) to SLEV (49). In May, 2003, 144 horses from Cozumel Island, Quintana Roo were tested for antibodies to WNV. Seventy five horses (52.1%) were positive for

WNV and 1 (0.7%) was positive for SLEV (59) by PRNT. From September 2003 to September 2004, 415 animals in the Merida El Centenario Zoo were tested for antibodies to WNV. Seven birds (2.7%) and 2 (3.8%) mammals (a jaguar and coyote) were seropositive for WNV antibodies by PRNT (59). Also, 7 farmed crocodiles from Ciudad del Carmen, Campeche were sampled in January 2004. Six out of 7 (86%) had antibodies to WNV (59).

### 1.2.5. Virology and Molecular Biology of WNV.

West Nile virus (WNV) is member of the genus Flavivirus, family Flaviviridae. The mature virion is a spherical enveloped particle with a diameter of about 50 nm (155). It contains a single-stranded, positive sense RNA genome approximately 11,000 nucleotides in length. It encodes 10 proteins in a single open reading frame (3 structural and 7 non-structural proteins) (11, 23). The open reading frame is flanked by 5' and 3' non-coding regions containing conserved secondary structures that play roles in genome replication (23). The proteins are derived from the translated polyprotein via cleavages by the viral protease and the host cell signalases. The three structural proteins are: capsid (C), pre-membrane/membrane (prM/M), and envelope (E). The C protein is predominantly a homodimer of alpha helical structure. One unit is formed by the association of four dimers, as revealed by crystallography analysis (16). Four roles have been attributed to the core protein: it participates in the structure of the virion; it interacts with the viral genome; it is involved in regulation of the genome replication; and it induces apoptosis in infected cells (11, 16, 48, 226). The prM acts as chaperone of the E protein for proper folding and protects E from inactivation during transport to the cell membrane (141, 225). PrM is cleaved by a host furin-like protease during virus

release from infected cells, leaving the non-glycosylated M protein (195). The E protein exists as a dimer, where each monomer has 3 structural domains that mediate viral attachment, entry, and assembly. Domain I is involved in low-pH-triggered conformational changes and elicits type-specific non-neutralizing antibodies (35); Domain II stabilizes the E glycoprotein dimer and contains the internal fusion peptide (3); and Domain III contains the cellular receptor binding ligand and is the target for virus type-specific neutralizing antibodies (3, 157). The NS1 glycoprotein is a highly conserved glycoprotein that exists as a dimer, but it is also secreted as hexamer at high levels from the infected cells. It is also found in association with cell surface membranes of the infected cells, and it is detected in the serum of WNV-infected animals (140, 221). NS1 contains three N-linked glycosylation sites (residues 130, 175, and 203) (203) that have been associated with WNV pathogenesis (1). The NS2B/NS3 protease complex plays an essential role in post-translational processing of the viral protein (37). The NS5 protein has two functional activities: a methyltransferase (50) and the viral RNA-dependent RNA polymerase (78).

### 1.2.6. Biology of Vector and Avian Hosts of WNV in North America.

The principal mosquito vectors of WNV in the US are: *Culex pipiens pipiens* (predominant in the northeast and northern midwest), *Cx. p. quinquefasciatus* (predominant in the south), and *Cx. tarsalis* (predominant in the western US, including California) (168). The first two species are considered to be responsible for up to 80% of the human WNV infections in the Northeastern USA (110). These species of mosquitoes are primarly ornithophilic, are very abundant, and have the highest infection rates for WNV in that region. The main transmission cycle for WNV involves

ornithophilic mosquitoes as vectors and birds as reservoirs. However, other mosquito species called bridge vectors (mosquitoes that feed on birds, and other vertebrate hosts including mammals and reptiles) may be responsible for most transmission to humans and horses. The potential of vectors to act as reservoirs needs to be investigated. There is not much experimental information describing the effects of life long infections in the vectors (133). It is commonly thought that arboviruses cause lifelong infections in vectors without major effects. However, apoptosis and severe cellular degeneration effects had been described in salivary glands of mosquitoes infected with WNV (210). If cell damage occurs in overwintering vectors, it would seem to reduce the fitness of the vectors and reduce overwintering efficiency.

The effects of WNV infections on birds vary widely in severity from relatively benign consequences to death. The possible participation of migratory birds and the potential impacts of WNV infections in bird species has been reviewed (162, 163). To understand the role of North American birds in WNV transmission, 25 bird species were exposed to WNV by infectious mosquito bite. The most competent bird reservoirs for WNV transmission (regarding their viremia titers) were species of the order Passeriformes: blue jay (family Corvidae), common grackle (family Icteridae), house finch (family Fringillide), American crow (family Corvidae), and house sparrow (family Passeridae). The durations of the viremia varied from 1 to 6 days (118).

### 1.2.7. Modes of WNV transmission to human.

The principal mode of transmission of WNV is by mosquito bite; however, alternative modes of transmission were also identified during the outbreaks in the US. These alternative modes included virus transmitted by: transplanted organs, blood

transfusions, breast-milk, transplacental (mother to child), and occupational exposure (as infections in laboratory technicians) (20).

### 1.2.8. Human clinical responses to WNV.

The clinical spectrum of WNV infections in humans has been characterized in great detail during the US outbreaks. In the initial New York City outbreaks, approximately 80% of the WNV infections in humans were asymptomatic (154), about 20% resulted in self-limited WN fever, and less than 1% developed neuroinvasive disease (187). WN fever symptoms are usually mild, characterized by fever, headache, fatigue, muscle pain, weakness, swollen lymphatic glands, and sometimes rashes on the trunk and extremities (218). WN fever lasts only a few days and does not cause long-term health effects. The more severe disease is West Nile encephalitis, which is characterized by: high fever, severe headaches, neck stiffness, disorientation, convulsions, muscle weakness, paralysis, and coma (185, 186). Sequelae in patients that had WNV encephalitis may persist for months and can be lifelong (185). In two follow-up studies, approximately 17% to 37% of patients with severe WN disease achieved a full recovery after one year. Common complaints reported from the remaining patients were: fatigue, memory problems, loss of concentration, confusion, word-finding difficulty, headaches, and tremors (28, 112). Major clinical signs during acute WNF illness or meningoencephalitis are profound weakness, stiff neck and flaccid paralysis (70, 178). These may be key clinical signs for differentiation between dengue and WNV infections in endemic dengue regions.

**Table 1.2.1 Surveillance studies in birds and horses to determine the introduction of WNV into México.**

| Period of collection | State | N°. of sites | N°. of animals | Characteristics of specimens | Tests | WNV positive | SLE positive | Date and site of first WNV confirmation | Autor (Ref.) |
|---|---|---|---|---|---|---|---|---|---|
| **Mar 2000-Apr 2003** | Yucatán | 3 | 8611 Birds | Migratory, resident and captive | PRNT, HI, b-ELISA | 5 (0.06%) | 1 | Dec 2002 (Hobonil, Yucatán) | Farfán (54) |
| **Dec 2001-Mar 2003** | Tamaulipas | 4 | 796 Birds | Migratory, resident | PRNT, b-ELISA | 4 (0.5%) | 0 | Mar 2003 (Laguna Madre, Tamaulipas) | Fernández (59) |
| **Jan 2002-Mar 2002** | Yucatán | 1 | 172 Birds | Migratory, resident | PRNT,IgG ELISA | 1 (0.58%) | 1 (0.58%) | ND | Dupois (44) |
| **Jul 2002-Oct 2002** | Yucatán | 14 | 252 Horses | Domestic | PRNT, b-ELISA | 3 (12%) | 1 (0.4%) | Jul 2002 (Tizimín, Yucatán ) | Loroño (116) |
| **Jul 2002-Mar2003** | 14 States | ND | 441 Horses | Domestic | PRNT, HI, ELISA | 97 (22%) | 0 | Jul 2002 (Coahuila) | Estrada (49) |
| **Dec 2002** | Coahuila | 3 | 24 Horses | Domestic | PRNT, b-ELISA | 15 (62.5%) | 0 | Dec 2002 (Ciudad Acuña, Coahuila) | Blitvich (16) |
| **Mar 2003-Apr 2003** | Nuevo león | 29 | 88 Horses | Domestic | PRNT, b-ELISA | 26 (29.5%) | 1 (1.1%) | Mar 2003 (Monterrey, Nuevo León) | Marlenee (124) |
| **May 2003** | Quintana Roo | 1 | 144 Horses | Domestic | PRNT, b-ELISA | 75 (52.1%) | 1 (0.7%) | May 2003 (Cozumel, Quintana Roo) | Farfán (56) |
| **Sep 2003-Sep 2004** | Yucatán | 1 | 257 Birds | Captive | PRNT, b-ELISA | 7 (2.7%) | 0 | Sep 2003 (Mérida, Yucatán) | |
| | | | 16 Horses | Captive | | 0 | 0 | | |
| | | | 36 Mammals | Captive | | 2 (0.05%) | 0 | | |
| | | | 106 Reptiles | Captive | | 0 | 0 | | |

### 1.2.9. Pathogenesis.

Humans are regarded as incidental in the mosquito transmission cycle and are considered dead-end hosts for WNV due to their inability to produce high titered viremia (22, 23). WNV infections in humans have multiple outcomes, ranging from inapparent infections to encephalitis. Most infections are inapparent; thus the host innate and adaptive immune responses typically reduce viremia, limit viral dissemination into the Central Nervous System (CNS), and reduce the number of patients who progress into a severe disease (111). Seroepidemiological surveys after the outbreak in New York in 1999 showed that one in five persons infected developed WN fever and one in 150 developed CNS disease (154). WNV is classified as a neurotropic virus that replicates first in the reticuloendothelial system (macrophage/monocyte/endothelial cells), and in some cases WNV also infects neuron cells of the CNS. Although CNS involvement is rare, the clinical results are often severe. The common clinical symptoms associated with CNS involvement generally include encephalitis and less frequently involve aseptic meningitis or paralytic poliomyelitis (66, 106). Severe infections of the basal ganglia and thalamus are typically confirmed by neuroimaging. Acute flaccid paralysis has been correlated with perivascular lymphocytic infiltration and neuronphagia of the anterior horn cell region (103).

### 1.2.10. Serological responses in humans infected with WNV.

Viral proteins are recognized by hosts as foreign, and antibodies of different types are elicited after infection. Humoral immune responses are essential for protection against WNV infections (26). The most abundant antibodies produced after

a viral infection are the immunoglobulins M, G and A. The immunoglobulin M antibody-capture enzyme-linked immunosorbent assay (IgM-ELISA) is the preferred technique for the diagnosis of acute WNV infections (144). When human WNV infections are suspected, serum or cerebrospinal fluid samples are tested for the presence of IgM antibodies to WNV using an IgM-ELISA. The WNV-reactive IgM can be detected within the first 20 days after onset of symptoms, and may persist more than 8 months in approximately 50% of persons with acute WNV infection (176, 200). The IgG antibodies to WNV have a longer serum half-life. The majority of neutralizing antibodies recognize regions of the envelope proteins of several flaviviruses and a subset of antibodies bind the prM protein (32, 57, 214). Generally, strong immune responses are induced to E, C, prM and NS1 proteins. Patients with WNV or DENV infections also produce significant NS1-specific antibody responses (2, 99, 140). The dengue serotype specificity of NS1-specific IgM antibodies was 80% and 50% for primary and secondary dengue infections, respectively (189). Interestingly, the DENV NS1 protein induces antibodies that cross-react with common epitopes in human blood clotting and integrin/adhesion proteins. This cross-reaction with platelet surface proteins may cause thrombocytopenia in persons with dengue infections. The titers of these cross-reactive IgM antibodies were found to be much higher in patients with dengue hemorrhagic fever/dengue shock syndrome than in those with dengue fever (56, 131, 217). Whether or not antibodies to NS1 condition the pathogenecity of WNV infections remains to be determined.

### 1.2.11. Clinical and serological responses in equines infected with WNV under experimental and natural infections.

Clinical signs and symptoms in horses with encephalitis include: fever, ataxia, weakness of limbs, and difficulty in rising (159, 183). One of the first studies of experimental infections of equines with WNV was conducted by Schmidt and Mansoury at the beginning of 1960's in Egypt (183). Six donkeys and 3 horses were inoculated with WNV, and the viremia titers and production of antibodies were studied. None of the 9 animals showed visible signs of illness, and only 2 donkeys developed detectable viremia, with very low titers that lasted only one day. Low titers of neutralizing antibodies were detected after the first viral inoculation and increased following multiple booster virus injections. After the boosters, the neutralizing antibody titers were similar to those obtained from naturally infected equines. Based on these results, the authors concluded that equines are natural hosts of WNV; however, the titer of the viremia was not enough to support their participation in a cycle for transmission of WNV. They were thus classified as a dead-end host. This classification, however, has been questioned (96). Recent studies demonstrated direct WNV transmission between mosquitoes that co-fed on a healthy non-infected mouse. The "donor" mosquitoes were infected with WNV and the "recipient" mosquitoes were uninfected. Donor and recipient mosquitoes had simultaneous access to the un-infected mouse for 1 hour. Interestingly, 18 out of 470 (3.8%) recipient mosquitoes became infected with WNV. These results support the concept that non-viremic transmission may play a role in amplifying the number of infected vectors (96).

In injected horses, anti-WNV IgM becomes detectable 8-10 days after infection and persists less than 2 months. IgM antibody levels decay similarly in naturally infected horses (158, 159). WNV neutralizing IgG antibodies may persist over 2 years, providing protection from reinfection with WNV. These IgG antibodies may also complicate the serological diagnoses of other suspected subsequent flavivirus infections.

### 1.2.12. Tests used for diagnosis and surveillance of WNV in humans.

Demonstration of IgM and 4-fold rise in serum neutralizing antibodies is sufficient for diagnosis of WNV infection. Isolation of the causal flavivirus agent is the definitive test for diagnosis of current flaviviral infections. Isolation of the virus is also beneficial for further characterization for evolutionary analyses, for reference collections, etc. The success of viral isolation, however, depends on several factors, for example: 1) samples must be collected and preserved properly in a cold-chain; 2) highly susceptible cell lines must be used to support the infection by the virus; 3) viral loads must be sufficient to infect the cells; and 4) reagents for detection may be too broadly reactive for differential diagnosis and may be too specific to detect a new virus variant. For example, when new variants emerge, the viruses may not be detected by existing primers for PCR assays. Attempts to propagate WNV in cell culture lines have three main disadvantages. First, viremia lasts 24 hours after onset of symptoms. Blood samples are rarely taken before day 3$^{rd}$ after onset of symptoms, reducing the opportunity to isolate the virus. Second, it takes at least one week to obtain laboratory results. The prolonged time between infection and identification of its cause effectively delays timely public health interventions by health authorities. Third, the propagation

of live WNV virus requires laboratories to institute expensive and demanding biosafety level 3 protocols. The sensitivities of cell cultures are also an issue; many animals do not develop high titered viremias. With the exception of alligators (102, 114), there is no evidence that animals, other than birds, naturally develop high enough WNV viremia titers to infect (www.nwhc.usgs.gov/disease_information/west_nile_virus/SpeciesAffected2004.pdf) a mosquito. As a result, isolation of WNV from humans and other mammals is very difficult (unless they die from encephalitis and tissue samples are collected and tested). Blood bank studies have shown humans with variable viremia (24, 29). In 2003, the estimated viremia levels in implicated donation plasma ranged from 0.06 to 0.5 PFU/mL, lower than the 0.8 to 75.1 PFU/mL range needed for transfusion-associated transmission (152).

Due to these problems of low and brief viremia (approximately only 1 day after onset of symptoms) and the lack significant levels of WN viral proteins, antibody detection offers a good alternative for an indirect detection of WNV infections. ELISAs and Microsphere based immunofluorescence assays are frequently used for IgM and IgG detection in the USA (40, 105, 146) and Europe (192). However, due to their significant costs, Microsphere based immunological assays are not practical for use by laboratories in the developing world. PRNT is the recommended test to confirm WNV infections, but PRNT has several requirements that restrict its routine usage. PRNT requires testing of acute and convalescent serum samples, it is laborious and time consuming, it requires BSL3 facilities, and it is very expensive. Because of the inherent challenges encountered in other methods, and due to its reasonable cost and ease of use,

the b-ELISA method has become very popular for WNV surveillance studies in animals (15, 60, 107, 129, 179, 194).

## 1.3. DENGUE VIRUS

### 1.3.1. Brief history of dengue virus in the New World.

The Pan American Health Organization (PAHO) has prepared a comprehensive description of the history of dengue in the New World, including information on the first appearances of dengue serotypes, outbreaks, and the increasing incidence of dengue hemorrhagic fever (DHF) in the Americas from 1635 to 2001 (see online document http:www.paho.org/English/AD/DPC/CD/dengue_finaltime.doc). Dengue was first suspected in the New World in Martinique and Guadalupe in 1635. Dengue-like illnesses were reported in Philadelphia in 1780. The first epidemic was reported in Perú in 1818. An epidemic between 1824-1828 was recorded as the first pandemic of dengue in the Caribbean-Gulf-Atlantic region. Reported symptoms included hemorrhaging from the gums, bowel, and stomach. Throughout the nineteenth century and half of the twentieth century, small outbreaks and large epidemics were reported in the Caribbean and the Americas. The first wide-spread concerted activities to control dengue transmission did not occur until much later. Between the 1950s and early 1960s, dengue transmission was suppressed by the *Aedes aegypti* eradication program coordinated by PAHO in an effort to prevent the emergence of yellow fever (YF) from jungle transmission cycles into urban environments. As a result of these programs, many countries were certified as free of *Ae. aegypti* mosquitoes between 1958-1963. Unfortunately, re-infestations of the mosquitoes were reported just a few years later, which was probably due to discontinuation of the vector control programs in the

affected countries and also potentially due to the lack of adequate control programs in neighboring countries and trading partners. In 1963-1964, the first epidemic of dengue 3 in the Caribbean was confirmed, and since then dramatic increases in epidemic dengue of all four serotypes and dengue hemorrhagic fever (DHF) cases have been documented (74-76). Currently, many countries experience annual dengue virus transmission with the co-circulation of all four serotypes.

### 1.3.2. History of dengue in México and Yucatán.

The first reported dengue occurrence in México was in 1927 in Veracruz during the 1924-1928 dengue pandemic in the Caribbean-Gulf-Atlantic region. It disappeared from México in 1928. The next dengue epidemic in México occurred in the same region between 1941-1946. In 1947, the Pan American Health Organization adopted a hemisphere-wide *Aedes aegypti* program to combat urban yellow fever. México participated in the Program, and in 1963 PAHO certified the eradication of *Ae. aegypti* in México. Re-infestation by *Ae. aegypti* was reported in 1967, and dengue cases caused by DENV-2 were subsequently reported in 1971. During the 1977-1980 DENV-1 epidemic in the Caribbean, the virus reached southern México in late 1978. By October 1979, the first isolate of DENV-1 from México was obtained from a febrile human resident from Mérida in Yucatán. DENV-4 was not reported in México until 1980, and DENV-2 was first reported in 1981. In 1984, the first DHF cases were reported in México. They were from the DENV-4 epidemic that occurred in Mérida and the rural areas of Yucatán (138). DENV-3 in México was first isolated from a resident of Mérida in September of 1995. The current status of dengue and DHF in México is alarming: all 4 dengue serotypes are circulating; every year the number and

distribution of dengue and DHF cases increases; and the cases become more severe. This situation is common in almost all countries of the Western Hemisphere, except Canada, USA, Cuba, Chile and Argentina.

The seroprevalence rate for antibodies to DENV in a population are determined by detection of specific immunoglobulin G (IgG) antibodies by Hemagglutination Inhibition (HI), Complement Fixation (CF), or IgG-ELISA tests. Since the arrival of the different strains of dengue viruses in the Yucatán, a steady increase in the seroprevalence of antibodies to dengue has been noted among its residents. In November of 1979, following an outbreak of dengue in Mérida, a seroepidemiological study was conducted in two neighborhoods. The two neighborhoods had seroprevalence rates of 12% and 51%, respectively (109). Subsequent serosurveys in the Yucatan state in 1985 (five years after the report of the first dengue cases) and again in 2006 (26 years later), found that 72.5% (136) and 81.5% (Gómez-Carro S, unpublished data) of residents had antibodies to dengue viruses, respectively. The prognosis is that the seroprevalence rates will continue to increase in the future. Clearly, seroprevalence rates of this magnitude could affect the epidemiology and the diagnosis of WNV infections in the Yucatan.

### 1.3.3. Molecular epidemiology of dengue viruses.

Dengue viruses are single-stranded positive-sense RNA viruses, classified as serotypes DENV-1 to DENV-4. The extent of the genetic diversity of all four serotypes has been determined through sequencing and phylogenetic analyses. Each serotype has well-defined phylogenetic groups (genotypes). DENV-1 occurs as 5 genotypes (I-V). DENV-1 genotypes I, II and IV comprise isolates from Asia and the Pacific; genotype

V contains isolates from Asia, Africa and the Americas; and genotype III contains one sylvatic strain from Malasia (69). DENV-2 genotypes are divided into American, Asian 1, Asian 2, Cosmopolitan, and American-Asian (209). DENV-3 contains 5 genotypes (I to V) (127, 222). All American DENV-3 isolates belong to genotype III. DENV-4 contains 3 genotypes (I, II and sylvatic). The DENV-4 genotype I contains strains from Asia; genotype II contains strains from around the world; and the DENV-4 sylvatic genotype comprises 3 isolates from mosquitoes and monkeys collected in Malasia in 1973 and 1975 (126, 216).

Changes in the incidence and severity of dengue diseases in recent years in México are associated with the introduction and circulation of different (new) serotypes and genotypes of DENV (46, 137). Detailed descriptions of dengue virus introduction, mutations, and movement across México have recently been published (46, 137) The investigations compared isolates mainly from the Yucatán with representative isolates from other parts of Mexico and around the world to determine the origins, persistence and geographical distribution of the four serotypes of dengue isolated in México. Using these isolates (from 1980 to 2002), the investigators developed phylogenetic trees from dengue E protein gene sequence data using Bayesian phylogenetic analyses (46, 137). The phylogenetic trees identify and demonstrate the evolutionary potential of the dengue genotypes. These trees are also useful in tracking the potential sources of the different dengue genotypes and their geographical dispersion across México.

### 1.3.4. Clinical outcomes following dengue infections.

Dengue fever is described as a severe illness that affects young children and adults, but is seldom fatal (htpp:\who.int/cdr/disease/dengue/en). Most dengue

infections are asymptomatic, although some children exhibit a non-specific febrile illness accompanied by rash. Classic dengue fever presents as: fever, severe headache, pain behind the eyes, muscle and joint pains, and sometimes rashes. DHF is characterized by high fever, hemorrhagic manifestations, occasional enlargement of the liver, and circulatory failure. The severity of the DHF is classified by World Health Organization into 4 grades. In patients classified as DHF grade I, the fever is accompanied by non-specific symptoms, and the only hemorrhagic manifestation is a positive tourniquet test. For classification of DHF grade II, in addition to the manifestations of Grade I, the patients also have spontaneous bleeding into the skin, gums, gastrointestinal tract and other sites (menorrhagia). DHF grade III patients will have signs of circulatory failure manifested by rapid and weak pulse, narrow pulse pressure, hypotension, cold and clammy skin, and agitation. DHF grade IV, also called dengue shock syndrome (DSS), is characterized by profound shock (undetectable pulse and blood pressure) and has a case fatality rate of approximately 10%. Maintenance of the circulating fluid volume is the central feature of effective DHF case management at the health care facilities (http://www.who.int/mediacentre/factsheets).

### 1.3.5. Serological responses to dengue virus infections.

In primary infections, human immune responses to dengue virus start with the production of immunoglobulin M (IgM) antibodies, which appear approximately 5 days post onset of symptoms. IgM antibodies are detectable for approximately 30-60 days. Immunoglobulin G (IgG) antibodies are detectable by the $14^{th}$ day of dengue virus infection and persist for the rest of the patient's life. In secondary dengue infections, the IgM antibodies may not be produced until 20 days after onset of symptoms, are

present at lower levels than in primary infections, or they may be even undetectable. They are also present for even shorter periods of time in secondary infections than in primary infections. Approximatly 30% of dengue patients do not produce detectable anti-dengue IgM antibodies by day 10 and must instead be diagnosed by dengue specific IgG antibodies. IgG antibodies rise rapidly in secondary infections (by day 1 or 2) after onset of symptoms, persist at high levels for 30-40 days, and then decline to similar levels as found in primary or past infections.

### 1.3.6. Diagnosis of dengue infections.

Laboratories around the world currently use three strategies for dengue diagnosis: isolation and characterization of the virus, detection of genome sequences, and detection of antibodies specific to dengue viruses (190, 201). The preferred type of dengue diagnosis is virus isolation in mammalian or mosquito cells, followed by immunofluorescence testing using monoclonal antibodies for identification of the specific dengue serotypes (77). The second type of dengue diagnosis is detection of DENV sequenced by polymerase chain reaction (RT-PCR) amplification of virus RNA, which can then be sequenced to genotype the isolate (89, 124). For both virus isolation and/or detection of the viral RNA genome, the serum or plasma must be collected during the first week after onset of symptoms because viremia lasts only 2 to 7 days. The third type of dengue diagnosis relies on serological tests that assay for the detection of specific antibodies to DENV. IgM and IgG capture ELISAs are frequently used for both detection and differentiation of primary and secondary infections (101). Collection and assay of both acute and convalescent blood samples helps in the interpretation for a correct diagnosis (e.g. if IgM or IgG are increasing or decreasing) and for the

differentiation between primary or secondary infection, which is useful for the analysis of data for epidemiological studies. Microsphere based assays offer new potential for rapid differential diagnosis of DENV and other flavivirus infections (213,214). The differential diagnosis capability of this assay will discussed in detail in section 1.5. Test results for the presence of IgM and IgG antibodies can be obtained in less than 3 hours.

Finally, it is worth mentioning several other significant serological methods that are either currently in limited use or that were useful in the past but are no longer commonly used. These methods include: hemagglutination inhibition (HI) test (31), complement fixation (CF) test (161), indirect immunofluorescent (IF) test (184), plaque reduction neutralization test (177), dot blot analysis (27), and immunochromatography test (36).

The HI test is the reference test recommended by the WHO to discriminate between primary and secondary dengue virus infections. The HI test has several disadvantages for diagnosis: it takes long time to perform, requires acute and convalescent phase samples, and the IgG antibodies strongly cross react with other flaviviruses (4). These disadvantages complicate and delay diagnoses. Due to these disadvantages, IgG-ELISA tests have been developed replace the HI test. ELISA tests can be designed for the diagnosis of primary and secondary dengue infections based on the IgM/IgG ratio (101, 213). However, the persistence of IgM for up to 8 months (38) can confound the diagnosis. The antigen-binding avidity of specific IgG can also be used to differentiate primary and secondary infections (43). Avidity is a measure of binding strength between IgGs and antigens. IgGs produced early after flavivirus infection have low avidity, while those produced several months later have high avidity

due to the memory immune response to flavivirus antigens. In addition, the IgGs resulting from secondary infections have stronger avidity for antigen than the avidities of those resulting from primary infections, and the level of avidity increases with increasing time after the infection. When using the IgG avidity index assay, a low index in a single serum sample indicates the diagnosis of a primary, acute dengue infection. One limitation of this assay is that blood samples collected up to 10 days after the onset of a primary dengue infection may be IgG negative (182). Also, a high avidity index can not discriminate between recent secondary and past dengue virus infections.

Even with the new technologies, definitive differential dengue diagnosis of flavivirus infectious is difficult, particularly when other flaviviruses circulate in the environment. New technologies, such as the blocking ELISA, need to be characterized for their capacity for the diagnosis of flavivirus infections in humans.

## 1.4. EPIDEMIOLOGICAL OUTCOMES OF WNV INTRODUCTION AND CIRCULATION IN DENGUE ENDEMIC AREAS.

### 1.4.1. Protection or immune enhancement of WNV infection by dengue antibodies?

Two possibilities can occur in humans when one flavivirus (WNV) is introduced into an area where other flaviviruses (DENV) are or have been circulating. Humans may be protected from being infected by the new virus because of cross reacting antibodies, or they may experience higher viremia titers and more severe disease, because of antibody-dependent enhancement (ADE). A discussion of each possibility follows a brief introduction.

Two years after the introduction of WNV into US, the virus was reported in Canada, and three years later in México. Each country had different experiences with WNV in their human and animal populations. The US experienced dramatic outbreaks of encephalitis, Canada experienced smaller outbreaks, and northern Mexico had a few cases but no real outbreaks. Canada employed sentinel chickens and monitored dead birds to check for the introduction of WNV. In México three Universities (Colorado State University [CSU], Universidad Autonoma de Yucatán [UADY] and Universidad Autonoma de Nuevo Leon [UANL]) collaborated with the support of three agencies (NIH, CDC and SEMARNAT) to monitor migrating and resident birds and resident horses for WNV, as described and referenced earlier in this chapter in section 1.2.4.: "The introduction of WNV into the Yucatán and México".

After 6 years, the USA has reported 23,967 human cases of WNV infections (http:\www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm). Canada has documented 2,300 human cases since their first detection in 2002 (htpp://www.phac-aspc.gc.ca/wnv-no/surveillance_e.html), and México has had only 7 documented cases (htpp://www.salud.gob.mx). These cases occurred in 2003 and 2004 in residents of Sonora, México (167). It is also worth noting there is no evidence of WNV infections subsequently increasing in México. One possibility is that people in México are protected by antibodies generated in response to dengue virus infections. All four dengue viruses have circulated in México for 25 years, and over 85% of the population has antibodies to DENV (68, 136, 164). WNV and DENV belong to the same family of flaviviruses, and antibodies generated against them cross-react in current diagnostic tests (1.5). This hypothesis is further supported by the other evidence. Protection

against WNV and SLEV in dengue-endemic populations has been reported previously (180). It is also well known that host factors influence the outcome of WNV infections (26, 44, 45, 180). Children, elders, and the immuno-compromised have higher risks for severe neurological disease as demonstrated in US and Europe (9, 204). It is reasonable to expect that these same groups in México should be the most vulnerable to severe WNV manifestations, because of their immature immunity or altered immunity. However, after four years, there is still no evidence of WNV in these groups, potentially due to the presence of previously acquired antibodies from prior dengue infections.

The effects of prior flavivirus infections (DENV in particular) may also present problems in the development of a safe vaccine for dengue. The problem of immune potentiation due to antibody-dependent enhancement (ADE) has not been resolved (80, 115). ADE replication of dengue virus has been demonstrated in human blood leucocytes carrying Fc receptors when the individuals has subneutralizing concentrations of antibodies to DENV (82). In any case, there are currently not enough data to either prove or disprove hypotheses that antibodies to dengue protect or enhance WNV infections in Mexico. This is due in part to the lack of a serological test to differentiate WNV infection in humans who have experienced previous DENV infections.

### 1.4.2. Possible factors involved in the differences in clinical outcomes in birds, humans and horses between USA and México.

Although WNV is circulating widely in México based on the detection of antibodies in horse and bird populations, there have been only seven reported infections in humans and these were in Northern Mexico (section 1.4.1.). In addition, there have

been almost no WNV-related deaths in birds or in horses, with the exception of some horses and crows in northern Mexico. The main vectors implicated in WNV transmission in the US are *Cx. pipiens, Cx. quinquefasciatus* and *Cx. tarsalis* (205). These mosquito species are also present in northern México. Indeed *C. quinquefasciatus* have been shown to be competent vectors in Nuevo León (51). The most abundant *Culex* sp. in México is *Cx. quinquefasciatus* (39, 208) The main amplifying bird hosts in New York were found to be the American crow and blue jay. Neither species is commonly found in the Yucatán, and corvids generally are not as common in Mexico as in the US, which may partially explain the low WNV infection rates in southern and eastern México. It is possible that the impact of WNV in Mexico has been diminished because there are few susceptible amplifying hosts with the potential for high viremia. It may also be possible that the impact of West Nile virus infections has been diluted because there are many different bird species present with variable susceptibilities to infection. The potential effects of high diversity of bird species on viral transmission rates have been published recently (55, 63). The authors propose that the systemic potential for vector-borne diseases is reduced when there are many different bird species present.

There is reasonable evidence that prior DENV infections may offer protection from subsequent WNV infections, (as discussed above in detail in section 1.4). Stated briefly, before WNV introduction, the US population was essentially "flavivirus-naïve", with a very low prevalence or almost no antibodies against any flaviviruses. As a result the US population was very susceptible to a flavivirus introduction, such as WNV. In contrast, the seroprevalence rate for antibodies to DENV were > 80% in the Yucatan,

and most individuals had experienced secondary infections (Gomez-Carro, S., unpublished data). These seroprevalence rates may well have protected challenged individuals as well as the population by increased herd immunity to WNV infections.

Finally, mutations in several genes were identified when comparing prototypes of the New York strain with other US WNV-isolates. Isolates were collected from different geographic regions from 2001 and 2002 and analyzed by nucleic acid sequence studies (12). A correlation was demonstrated between mutations and the emergence of WNV isolates with attenuated neuroinvasiveness in a mouse model (42). These studies suggested an attenuated virus hypothesis. Following inoculation with mutated US WNV isolates, there were up to 100,000 fold increased attenuations of neuroinvasivenss when compared to the prototypic (positive control) WNV isolate from New York in 1999. Komar and Clark have hypothesized that migratory birds infected with avirulent strains of WNV may survive and spread those avirulent WNV strains to new transmission foci along their migratory routes (117). These hypotheses could partly explain the differences between clinical outcomes in the WNV infections in USA and the rest of the Western Hemisphere.

## 1.5. DIFFERENTIAL DIAGNOSIS OF FLAVIVIRUS INFECTIONS.

Early and accurate diagnosis of flaviviruses causing outbreaks or epidemics is critical for successful prevention and control of the diseases. Flaviviruses such as WNV and DENV have different vectors, different control measures, and pose different risks to populations. Tests capable of differential diagnosis are a critical tool in the fight against flaviviruses. As mentioned previously and reviewed below, the close antigenic relationships of the flaviviruses confound most serological tests The problems with

flavivirus diffential diagnosis are increasing because of the hyperendemicity of DENVs throughout much of the tropical world and the trafficking and circulating of other flaviviruses, eg, WNV and JEV, into dengue endemic areas. Development of a serologic test that could differentiate flavivirus etiological agents in tropical regions and in secondary infections would be a significant contribution to public health. The test should also be rapid, sensitive, specific and economical for use in laboratories around the world.

### 1.5.1. Serological relationships of flaviviruses complicate diagnosis.

Because the viruses of the genus Flavivirus are closely related, infected avian and mammalian species generate antibodies that serologically cross-react with other flaviviruses in diagnostic tests (25, 87, 180). As a result, current diagnostic methods are prone to false positive results due to serological cross-reaction, when the individual has been previously infected with a different flavivirus. These serologic cross-reactions among members of the genus Flavivirus have made differential serologic diagnosis of the etiological agent nearly impossible in secondary infections. As noted previously, serological studies of infected humans, birds, and many other animals, have revealed the phenomenon of "original antigenic sin" (OAS) (62, 81). The OAS hypothesis postulates that many B-cell clones responding to a first flavivirus infection will be re-stimulated to synthesize antibodies with greater affinity for the first infecting virus than for the current infecting virus (81). OAS was clearly demonstrated in a recent study of sequential infections with members of the Japanese encephalitis serocomplex in pigs (220). As a result, the authors were unable to make an unequivocal diagnosis due to variation in immune responses and broadened anamnestic responses. The same

problems of cross-reactions and potential "original antigenic sin" occur with dengue and WNV. The presence of multiple flaviviruses in the tropical areas of the Western Hemisphere complicates current serological tests. This is an important factor driving the development of new highly specific serological test methods for individual flaviviruses, which will be discussed below and in the ensuing chapters.

When evaluating serological test results for flavivirus antibodies, the effects of potential prior and current primary, secondary, or multiple infections need to be considered. In primary infections, the titers of antibodies are generally low or moderate, but specific to the infecting virus. In contrast, there are immediate anamnestic responses following secondary, tertiary, or multiple infections (188). Cross-reacting antibodies to flaviviruses also increase rapidly during the first days of illness. In secondary infections, high titers to many flaviviruses make it nearly impossible to make a specific diagnosis of the infecting flavivirus by current serological methods.

### 1.5.2. Current diagnostic methods for dengue and WNV infections in humans.

As described in section 1.2.12 and 1.3.6, laboratory diagnosis of WNV and dengue infections in humans is currently made by the detection of: infectious virus, viral antigens, genomic sequences, and/or antibodies (79, 190). The three approaches currently used by most laboratories are: viral isolation in cell culture, followed by monoclonal antibody differential diagnosis (77); detection of dengue virus sequences by RT-PCR (124); detection of specific IgM and IgG antibodies by ELISA (122, 144).

Serologic tests, especially ELISA tests, are the mainstay for diagnosis of flavivirus infections in humans in most of the developing world. ELISA tests can also be designed to distinguish between recent and past flavivirus infections (130, 166) or

primary and secondary infections (43, 145). These new ELISA tests measure the avidity of IgGs. Avidity is a measure of binding strength between IgG's and antigens. IgG's produced early in the infection of flaviviruses have low avidity, but avidity increases after several months due to the memory immune response to flavivirus antigens. Thus when IgG avidity is low the infection presumably occurred in the previous 4 months.

Despite advances in serological tests, the IgM-ELISA remains the most commonly used routine test for WNV infections. However, it is confounded by false positive reactions in flavivirus endemic areas of the world and the long time persistence of the IgM antibodies from multiple flavivirus infections. Some laboratories are exploring new serological diagnostic approaches, such as microsphere immunofluorescence assay (10, 224), and immunochromatography (36, 181) to address problems in in differentiating flavivirus etiological agents, especially in secondary flavivirus infections. The MIA is based on covalent coupled recombinant E, NS1, NS3 and NS5 WNV proteins attached to fluorescent polysterene microspheres (223, 224). A MIA based on the NS5 protein discriminated between WNV and SLEV infections, and it also differentiated between recent and old infections and between natural infection and vaccination. Unfortunately, this test is a flow cytometry based assay, and most laboratories in developing countries would be unable to purchase the expensive equipment. Despite the costs, the MIA was a promising test for differential diagnosis of DEN and WNV infections.

### 1.5.3. Pilot studies of the MIA and IgM-ELISA tests for differential diagnosis of flavivirus infections.

I conducted pilot studies to determine if the MIA (213) or a new CDC IgM-ELISA protocol for differentiation of WNV infections (138) could differentiate between WNV and DENV infections in sera from the Yucatan, where most people have experienced secondary dengue infections

The MIA used in the pilot study was developed at the Diagnostic Immunology Laboratory at the New York State Department of Health - Wadsworth Center (NYSDHWC) to detect total human antibodies (IgG + IgA + IgM) to WNV. In this technique, each fluorescent polystyrene microsphere or bead was coated with several recombinant eukaryotic-expressed antigens, allowing the detection of antibodies in a sample. The Luminex analyzer uses two lasers to excite the internal dyes that identify activity on each microsphere particle. At the NYSDHW, recombinant WNV proteins (E, NS1, NS3 and NS5) and recombinant DENV proteins (NS5) were individually attached to the beads to detect antibodies for WNV and DENV. The NYSDHWC group demonstrated that the NS5-based MIA assay discriminates between WNV infections and dengue virus or St. Louis encephalitis virus infections, differentiates between flavivirus vaccination and natural WNV infection, and indicates recent infections (223).

They also demonstrated that in the sera of WNV patients (confirmed by PRNT) NS5-reactive signals appeared on the 6th day after onset of symptoms and that 92% (35 of 38) of serum samples were positive for WNV. However, a cross-reactivity of 8.8% (3 out of 34 of DENV patients) and 5% (2 out of 40 SLEV patients) was also observed.

I then collaborated with the NYSDHWC scientists to determine if the MIA would be able to detect WNV infections among patients living in DENV endemic regions, especially in those with confirmed secondary infections. Samples from Mexico were chosen to investigate this objective. WNV circulation was confirmed in Mexico from DENV endemic areas in July 2002 based upon the detection of WNV antibodies from many different species of animals, as previously described in this Chapter (section 1.2.4.). The human serum samples used in this study were from patients with clinical symptoms of dengue infections, but who had also tested negative by the IgM-capture ELISA for DENV infections. The MIA protocols are presented in Appendix 6.3. Cut-off values for each recombinant protein were established, based on the arithmetic mean of WNV negative serum samples plus 3 Standard Deviations. All serum samples tested were negative with WNV NS5-based MIA, indicating no evidence of WNV antibodies in patients from Yucatán, Mexico (Table 6.3.1. in Appendix 6.3). Nine out of 139 serum samples were positive for both DENV 1 and 2 based on the reaction with NS5 proteins. The reactivity of the polyvalent recombinant WNV-E and rWNV-NS1 can be explained by cross reaction with antibodies to DEN.

A pilot study was also conducted determine if the CDC protocol for IgM-capture ELISA could be used for differential diagnoses of WNV infections (144). All specimens tested had been submitted to the Laboratorio de Arbovirología del Centro de Investigaciones Regionales, Universidad Autónoma de Yucatán for diagnostic testing for dengue infections. The serum samples had been tested by: IgM-ELISA for dengue (122), RT-PCR (124), or virus isolation (77). Subsequent plaque reduction neutralization tests (90% endpoints) were conducted at the Arthopod-borne Infectious

Diseases Laboratory, Colorado State University. The samples were further characterized by complement fixation (161) and hemagglutination inhibition tests (14) conducted at the University of Texas, Medical Branch at Galveston. Thirty two serum samples were tested to detect IgM against three different antigens (WNV, DEN and SLE) in an IgM-capture ELISA. Detailed information on materials, procedure, and interpretation with IgM-ELISA for differential diagnoses of WNV, DEN and SLE are described in Appendix 6.4. Of the 32 serum samples tested, 13 showed higher P/N ratios to DEN antigens; 2 did not react; and the remaining 17 samples showed similar P/N ratios among the three antigens (Tables 6.4.1 and 6.4.2 in Appendix 6.4). These very similar P/N ratio values demonstrated the difficulties of diagnosing human WNV infections, when antibodies cross react among flaviviruses.

Based upon these preliminary results, it seemed that neither the MIA nor the IgM-ELISA tests would be useful for differential diagnosis of flavivirus infecting agents in areas, such as the Yucatan, where most patients would have secondary dengue virus infections. The very high titers of flavivirus antibodies in these patients seemed to confound the tests. Thus, other tests were investigated for differential diagnosis of flavivirus infections.

### 1.5.4. Other problems with currently used tests for Flavivirus diagnosis of human infections.

In addition to the issues of test specificity, the current methods of WNV testing and diagnosis do not meet the needs of public health officials, individual patients and physicians in the developing world. Laboratories outside the USA and European Union face additional challenges. For example in México, biological reagents necessary for

the common tests require an additional 30-45 days for shipping and clearing Customs, and they typically cost between 30% and 300% more than in the USA. In addition, improving the quality of the surveillance programs is essential for the detection and control of arthropod-borne viruses, for example WNV. The following changes are needed to improve the diagnosis of WNV in humans and animals: 1) the institution of practical management programs of human and animal surveillance; 2) the use of validated diagnostic tests for rapid and sensitive screening of samples; 3) the establishment of systems for adequate preservation of samples during delivery from collection sites to the laboratory; and 4) encouragement of public health agencies and foundations to support research on issues related with WNV infections.

### 1.5.5. Need for new diagnostic tests for WNV diagnosis in tropical regions.

For all of these reasons, new test methods are needed to rapidly, easily, inexpensively and accurately detect and diagnose WNV infections. The criteria for an ideal detection and diagnostic method include the following qualities. The test should reliably detect WNV or antibodies within the first days after infection. The testing should be both sensitive enough to detect low levels of virus or antibodies and be specific enough to distinguish WNV infections from other flavivirus infections. The test should offer rapid turn-around times (at bedside or one day) to allow physicians and public health officials to make good decisions before disease or epidemic management problems become significant. The test should not require exotic, expensive, fragile, dangerous, or short-lived reagents. The test methods further should be robust and easily adapted in a broad range of laboratories and by typical laboratory personnel. Virus isolations and PRNT methods are hampered by the necessity of using live virus, which

severely limits the number of qualified laboratories to only those capable of implementing biosafety level 3 or 4 protocols. New test methods should require only biosafety level 2 protocols, to allow routine testing by existing laboratories in both the developed and developing world. Currently, there are no methods available that meet all of the desired qualities listed. One of the goals of this research program was to develop a test method that would meet many, if not all of the requirements, and would provide differential diagnosis of dengue and WNV infections in the Yucatan and other flavivirus endemic areas.

### 1.5.6. The b-ELISA for zoonotic serosurveys for WNV infection.

For large serosurveys of many different animal species infected with WNV, the epitope-blocking ELISA is typically the test of choice. The main advantage of this test is that its results are species-independent. This characteristic is particularly important in identifying outbreaks of WNV infections that simultaneously involve many different species of animals. Briefly, the sera or CSF are incubated in 96-well ELISA plates previously coated with WNV-infected cells. The monoclonal antibody specific for the NS1 protein of WNV is added and competes in antigen binding with the antibodies of the sample. An anti-mouse conjugate is then added to reveal the reaction. The percentage of inhibition of the WNV-specific monoclonal antibody (MAb) binding is calculated to determine whether the sample is positive or negative for WNV. Detailed protocols of the e-blocking ELISA (Appendix 6.1) and for the preparation of antigens for the ELISA (Appendix 6.2) are provided at the end of this dissertation.

The b-ELISA test is now widely used in the USA and Latin America for diagnosis of WNV infections in animals. It is rapid, inexpensive, sensitive and specific

for detection of antibodies to WNV in animals. Thus, it does meet many of the needs for a test for use in developing countries. However, it has not been properly characterized in terms diagnosis of WNV infections in humans.

## 1.6 DISSERTATION AIMS AND ORGANIZATION.

My research projects focused on the differential diagnosis of West Nile virus infections of humans and horses. Although much progress has been made in the development of WNV diagnostic tests, current methods are not capable of reliably diagnosing WNV rapidly in flavivirus endemic areas of the developing world. Clearly, existing serological tests have not proven to be satisfactory for differentiation of WNV with other flaviviruses in clinical diagnostic laboratories in disease endemic countries. The inability of current flavivirus tests to make accurate differential diagnoses in patients who may have experienced multiple flavivirus infections mandates exploration of new analytical alternatives. The aim of this dissertation was to evaluate blocking- and peptide-based ELISA tests for their abilities to accurately diagnose WNV infections in human and horse populations sequentially infected with several different flaviviruses.

The b-ELISA had previously been proven to be efficacious for diagnosis and surveillance of WNV infections in animals and is readily applied in disease endemic country laboratories. The rationale for selecting the b-ELISA format specifically was that the test design and specificity for diagnosing WNV infections is based upon blocking of monoclonal antibody detection of a specific epitope on the NS1 protein of WNV. The hypothesis was that the epitope was specific for WNV, and thus would induce WNV specific antibodies, which could provide a WNV diagnosis, even if the host had previously been infected with dengue virus or an alternate flavivirus.

In the following chapters, the diagnostic efficacy of the b-ELISA for differential diagnosis of WNV and dengue virus infections was investigated. In Chapter II, the ability of the b-ELISA to diagnose WNV infections in humans in flavivirus-naïve and flavivirus endemic regions was examined. In Chapter III, synthetic peptides representing the envelope and the NS-1 glycoproteins were evaluated as diagnostic reagents in a peptide-based ELISA. Finally, in Chapter IV the efficacy of the b-ELISA for the diagnosis of WNV infections in horses sequentially infected with flaviviruses was determined.

# CHAPTER II

## EVALUATION OF THE EPITOPE-BLOCKING ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DIAGNOSIS OF WEST NILE VIRUS INFECTIONS IN HUMANS.

### 2.1. ABSTRACT.

An epitope-blocking enzyme-linked immunosorbent assay (b-ELISA) was evaluated for the diagnosis of West Nile virus (WNV) infections in humans. Sera from patients diagnosed with WNV infections from an outbreak in 2003 in Colorado, U.S.A. and from dengue patients from Mexico and Thailand were tested by b-ELISA. The b-ELISA tests were performed using the WNV-specific monoclonal antibody (MAb) 3.1112G and the flavivirus-specific MAb 6B6C-1 (14, 17, 18). The WNV-specific b-ELISA was effective in diagnosing WNV infections in humans from Colorado, but it was determined to be less effective in detecting WNV in humans from México and Thailand. The data indicate antibodies to other flaviviruses confound the b-ELISA results. Specifically, the high flavivirus seroprevalence rates likely attributable to dengue virus (DENV) and/or Japanese encephalitis virus (JEV) infections in samples from Mexico and Thailand are the most likely cause of unacceptably high false positive results in the b-ELISA results.

In serum specimens from patients from Colorado, the WNV b-ELISA and the WNV plaque reduction neutralization test ($PRNT_{90}$) showed an overall agreement of

91%. The sensitivity (true positive rate) and specificity (true negative rate) of the WNV b-ELISA were 89% and 92%, respectively for the Colorado samples. In specimens from Mexico and Thailand, the WNV b-ELISA false positive rates were 79% and 80%, respectively. Thus, in regions where people have experienced previous or multiple flavivirus infections, the use of the b-ELISA for WNV diagnosis is contraindicated.

## 2.2. INTRODUCTION.

The most medically important flaviviruses include dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), yellow fever virus (YFV), tick-borne encephalitis virus (TBEV), and Saint Louis encephalitis virus (SLEV) (76, 142, 193). Flaviviruses are positive-strand RNA viruses with genomes of approximately 11 kb that encode 3 structural and 7 non-structural (NS) proteins in the gene order: C (capsid), M (membrane), E (envelope), NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. WNV is a member of the JEV serocomplex within the genus *Flavivirus*, family *Flaviviridae*. The virus has been isolated in Africa, Australia, Eastern Europe, the Middle East, North America and South America (41, 92, 117). WNV was first detected in the United States in July 1999 and spread rapidly throughout the country causing large numbers of infections in humans, horses and birds (91, 142). Prior to 1999, flavivirus infections in humans in the U.S. were infrequent, and were mostly attributed to sporadic cases of SLEV and travel-associated cases of DENV (211). In Thailand, all four DENV serotypes and JEV circulate (198), resulting in very high flavivirus transmission and seroprevalence rates. In the Yucatán Peninsula of México, all four DENV serotypes circulate and seroprevalence rates are very high (46). Serological diagnoses of WNV are complicated by the high rates of both primary DENV infections

and secondary DENV infections in inhabitants of Thailand and Yucatan, Mexico, with seroprevalence rates > 85% in Thailand (6) and 72% in the Yucatán (61). The introduction of WNV into the Yucatán in 2002 was revealed by detection of antibodies in horses (135), then later in migratory and resident birds (59) and in zoo animals (60). However, no WNV infections of humans have been diagnosed in the Yucatán.

The IgM-capture ELISA is the preferred test used for diagnosis of WNV in humans in the USA (144). The test is used to detect antibodies to WNV in serum and/or cerebrospinal fluid. Plaque reduction neutralization test (PRNT) is the gold standard for serodiagnosis of flavivirus infections and for identifying the infecting agent (14). However, both of these tests can be confounded if patients have had previous flavivirus infections. Indeed, diagnosis of flavivirus infections in humans is very difficult in geographic areas where multiple flaviviruses are circulating and causing sequential infections. Because of "original antigenic sin" the highest antibody titer may be due to a previous flavivirus infection rather than to the current etiologic agent (81, 123). Serological diagnosis of WNV, SLEV and YF virus infections is extremely difficult in patients from areas where DENV is hyperendemic.

Previously, our group developed and evaluated an epitope-blocking ELISA that reliably detected antibodies to WNV in diverse species of avians and domestic animals (17, 18). The WNV b-ELISA measures the ability of the antibodies present in sera to block the binding of a monoclonal antibody (MAb) to a WNV-specific epitope on the NS1 protein (83). The WNV b-ELISA had not been previously evaluated for use in humans. In this study, WNV specific and flavivirus broadly-reactive b-ELISAs were evaluated for their abilities to detect antibodies against WNV in human serum

specimens from the USA, Thailand and México, countries with differing levels of flavivirus endemnicity. The objectives of this study were: a) to determine the ability of the b-ELISA to detect antibodies to WNV in human serum samples, and b) to determine the effects of previous flavivirus infections of patients (eg, DENV and JEV) on the diagnostic efficacy of the WNV b-ELISA.

## 2.3. MATERIALS AND METHODS.

### 2.3.1. Serum samples.

Seven hundred twenty five (725) sera samples from patients diagnosed with WNV infections from an outbreak in 2003 in Colorado, U.S.A. and from dengue patients from Thailand and México were tested by b-ELISA for the diagnosis of WNV infections. General information about these patients is described in Results Section 2.4.1.

### 2.3.2. Preparation of b-ELISA antigen.

The antigen used for the WNV b-ELISA was prepared from *Aedes albopictus* C6/36 cells that had been infected with WNV (NY-99 strain) at a multiplicity of infection of 0.1 (Appendix 6.1). At 120 hours post infection (p.i.), the cells were scraped from the flask and pelleted by centrifugation at 4,000 rpm for 10 minutes at 4°C. Cell pellets were washed 4 times with borate saline (1.5 M NaCl, 0.5 M $H_3BO_2$, 1.0 M NaOH, pH 9.0) and the final pellet was resuspended in 0.1% SDS and 1% Triton X-100. The cells were sonicated on ice at 20% output setting for 30 seconds, and centrifuged 8,000 rpm for 10 minutes at 4°C. Supernatants were aliquoted and stored at -70°C until use.

### 2.3.3. b-ELISA testing.

The b-ELISA tests were performed using either MAb 3.1112G (Chemicon International, Inc., Temecula CA) or MAb 6B6C-1 (CDC, Fort Collins, CO). The MAb 3.1112G is specific for the NS1 glycoprotein of WNV. The MAb 6B6C-1 is specific for the flavivirus E protein (94, 174) (Appendix 6.2). Briefly, coating antigen, conjugated antibodies, and monoclonal antibodies were independently titrated against negative and positive control serum samples. Optimal dilutions that yielded an $A_{415}$ of 0.30 with negative controls were chosen. Percent of inhibitions of MAb binding in the b-ELISA were calculated using the following formula:

$$\% \text{ Inhibition} = 100\% * \{1 - [\text{O.D. (Smp.)} - \text{O.D.(Bkg.)}]\} / [\text{O.D.(Neg. Ctrl.)} - \text{O.D.(Bkg)}]$$

Where: O.D.(Smp.) = Optical Density of the Sample, O.D.(Bkg.) = Optical Density of the Background, and O.D.(Neg. Ctrl.) = average Optical Density of the six (6) Negative Controls (83).

Optical density data collected were entered into Microsoft Excel (Microsoft Corp., Bellingham, WA, USA), and the percent of inhibition was calculated using the previous formula. A selected pool of 50 human serum samples from Colorado was used as negative control sera. These samples tested negative for presence of antibodies to WNV, SLEV and DENV viruses by plaque reduction neutralization test (PRNT) and by IgM-ELISA.

### 2.3.4. IgM-capture ELISA.

Serum samples from Mexican patients were assayed for the presence of IgM antibodies to WNV, SLEV and DENV as described elsewhere (144) and in Appendix

6.4. Briefly, plates were coated with antibodies to human IgM, incubated overnight at 4°C, and blocked with phosphate buffered saline (PBS) containing 0.5% Tween 20 and 5% nonfat dry milk. Serum samples were tested at 1:400 dilutions. Positive antigens (cell lysates infected with WNV, SLEV, or DENV) and negative antigens (non-infected cell lysates) were added to the samples and incubated overnight at 4°C. The following day, peroxidase-labeled MAb 6B6C-1 was added. The conjugate was detected using Enhanced K-blue substrate, which contains both 3,3',5,5' tetramethylbenzidine and hydrogen peroxide. Readings of the spectrophotometric absorbance values were conducted at $A_{450}$. The positive and negative antigens used for the test were the following: 1) recombinant WNV envelope protein expressed in COS-1 cells; 2) normal COS-1 cells; 3) suckling mouse brain infected with SLEV; 4) normal control suckling mouse brain; 5) pooled tissue culture supernatants of C6/36 cells infected with each of the 4 dengue serotypes, and 6) tissue culture supernatant of non-infected C6/36 cells. The first 4 reagents were provided by Dr. Barbara Johnson, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado, USA.

### 2.3.5. Plaque reduction neutralization test.

Serum samples were tested by plaque reduction neutralization test (PRNT) to detect the presence of neutralizing antibodies to WNV, SLEV and DENV (14). PRNTs were conducted using Vero cells in the BSL-3 facilities at CSU. The following prototype virus strains were used in this test: WNV (NY99 35261-11), DENV-1 (Hawaii), DENV-2 (Jamaica 1409), DENV-3 (H-87), DENV-4 (H-241) and SLEV (TBH-28). Neutralizing antibody titers were calculated as the reciprocal of the serum

dilution with a 90% reduction of the number of plaques ($PRNT_{90}$). Two fold dilutions of serum samples were tested starting at a 1:10 dilution. Primary and secondary infections were assigned based on PRNT titers and AFRIMS criteria for IgM and IgG-capture ELISA titers in Thai samples (52, 212) .

### 2.3.6. Methods commonly used to determine cut-off values in ELISA tests.

The determination of the diagnostic cut-off value is essential to any serological assay to define positive and negative results and can be calculated using several different statistical approaches. Cut-off values for ELISA tests can be determined by: 1) measuring values using sera from non-infected individuals and adding 2 or 3 Standard Deviations to the mean negative control value; 2) doubling the mean optical density readings of the negative serum samples; 3) comparing the optical densities of samples relative to a negative control (Positive/Negative ratio); or 4) determining the receiver-operating characteristic (ROC) analysis that distinguishes between the negative and positive groups based on the value obtained in the area under a curve to simultaneously minimize both false positive and false negative rates (73, 93, 132, 156, 170).

### 2.3.7. Statistical software packages used for the analysis.

The ROC curve analysis and cut-off values, sensitivity, specificity, positive predictive value, negative predictive value and other statistical analyses were determined using the following programs: MedCalc for Windows, version 8.2 (Mariakerke, Belgium) and Diagnostic Agreement Statistics (www.mhri.edu.au/biostats/DAG_Stat/). The levels of discrepancy between b-ELISAs and other serological tests were determined using the McNemar test with Yates

correction. The SAS program version 9.1 (Cary, North Carolina, U.S.A.) was used for logistic regression analysis on primary and secondary infections. Significance was defined as $p < 0.05$. Microsoft Office Excel 2003 was used to calculate the mean, the variance, and 2 and 3 SD values for the negative control samples.

**2.3.8. Description of the Statistical Methods for Receiver Operating Characteristic curve and Dot Plots for graphical determination of cut-off values.**

The ROC curve utilizes a graphical method to identify the cut-off values that simultaneously minimize false positive rates and false negative rates. The false positive and false negative rates for a given process are inherently inversely linked. As the false positiverate increases, the false negative rate decreases for normally distributed data, and vice versa. The false positive rate for a particular cut-off value can be described as the "Specificity" or True Negative Rate, where:

Specificity = 100% - False Positive Rate = True Negative Rate

The False Negative Rate for a particular cut-off value is described as the "Sensitivity" or True Positive Rate, where:

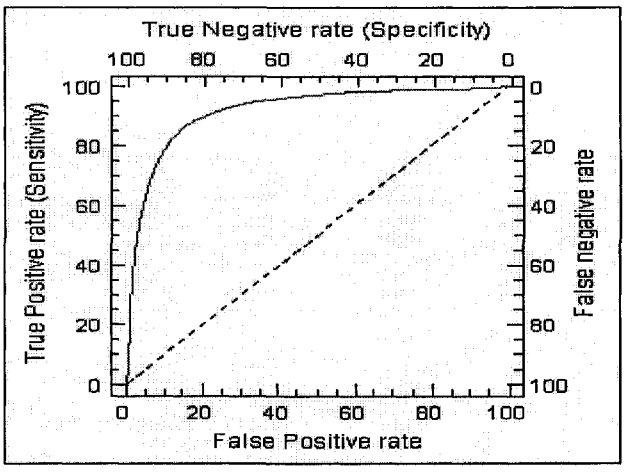
Sensitivity = 100% - False Negative Rate = True Positive Rate

A cut-off value is the threshold value, where test results that fall below (are less than) the cut-off value are deemed to be negative test results, and values that are greater than the cut-off value are deemed to be positive test results. So, the ROC analysis plots Sensitivity vs. Selectivity for many different cut-off values, graphically displaying the minimum false positive and minimum false negative pairs.

In the ROC analysis false negative rates are represented on the y axis versus false positive rates on the x axis. Specifically, a ROC curve is a graph that plots the true positive rates (Sensitivities) relative to the false positive rates, calculated individually for different cut-off values. (See the following "Theoretical Example Plot of Typical ROC Analysis Results.) Each point on the plot represents a Sensititivity/Specificity pair corresponding to a particular cut-off value. A test method that discriminates perfectly between positive and negative sample results has a curve plot that reaches the upper left corner (100% Sensitivity, 100% Specificity), and the area under the curve equals 1. In the theoretical example shown below, the area under the curve is 0.84, meaning that 84% of the individuals from the positive group have a test value higher than the individuals from the negative group. When the test can not distinguish between the positive and the negative groups the area will be equal to 0.5 and the curve will coincide with the diagonal line on the graph.

Theoretical Example Plot of Typical ROC Analysis Results

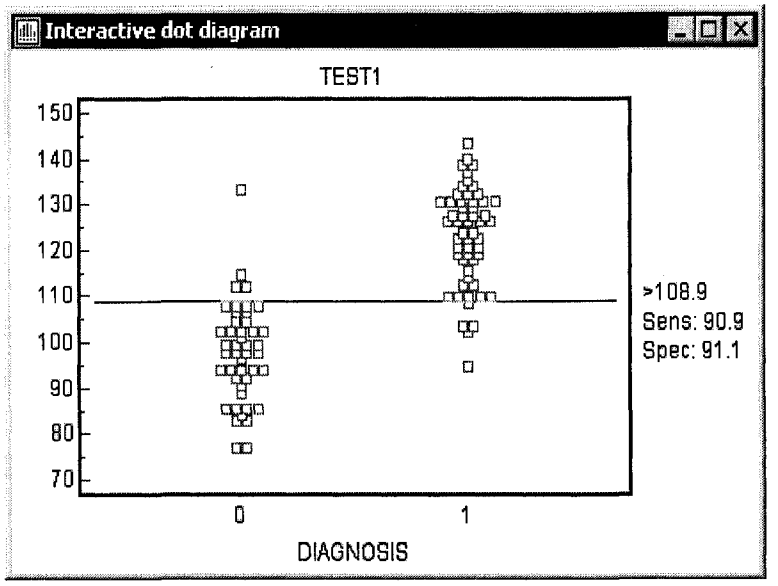
(Figure Provided by MedCalc for Windows v.8.2)




Dot Plots are another useful method for graphically representing and determining cut-off values. When constructing Dot Plots, the data sets of the negative

and positive groups are represented as separate groups of data points presented as vertical groups of dots in a dot diagram (see below). The horizontal line indicates the cut-off point with the best separation between the two groups. The sensitivity and specificity corresponding to the test is indicated at the right side of the graph. The data shown below are purely hypothetical training examples provided by the software manufacturer: MedCalc for Windows Version 8.2.

Theoretical Example Plot of Typical Dot Plot Results
(Figure Provided by MedCalc for Windows v.8.2)




## 2.4. RESULTS.

### 2.4.1. Patient serum samples.

Overall, 725 serum samples from patients from three countries were analyzed in this study. The general information about the patients was obtained from collaborating laboratories in the USA, Thailand and México (Table 2.4.1). Most of the serum samples obtained from Thailand were from children averaging 8 years old. The Colorado samples were mainly from middle aged adults, averaging 50 years old. A

broader age range was obtained from the patients from Yucatán with a mean age of 29 years. All serum samples were heat-treated (56°C, 30 minutes) before testing. A total of 366 serum specimens (292 positive and 74 negative) were obtained from patients who presented with West Nile fever symptoms during the 2003 outbreak in Colorado, USA (30). All samples had been previously analyzed by WNV IgM-ELISA (144) at the Colorado Department of Public Health and Environment and by plaque reduction neutralization test (PRNT) in BSL-3 facilities at Colorado State University (CSU). The WNV positive controls were sera that were positive in both tests; the negative control samples were only sera that were negative in both tests. A total of 195 serum samples were obtained from Thai children with suspected DENV infections (157 positive and 38 negative). Sera were previously tested by DEN IgM- and IgG-capture ELISA (101) and RT-PCR (124) in the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand, and by PRNT in the BSL-3 facilities at CSU (Table 2.4.1). In addition to the samples from Thailand and USA, 164 serum samples from patients from México with suspected dengue infections were included in this study. Among these 164 Mexican serum samples, fifty cases were diagnosed as dengue positive by IgM-capture ELISA, RT-PCR and/or virus isolation, and the remaining 114 samples were classified as negative. For the b-ELISA analysis, 19 samples were included from serum collections between 1997 and 2001 before the introduction of WNV into México. Of these, 16 samples were confirmed as dengue positive. An additional 145 serum samples were collected between 2002 and 2005 after the introduction of WNV in México, 34 of the samples were confirmed as dengue positive (Table 2.4.1). It is important to highlight that the first evidence of WNV transmission in the Yucatán

Peninsula was the detection of antibodies to WNV in horses in mid 2002 (135). WNV, DENV, and SLEV IgM-capture ELISAs were also conducted at CSU to confirm diagnostic results. Collection and testing of human sera was approved by the Institutional Ethical Review Boards of the participating institutions.

DENV infections were confirmed by AFRIMS of Thailand in 80.5% (157/195) of serum specimens from Thailand, and the Yucatán laboratory confirmed DENV infections in 30.5% (50/164) of serum specimens from México. When tested by the laboratories in Thailand and Mexico, antibodies to general flaviviruses were detected in 98.5% (193/195) and 86.6% (142/164) of the Thai and Mexican samples, respectively. These percentages confirm the high prevalence of antibodies to flaviviruses among the people from Thailand and México.

### 2.4.2. Evaluation of the WNV b-ELISA using serum samples from WNV-positive patients from Colorado.

The diagnostic efficacy of the b-ELISA for the diagnosis of WNV infections was evaluated using sera collected from 366 human patients presenting with WNV-like symptoms in Colorado in 2003. Two hundred and ninety two patients were classified as WNV positive and 74 were negative by IgM-capture ELISA. Those samples were chosen because the PRNT and the IgM-capture ELISA results were in perfect concordance. An optimized cut-off value that maximizes both the diagnostic sensitivity and the diagnostic specificity of the b-ELISA for the WNV-infected patients from Colorado was determined by the ROC analysis (65, 72).

**Table 2.4.1. History and Information about 725 laboratory confirmed serum samples from patients infected with WNV, DEN or without evidence of flavivirus infections.**

| Country | Colorado, U.S.A. | Bangkok, Thailand | Yucatán, México | |
|---|---|---|---|---|
| **Institution** | Colorado Department of Public Health and Environment | Armed Forces Research Institute of Medical Sciences | Universidad Autónoma de Yucatán | |
| **Laboratory techniques applied for diagnosis** | IgM-ELISA for WNV | IgM and IgG-ELISA for DEN and JE, RT-PCR for DEN | IgM-ELISA for DEN and RT-PCR for DEN and/or Virus isolation | |
| **Year of collection** | 2003 | 2001 | 1997-2001 | 2002-2005 |
| **Age Range (years)** | 6 – 95 | 2 – 28 | 2 – 67 | 0 – 85 |
| **Mean Age (years)** | 50 | 8 | 29 | 29 |
| **No. Samples tested (n)** | 366 | 195 | 19 | 145 |
| **No. Positive for WNV** | 292 | 0 | 0 | 0 |
| **No. Positive for DENV** | 0 | 157 | 16 | 34 |

### 2.4.3. Results obtained using ROC analyses.

A complete sensitivity/specificity report (Table 2.4.2.) and a ROC curve (Figure 2.4.1.0 were created entering the percentages of blocking of the WNV b-ELISA and the diagnosis classification by PRNT (as WNV positive or WNV negative).

The complete sensitivity/specificity report was prepared using the MedCalc program. (Table 2.4.2). The report includes the number of positive and negative samples introduced in the analysis, the area under the curve reached by all samples at the 95% confidence interval (95% CI), the p value, and a list of cut-off values

(criterion) with their corresponding sensitivities, specificities of the test and the positive (+LR) and negative likelihood ratio (-LR).

**Table 2.4.2. MedCalc display of the ROC analysis of the sensitivity and specificity of the WNV b-ELISA.** Human serum specimens (n = 366) from Colorado. The report is reduced for simplicity purposes.

| | |
|---|---|
| Variable | MAb_3.1112G |
| Classification variable | WNV_diagnosis |
| Positive group | |
| WNV diagnosis | = Positive |
| Sample size | 292 |
| Negative group | |
| WNV diagnosis | = Negative |
| Sample size | 74 |
| Disease prevalence (%) | unknown |
| Area under the ROC curve | 0.962 |
| Standard error | 0.009 |
| 95% Confidence interval | 0.937 to 0.979 |
| Significance level P (Area=0.5) | 0.0001 |

| Criterion | Sensitivity | 95% CI | Specificity | 95% CI | +LR | -LR |
|---|---|---|---|---|---|---|
| >-35 | 100.0 | 98.7 - 100.0 | 1.4 | 0.2 - 7.3 | 1.01 | 0.00 |
| >0 | 97.3 | 94.7 - 98.8 | 60.8 | 48.8 - 72.0 | 2.48 | 0.05 |
| >1 | 95.5 | 92.5 - 97.6 | 68.9 | 57.1 - 79.2 | 3.07 | 0.06 |
| >2 | 94.2 | 90.8 - 96.6 | 73.0 | 61.4 - 82.6 | 3.48 | 0.08 |
| >3 | 93.8 | 90.4 - 96.3 | 74.3 | 62.8 - 83.8 | 3.65 | 0.08 |
| >4 | 92.5 | 88.8 - 95.2 | 77.0 | 65.8 - 86.0 | 4.02 | 0.10 |
| >5 | 91.8 | 88.0 - 94.7 | 79.7 | 68.8 - 88.2 | 4.53 | 0.10 |
| >6 | 91.4 | 87.6 - 94.4 | 83.8 | 73.4 - 91.3 | 5.64 | 0.10 |
| >7 | 90.8 | 86.8 - 93.8 | 89.2 | 79.8 - 95.2 | 8.39 | 0.10 |
| >8 | 89.0 | 84.9 - 92.4 | 91.9 | 83.2 - 96.9 | 10.98 | 0.12 |
| >9 | 87.3 | 83.0 - 90.9 | 93.2 | 84.9 - 97.7 | 12.92 | 0.14 |
| >10 | 84.9 | 80.3 - 88.8 | 95.9 | 88.6 - 99.1 | 20.95 | 0.16 |
| >11 | 83.9 | 79.2 - 87.9 | 97.3 | 90.6 - 99.6 | 31.04 | 0.17 |
| >12 | 82.9 | 78.1 - 87.0 | 98.6 | 92.7 - 99.8 | 61.33 | 0.17 |
| >20 | 72.9 | 67.5 - 78.0 | 100.0 | 95.1 - 100.0 | | 0.27 |
| >21 | 71.2 | 65.7 - 76.4 | 100.0 | 95.1 - 100.0 | | 0.29 |
| >22 | 70.9 | 65.3 - 76.0 | 100.0 | 95.1 - 100.0 | | 0.29 |
| >23 | 69.2 | 63.5 - 74.4 | 100.0 | 95.1 - 100.0 | | 0.31 |
| >24 | 68.5 | 62.8 - 73.8 | 100.0 | 95.1 - 100.0 | | 0.32 |
| >26 | 67.1 | 61.4 - 72.5 | 100.0 | 95.1 - 100.0 | | 0.33 |
| >27 | 65.8 | 60.0 - 71.2 | 100.0 | 95.1 - 100.0 | | 0.34 |
| >28 | 65.4 | 59.6 - 70.9 | 100.0 | 95.1 - 100.0 | | 0.35 |
| >29 | 64.7 | 58.9 - 70.2 | 100.0 | 95.1 - 100.0 | | 0.35 |
| >30 | 62.7 | 56.8 - 68.2 | 100.0 | 95.1 - 100.0 | | 0.37 |
| >95 | 0.0 | 0.0 - 1.3 | 100.0 | 95.1 - 100.0 | | 1.00 |

+LR : Positive likelihood ratio

-LR : Negative likelihood ratio

The ROC curve (solid line in the Fig. 2.4.1. graph) was calculated using the percentage of blocking from the 292 WNV-positive and the 74 WNV-negative samples, and indicates that 8% blocking is the optimal cut-off value for maximum diagnostic Sensitivity and Specificity (simultaneously minimizing false positive and false negative rates). In the graph, the area under the curve is 0.962, meaning that 96% of the individuals from the WNV-positive group had test results that were greater than the individuals from the WNV-negative group.

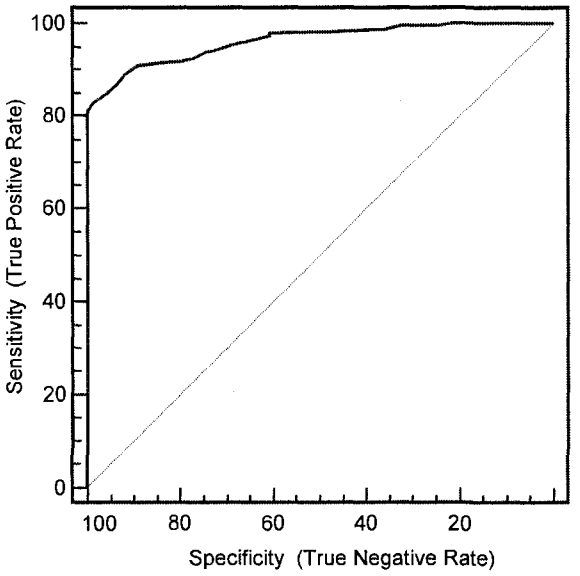



**Fig. 2.4.1 ROC Analysis Operating Curve for MAb 3.1112G b-ELISA Results.**

### 2.4.4. Results obtained using Dot Plot analyses.

As discussed above, Dot Plots are also useful to graphically present and determine cut-off values. The horizontal line indicates the cut-off value with the best separation between the negative and positive groups. The Dot Plot analysis in Fig. 2.4.2 presents the WNV b-ELISA test results with the monoclonal antibody and the human serum samples from the WNV outbreak that occurred in Colorado in 2003.

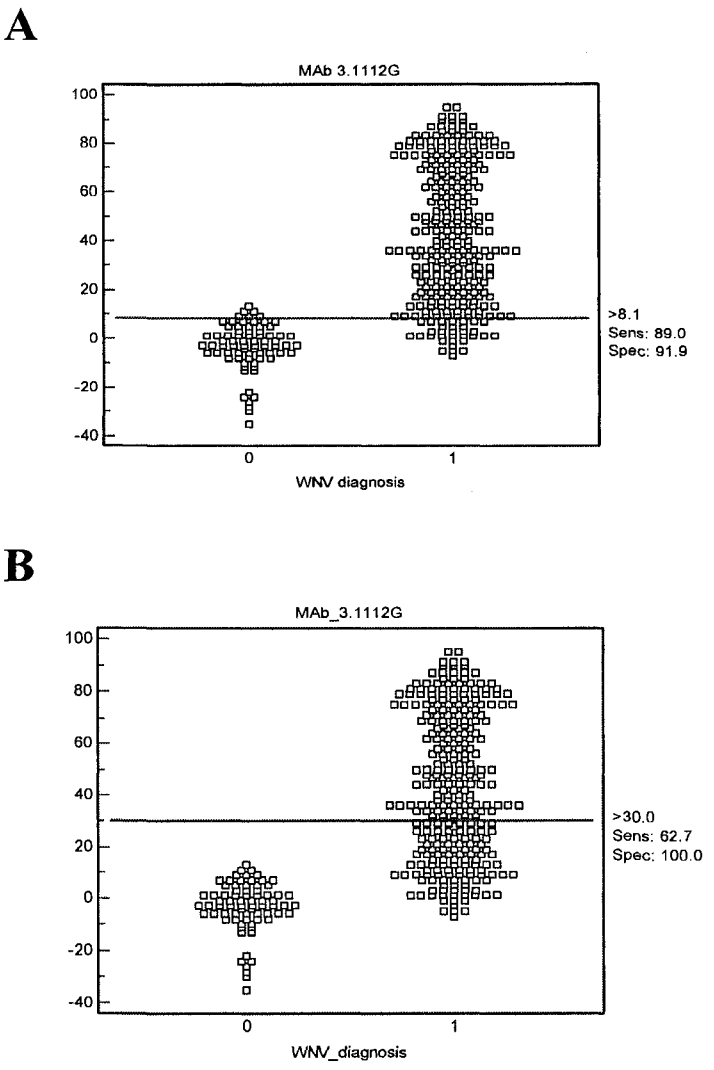



**Fig.2.4.2. Dot plot tests results with 292 WNV-positive and the 74 WNV-negative sera from patients from Colorado, USA.** Samples were analyzed by WNV-specific b-ELISA. The cut-off value used for the upper dot plot A was 8% (as determined by ROC analyses) and 30% for the lower dot plot B. The y-axis scale is: percentage obtained with b-ELISAs.

When the cut-off value was 8% (Fig. 2.4.2.A), the sensitivity and specificity were 89% and 91.9%, respectively. When the cut-off value was set at 30% (Figure

2.4.2.B), which is the value used when testing sera from other vertebrate animals (17, 18), the sensitivity and specificity were 62.7% and 95%, respectively (Table 2.4.3.).

**Table 2.4.3. Sensitivity and specificity of b-ELISA using different diagnostic criteria.** Diagnostic results using the b-ELISA for the detection of antibodies to WNV and other flaviviruses in 366 human serum samples from Colorado, USA. Results were compared with $PRNT_{90}$, which is the gold standard for WNV diagnosis. Cut-off values were determined by the different methods described above.

| MAb[a] | Method of calculation of cut-off values | Cut-off value | Overall agreement with $PRNT_{90}$ % | Sensitivity % | Specificity % |
|---|---|---|---|---|---|
| 3.1112G | 2 SD[b] | 0.278 | 79 | 73 | 100 |
| | 3 SD | 0.245 | 70 | 63 | 95 |
| | ROC[c] | 8 % | 91 | 89 | 92 |
| | ROC | 30 % | 70 | 63 | 95 |
| 6B6C-1 | 2 SD | 0.254 | 70 | 63 | 97 |
| | 3 SD | 0.192 | 56 | 45 | 100 |
| | ROC | 13 % | 79 | 75 | 93 |
| | ROC | 30 % | 64 | 57 | 97 |

[a] The MAb 3.1112G is WNV-specific and the MAb 6B6C-1 is flavivirus specific; [b] 2 SD, 2 standard deviation for the noninfected reference population based on optical densities obtained with b-ELISA; [c]ROC, receiver operating characteristic curve analysis.

The overall agreement between the $PRNT_{90}$ values and WNV b-ELISA values was 91%, using an 8% cut-off value from ROC analyses. The overall agreement fell to

just 70% when using the historical 30% cut-off value (Table 2.4.3.). This difference demonstrates a significant improvement in test efficacy after using ROC analyses to determine the cut-off value for the WNV b-ELISA versus using the historical 30% cut-off value. This pattern of ROC analyses producing better cut-off values for b-ELISA results is confirmed when using the flavivirus-specific MAb 6b6C-1 in the b-ELISA (Table 2.4.3.).

**2.4.5. Calculation of cut-off values, overall agreements between b-ELISA and PRNT, sensitivities and specificities using different diagnostic criteria.**

To determine the best cut-off values for the b-ELISA tests using the WNV-specific MAb 3.1112G and the flavivirus-specific MAb 6B6C-1, three diagnostic criteria were compared. These were: 1) the mean (X) result + 2 SD for the negative serum samples based on optical densities obtained with b-ELISA; 2) the mean (X) results + 3 SD for the negative serum specimens based on the optical densities with b-ELISA; and 3) the ROC analyses described above.

The human sera from patients diagnosed as WNV-positive or negative from the 2003 WNV-outbreak in Colorado in 2003 were tested using all three diagnostic criteria (Table 2.4.4.). The cumulative statistics obtained using 2 and 3 SDs cut off values are described in Table 2.4.4.

The overall agreement of b-ELISA results with the gold standard test for flaviviruses and the sensitivities and specificities of each diagnostic criterion are summarized in Table 2.4.3. Using a 2 SD cut-off value in the WNV b-ELISA, the overall agreement with PRNT and the sensitivity and specificity were 79%, 73% and 100%, respectively (Table 2.4.3.).

**Table 2.4.4. Cumulative statistics for evaluating of b-ELISA cut-off values.**

| Parameters | WNV b-ELISA using MAb 3.1112G | Flavi b-ELISA using MAb 6B6C-1 |
|---|---|---|
| **Sum of all negative results** | 25 | 28 |
| **Mean (average) of all negative results** | 0.34324 | 0.37831 |
| **Standard Deviation of all negative results** | 0.032866 | 0.061947 |
| **2 Standard Deviations of all negatives** | 0.065732 | 0.123893 |
| **3 Standard Deviation of all negatives** | 0.098597 | 0.185840 |
| **Cut-off value for 2 Standard Deviations** | 0.278 | 0.254 |
| **Cut-off value for 3 Standard Deviations** | 0.245 | 0.192 |
| **Total WNV positive values using 2 Standard Deviations** | 212 | 184 |
| **Total WNV positive values using 3 Standard Deviations** | 184 | 130 |
| **Total WNV samples** | 292 | 292 |

Using the 3 SD cut-off value, the overall agreement with PRNT and the sensitivity and specificity were 70%, 63% and 95%, respectively (Table 2.4.3.). Comparison of the percent overall agreement with PRNT and sensitivities and specificities obtained using the cut-off values of 2 SD, 3 SD, and ROC analysis revealed that the latter yielded the best diagnostic criteria and results (Table 2.4.3.). Thus, in all subsequent characterizations of the b-ELISA the 8% cut-off value was used with the WNV-specific MAb 3.1112G, and the 13% cut-off value was used the MAb 6B6C-1.

The diagnostic efficiencies of the WNV b-ELISA using the 8% cut-off value obtained by ROC criteria, WNV-$PRNT_{90}$, and IgM-capture ELISA were then compared using $X^2$ analyses (Table 2.4.5).

**Table 2.4.5. Comparisons of serologic tests for diagnosis of WNV infections using sera from patients from Colorado, USA by chi square analysis.**

| Comparisons* | Positive in both tests | Positive in $PRNT_{90}$ & Negative in ELISA | Positive in ELISA & Negative in $PRNT_{90}$ | Negative in both tests | p value, $x^2$ analysis |
|---|---|---|---|---|---|
| IgM-ELISA vs. WNV-$PRNT_{90}$ | 292 | 0 | 0 | 74 | - |
| WNV b-ELISA vs. WNV-$PRNT_{90}$ | 265 | 27 | 6 | 68 | 0.0005 |

*Cut-off values of tests: for $PRNT_{90}$ WNV titer $\geq$ 1:10; for IgM-ELISA positive/negative ratio $\geq$ 3.0; for b-ELISA-WNV 8%.

The IgM-ELISA and WNV-PRNT test results for Colorado serum samples were completely concordant, since there were no false positive or false negative results. Using the WNV-$PRNT_{90}$ as the gold standard, the WNV b-ELISA percentages of false positive and false negative results were 9.25% and 8.11% (data not shown), respectively, which correspond to a sensitivity and specificity of 90.75% and 91.89%, respectively (Table 2.4.5.). The false positive and false negative rates as well as sensitivity and specificity of the three tests are more clearly presented in the following two by two table analyses comparing IgM-ELISA and b-ELISA results with PRNT results (Table 2.4.6.). The results presented in Table 2.4.5 were further evaluated using the McNemar test with Yates' correction. These results are shown in two separate 2 x 2

tables to evaluate the discrepancy levels between paired proportions obtained between the three tests (Table 2.4.6 A & B).

**Table 2.4.6. Comparison of serologic tests for diagnosis of WNV infections using sera from patients from Colorado, USA.**

A

| TEST | WNV-PRNT POSITIVE | WNV-PRNT NEGATIVE |
|---|---|---|
| IgM-ELISA POSITIVE | 292 | 0 |
| IgM-ELISA NEGATIVE | 0 | 74 |
| TOTAL | 292 | 74 |

Sensitivity: 292/292 x 100% = 100% Sensitivity => 0% False negative rate
Specificity: 74/74 x 100% = 100% Specificity => 0% False positive rate

B

| TEST | WNV-PRNT POSITIVE | WNV-PRNT NEGATIVE |
|---|---|---|
| WNV b-ELISA POSITIVE | 265 | 6 |
| WNV b-ELISA NEGATIVE | 27 | 68 |
| TOTAL | 292 | 74 |

Sensitivity: 265/292 x 100% = 90.75% Sensitivity => 9.25% False negative rate
Specificity: 68/74 x 100% = 91.89% Specificity => 8.11% False positive rate

The high levels of agreement between WNV-PRNT results and the IgM-ELISA results (Table 2.4.6.A.) and the WNV-PRNT and WNV b-ELISA results (Table

2.4.6.B.) demonstrate the efficacy of both ELISA assays for diagnosing WNV infection in patients that have a low probability of prior flavivirus infections. These serum samples were from Colorado and thus would be unlikely to contain antibodies to other flavivirus.

### 2.4.6. Evaluation of WNV b-ELISA using serum samples from México and Thailand.

The purpose of using serum samples from patients with previously confirmed DENV or JEV infections was to determine the ability of the WNV b-ELISA to discriminate new WNV infections from antibodies from prior flavivirus infections. If the antibodies from prior flavivirus infections yield positive results in WNV b-ELISA but there is no WNV infection as determined by the gold standard test, PRNT, then it is a false positive WNV b-ELISA result. False positive results are often called Type I errors or α errors.

When the samples were assayed at AIDL by WNV b-ELISA (using a cut-off value of 8%), 81% (156/195) of the specimens from Thailand and 100% (19/19) of the specimens from México collected before 2001 prior to the introduction of WNV and 76% (110/145) of the samples collected from Mexico between 2002-2005 yielded false positive results for the diagnosis of WNV infections (Table 2.4.7.). In contrast the false positive rate for serum specimens from the US was only 5%.

The dramatic difference in false positive rates in sera from the US and the dengue endemic countries of Thailand, and México is most likely due to interference from antibodies to previous dengue infections (and/or JEV infections in the samples from Thailand). In other words, there was a much high probability for false positive

diagnoses of WNV using WNV b-ELISA (MAb 3.1112G) in patients from Thailand and Mexico who had likely experienced primary or secondary DENV infections. The false positive rate was much lower for patients who likely had not experienced previous flavivirus infections (as seen in the negative samples from US). Due to the low incidence of WNV infections or other flaviviruses in Colorado, the risk of false positive diagnosis for WNV is very low (Tables 2.4.5, 2.4.6, and 2.47).

**Table 2.4.7 False positive WNV b-ELISA test diagnostic results obtained with WNV or DEN antibody positive and negative patients from USA, Thailand, and México.** All serum samples were tested by WNV-b-ELISA test at AIDL (Colorado State University).

| **Country of origin of the samples** | **USA** | **Thailand** | **México** | |
|---|---|---|---|---|
| **Year of samples collection** | 2003 | 2001 | 1997-2001 | 2002-2005 |
| **No. of samples classified as WNV-negative** | 74 | 195 | 19 | 145 |
| **No. (%) of false positive samples for WNV infections[1]** | 4 (5%) | 156 (80%) | 19 (100%) | 110 (76%) |
| **False positive rate[2]** | 0.054 | 0.80 | 1.0 | 0.758 |

[1] The WNV b-ELISA test was conducted using the MAb 3.1112G; [2] The false positive rate is the proportion of WNV-negative samples that were positive by b-ELISA.

### 2.4.7. Surveillance of flavivirus infections by b-ELISA with the monoclonal antibodies 3.1112G and 6B6C-1.

In routine surveillance for WNV and other flavivirus infections in animals, b-ELISA tests are conducted using two different monoclonal antibodies (17) to either detect antibodies specific for WNV (MAb 3.1112G), or to detect antibodies that cross react between flaviviruses (MAb 6B6C-1). To evaluate the ability of the b-ELISA to detect antibodies to flaviviruses and to WNV in humans in areas that differ in flavivirus endemicity, we conducted ROC analyses with data obtained from patients from the US, Thailand and México. The ability of the test to distinguish between the positive and negative groups is based on the value obtained in the area under the curve as indicated after ROC analysis.

For the serum samples from Colorado, the WNV-specific and the flavivirus-specific b-ELISAs distinguished between positive and negative groups. The highest area under the curve was 0.962 when using the MAb 3.1112G and was 0.903 when using MAb 6B6C-1. The sensitivity rate was 89% and the specificity rate was 92% with MAb 3.1112G, and 75% and 93%, respectively, with MAb 6B6C-1 (Table 2.4.8).

In contrast, the b-ELISA test results were unsatisfactory for the samples from Thailand and México. The areas under the ROC curves ranged from 0.767 to 0.549 (Table 2.4.8.) as compared to >0.9 for the samples from Colorado. Similarly the sensitivity and specificity of the WNV b-ELISA were 63% and 81% for the Thailand samples and 65% and 74% for the Mexico samples.

The ability of the b-ELISA test to distinguish between the positive and negative groups is reflected in the values obtained for the areas under the curves. A value of 1

indicates that the test perfectly distinguishes between positive and negative groups. This evaluation confirmed the previous results that the b-ELISA methods tested did not work well in the likely presence of antibodies from prior flavivirus infections.

**Table 2.4.8. Receiver Operating Curve analysis obtained from the serum samples from USA, Thailand and Mexico with the b-ELISA using two monoclonal antibodies, the WNV-specific MAb 3.1112G and the flavivirus-broad reactive MAb 6B6C-1.**

| Geographic location | Colorado, USA | | Bangkok, Thailand | | Yucatan, Mexico | |
|---|---|---|---|---|---|---|
| **MAb used** | **3.1112G** | **6B6C-1** | **3.1112G** | **6B6C-1** | **3.1112G** | **6B6C-1** |
| **Cut-off value** | 8 % | 13% | 30% | 74% | 28% | 93% |
| **Sensitivity** | 89% | 75% | 63% | 89% | 65% | 76% |
| **Specificity** | 92% | 93% | 81% | 47% | 74% | 40% |
| **Area under the curve[1]** | 0.962 | 0.903 | 0.767 | 0.685 | 0.752 | 0.549 |
| **Significance level [2]** | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.1454 |

[1] When the test cannot distinguish between positive and negative results, the area under the curve will be equal to 0.5; [2] Significance level between groups was determined by using $P < 0.05$ confidence limits.

## 2.5. DISCUSSION.

The b-ELISA test was evaluated in its performance for detecting WNV and flavivirus antibodies in 366 human serum samples from Colorado. The ROC analysis of WNV b-ELISA results determined an optimal cut-off value of 8% that yielded a sensitivity of 89% and specificity of 92%, corresponding to an 11% false negative rate and an 8% false positive rate (Table 2.4.3.). The ROC analysis provided superior diagnostic results; application of the 30% cut-off value used previously in animal studies, yielded an unacceptably high 37% false negative rate. The ROC analysis applied to flavivirus b-ELISA results for 366 human sera samples from Colorado determined an optimal cut-off value of 13% that yielded a sensitivity of 75% and specificity of 93%, corresponding to a 25% false negative rate and a 7% false positive rate (Table 2.4.3.). The ROC analyses' cut-off values also significantly outperformed both 2 SD and 3 SD statistical approaches in other WNV b-ELISA tests (Table 2.4.3). These evaluations demonstrate that the WNV b-ELISA method worked well using ROC analysis cut-off values for both WNV positive and negative Colorado human sera samples, and the ROC cut-off values definitively outperformed other accepted statistical approaches.

The flavirus b-ELISA provided an unacceptably high false negative rate for Colorado human sera samples. The reason for this is unknown, but the flavivirus b-ELISA for WNV infections would not be the test of choice for screening for WNV infections. Too many cases would be missed. It is also worth considering the potential consequences of the 8% false positive rate in the WNV b-ELISA testing. False positive results may cause harm by misleading health caregivers and Public Health officials into

taking inappropriate WNV oriented palliative actions or interventions. False positive WNV diagnoses would also likely cause physicians to stop looking for the real causes of infection and disease. False positive results generally arise from anomalies in either the samples or the methodology. The false positive results with the Colorado specimens are likely real, since the sample size (n) was reasonably large (366 samples). These false positive WNV b-ELISA may arise from the inclusion of specimens from patients who were originally from Latin America or tourists who had been infected when visiting dengue endemic areas. There are significant Hispanic immigrant populations in the Denver, Greeley, and ski resort areas of Colorado. Unfortunately, the samples were coded, so we could not assess these possiblilities. This potential mechanism to account for the false positive results is supported by the high rates of false positive WNV b-ELISA results for samples from Mexico and Thailand (Table 2.4.8).

Unfortunately, the WNV b-ELISA can not be used to accurately diagnose infections in humans in areas where other flaviviruses are endemic due to unacceptably high rates of false positive test results (Table 2.4.7). The seroprevalence rates for flavivirus infections are very high in many regions in the tropics, and secondary flavivirus infections, which induce very high titers of cross reactive antibodies to flaviviruses, make differentiation of these antigenically closely related viruses even more difficult. In addition, secondary infections can induce antibodies to previous viral infections. Studies of antibody responses in hamsters sequentially infected with WNV, JEV, SLEV and YFV clearly demonstrated that it is difficult to make serologic diagnosis of WNV infections in animals with preexisting flavivirus immunity (202).

As noted previously in Chapter I (section 1.3.2 - History of dengue in México and Yucatán), the DENV seroprevalence rates are extremely high in the Yucatán (81.5%), and secondary infections are also very common (61). We have not detected WNV infection in humans in the Yucatán, despite serological evidence that the virus is circulating there in birds, horses and other animals (59). The reason for the lack of human infections in the Yucatán is unknown. Human infections (7 cases) have been diagnosed in northern México (www.salud.gob.mx). It is possible that the high DENV seroprevalence rates in the Yucatán protect against WNV infection. Studies conducted in the 1960's suggested that infection with DENVs confers cross protection against WNV and SLE infections (19, 165, 180).

The WNV b-ELISA test readily detects the infecting virus in primary infections; however, viruses causing secondary infections are very difficult to identify because of cross reactions and original antigenic sin. Even in primary infections, b-ELISA positive samples should be confirmed using PRNT or complement fixation (CF) to confirm the etiologic agent (161, 180).

In summary, the WNV b-ELISA could potentially be used for the diagnosis of WNV infections in humans in areas where only WNV circulates, but should not be used in areas where other flaviviruses are circulating. Currently we are trying to identify specific WNV and dengue epitopes that are could potentially be provided as peptide or recombinant antigens in the b-ELISA or Western blot analyses to differentiate secondary infecting flaviviruses. Development of a serological test to identify flaviviruses causing secondary infections would be a significant public health contribution.

## CHAPTER III

# EVALUATION OF SYNTHETIC PEPTIDES OF NON-STRUCTURAL-1 AND THE ENVELOPE GLYCOPROTEINS OF WEST NILE VIRUS FOR DIFFERENTIAL DIAGNOSIS OF WEST NILE AND DENGUE VIRUS INFECTIONS.

## 3.1. ABSTRACT.

The main goal of this study was to evaluate arrays of synthetic peptides of the non-structural-1 (NS1) and the envelope (E) proteins of West Nile virus (WNV) as diagnostic reagents for peptide-based ELISA for WNV differential diagnosis. Dot blot analyses and peptide-based ELISA were used to evaluate the reactivities of two monoclonal antibodies, 195 human serum samples and 2 horse serum samples with 67 synthetic peptides spanning the E and 46 peptides spanning the NS1 proteins of WNV. Serum samples from WNV and DEN infected and non-flavivirus infected humans and monoclonal antibodies were tested in dot blot assays for reactivity to the peptides.

The NS1-27 peptide of the WNV was recognized by the monoclonal antibody 3.1112G specific for NS1 of WNV, but it also reacted with all human serum samples tested, including patients with WNV or DENV infections, plus those with no serologic evidence of previous flavivirus infection. The NS1-1 was recognized only by serum samples from patients with recent DENV infections. NS1-1 was not recognized by neither the MAb 3.1112G, nor serum samples from patients with WNV infections, nor

any of the flavivirus negative samples, (samples with no measurable antibodies in previous flavivirus testing). The peptides NS1-2 and NS1-26 were only recognized by serum samples from patients with previous DENV infections.

None of the synthetic peptides of the E glycoprotein were recognized by the monoclonal antibody 6B6C-1, as determined by Dot Blot analyses. The peptide E-44 was recognized by all serum samples tested, including human serum samples with WNV or DEN infections, human sera without antibodies to other flaviviruses, and by both WNV positive and WNV negative horse sera.

The performance of the peptide-based ELISA for each group of samples was evaluated using Receiver Operating Characteristic (ROC) analyses. ELISA testing of the WNV peptide NS1-1 and serum from patients with recent DENV infections produced ROC results of sensitivity of 60% (corresponding to a 40% false negative rate), and specificity of 95.8% (corresponding to a 4.2% false positive rate). The WNV NS1-1 peptide was also determined to be the best peptide evaluated for distinguishing between recent dengue infections and sera classified as flavivirus-negative by ROC analyses. The overall conclusion was that none of the WNV peptides were effective for WNV diagnoses.

## 3.2. INTRODUCTION.

Several closely related flaviviruses are important human pathogens, including dengue (DEN), West Nile (WN), yellow fever (YF), tick-borne encephalitis (TBE), Japanese encephalitis (JE), and Saint Louis encephalitis (SLE) viruses. WNV is a member of the JEV serocomplex of flaviviruses (25). WNV is transmitted by

mosquitoes and can cause febrile illness or fatal encephalitis in humans, other mammals and birds. The WNV genome is a positive-stranded RNA that encodes a single polyprotein. After processing, the polyprotein produces 3 structural proteins, (capsid, pre-membrane and envelope), and 7 non-structural proteins. The E-glycoprotein homodimer of the flaviviruses folds into three distinct domains called I, II and III, as first determined by X-ray crystallography using TBE, and later confirmed with DEN and WNV (108, 151, 169). Peptides corresponding to the antigenic determinants of E (57, 149, 172) and NS1 (58, 64) of DENV have been extensively mapped, but not much information regarding the peptides of WNV has been published. Several research groups have recently explored diagnosing DENV, tick-borne encephalitis, and WNV infections using synthetic peptides derived mainly from the E and the NS1 glycoproteins in different ELISA formats (5, 95, 160, 215).

Synthetic peptides offer several advantages over biological proteins, and they are now commonly used for the detection of antibodies against viral proteins (150). This can be extremely important if preparation of biological molecules requires biosafety level 3 or 4 protocols. Laboratories certified for and capable of executing biosafety level 3 and 4 protocols are not common in developed countries and are almost non-existent in developing countries. Ironically, the countries that have the greatest need for detection of flaviviruses frequently have the most limited capabilities, due to the hazards of handling live viruses. Synthetic peptides present few of the hazards associated with handling live viruses, so analytical methods that employ synthetic peptides could be safely used in the developing world. Further, synthetic peptides cover

B-cell epitopes which typically comprise a small number of amino acids (between 12 to 20 amino acids); thus they can be readily and inexpensively synthesized.

The main hypothesis for these studies was that the epitopes recognized by monoclonal antibodies could be identified and exploited for specific flavivirus diagnoses. The best analytical candidate for exploiting this property would be the epitope-blocking enzyme-linked immunosorbent assay (ELISA). The epitope-blocking ELISA (b-ELISA) test was developed by Hall et al (83) and validated by Blitvich et al (17, 18). The b-ELISA is a highly sensitive and specific test to detect antibodies to WNV in any species of birds, mammals or other animals (17, 18). The b-ELISA format is a competitive ELISA and employs WNV-infected cell lysates as antigens and one monoclonal antibody, MAb 3.1112G, specific for the NS1 protein of WNV (84). One drawback to the test is that the WNV-infected cell lysate antigen must be prepared in a Biosafety Level 3 Laboratory (BSL-3) protocols. Since few laboratories in disease endemic countries have these capabilities, we explored the safer alternative of potentially using synthetic peptides as antigens in the b-ELISAs for diagnostic testing.

The main objective of this study was to identify linear epitopes, using synthetic peptides of the E and NS1 proteins of WNV, recognized by monoclonal antibodies and human serum samples from patients infected with WNV. A second objective was to determine if those peptides could be used as diagnostic reagents in an ELISA for the differential diagnoses of WNV and DENV infections.

To determine if synthetic peptides could be used in place of the cell lysate antigen for WNV diagnosis, 46 different NS-1 peptides and 67 different envelope

protein peptides were characterized for diagnostic utility. These peptides were screened using dot blot and/or a peptide-based direct ELISA to determine if the peptide was recognized by the MAb or by human immune serum samples. Identification of peptides that were specific for one flavivirus (WNV, SLEV, DENV or other) would not only provide synthetic peptides for the test but would also permit production of monoclonal antibodies against these peptides, thereby providing the best reagents for flavivirus type specific b-ELISAs.

## 3.3. MATERIALS AND METHODS.

### 3.3.1. Synthetic peptides.

Peptides used in this study were 15 to 19 mers, with 10 or 11 amino acid overlaps. The 46-peptide array spanned the NS1 gene, and the 67-peptide array spanned the E gene of the WNV strain NY99-flamingo382-99 (GenBank accession number AF196835). The reagents were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, Manassas, VA: Peptide Array West Nile Virus Gene E, NR- 435 and Peptide Array West Nile Virus Gene NS1, NR-436. All peptides were provided lyophilized at 1 mg per vial. Information about the length and sequence of the individual peptides of the NS1 and E proteins are listed in Tables 3.3.1 and 3.3.2, respectively.

### 3.3.2. Viral antigen preparation.

WNV, DENV and SLE antigens were produced by infecting *Aedes albopictus* C6/36 cells with WNV (NY-99-flamingo382-99), DEN-1 (Hawaii), DEN-2 (Jamaica 1409), DEN-3 (H-87), DEN-4 (H-247), or SLE (TBH-28) viruses, respectively. The

protocol used to prepare viral antigens for ELISAs is described in Appendix 6.1. Briefly, 5-10 days post-infection, depending on the virus, cells were harvested and centrifuged for 10 minutes at 4,000 rpm. The resulting pellets were re-suspended in pH 9.0 borate saline buffer (1.5M NaCl, 0.5M $H_3BO_2$, 1.0M NaOH), and 0.1% SDS and Triton X-100. The antigen was sonicated and centrifuged to eliminate pellets, and the resultant supernatants were stored at -70C for later use (17).

### 3.3.3. MAb and human serum samples used to characterize the tests.

The reagent 3.1112G (Chemicon International, Inc., Temecula, CA) is a monoclonal antibody specific for the NS1 glycoprotein of WNV/Kunjin virus (84). The MAb 3.1112G was prepared by immunizing BALB/c mice with Kunjin virus (strain MRM 16). Spleen cells from immunized mice were fused with myeloma cells, followed by cloning and characterization of the secreted MAb (83, 85, 86). The MAb 6B6C-1 was provided by Dr. John T. Roehrig (Division of Vector-Borne Infectious Disease, Centers for Disease Control and Prevention, Fort Collins, Colorado, USA). This MAb, produced using SLEV, is a flavivirus group-reactive antibody that has been well characterized (34, 175). The human sera from WNV infections were provided by Dr. James L. Beebe, (Laboratory Services Division, Colorado Department of Public Health and Environment, Denver, Colorado, USA). The human sera from dengue infections were provided by Dr. Jose Farfán, (Centro de Investigaciones Regionales, Universidad Autónoma de Yucatán, Mérida, Yucatán, México). The horse sera that were positive for WNV and negative for other flavivirus infections were provided by Dr. Richard A. Bowen, (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colorado, USA).

**Table 3.3.1. Amino acid sequences of the 46 peptides in the array of the West Nile NS1-protein.**

| Peptide number | Length | Amino acid sequence of the peptide | Location* | | Peptide | Length | Sequence | Location |
|---|---|---|---|---|---|---|---|---|
| 1 | 19 | DTGCAIDISRQELRCGSGV | 1-19 | | 24 | 18 | NTTECDSKIIGTAVKNNL | 175-192 |
| 2 | 18 | RQELRCGSGVFIHNDVEA | 14-27 | | 25 | 18 | IIGTAVKNNLAIHSDLSY | 183-200 |
| 3 | 18 | GVFIHNDVEAWMDRYKYY | 18-35 | | 26 | 16 | NLAIHSDLSYWIESRL | 191-206 |
| 4 | 18 | EAWMDRYKYYPETPQGLA | 26-43 | | 27 | 18 | DLSYWIESRLNDTWKLER | 197-214 |
| 5 | 18 | YYPETPQGLAKIIQKAHK | 35-51 | | 28 | 17 | RLNDTWKLERAVLGEVK | 205-220 |
| 6 | 17 | LAKIIQKAHKEGVCGLR | 42-58 | | 29 | 18 | LERAVLGEVKSCTWPETH | 212-229 |
| 7 | 17 | AHKEGVCGLRSVSRLEH | 49-65 | | 30 | 18 | KSCTWPETHTLWGDGIL | 221-237 |
| 8 | 17 | GLRSVSRLEHQMWEAVK | 56-72 | | 31 | 18 | THTLWGDGILESDLIIPV | 228-245 |
| 9 | 18 | LEHQMWEAVKDELNTLLK | 63-80 | | 32 | 16 | ILESDLIIPVTLAGPR | 236-251 |
| 10 | 18 | VKDELNTLLKENGVDLSV | 71-88 | | 33 | 16 | IIPVTLAGPRSNHNRR | 242-257 |
| 11 | 18 | LKENGVDLSVVVEKQEGM | 79-96 | | 34 | 18 | AGPRSNHNRRPGYKTQNQ | 248-265 |
| 12 | 18 | SVVVEKQEGMYKSAPKRL | 87-104 | | 35 | 18 | RRPGYKTQNQGPWDEGRV | 256-273 |
| 13 | 17 | GMYKSAPKRLTATTEKL | 95-111 | | 36 | 16 | NQGPWDEGRVEIDFDY | 265-279 |
| 14 | 17 | KRLTATTEKLEIGWKAW | 102-118 | | 37 | 18 | EGRVEIDFDYCPGTTVTL | 270-287 |
| 15 | 17 | EKLEIGWKAWGKSILFA | 109-125 | | 38 | 17 | DYCPGTTVTLSESCGHR | 279-294 |
| 16 | 18 | KAWGKSILFAPELANNTF | 116-132 | | 39 | 15 | VTLSESCGHRGPATR | 285-299 |
| 17 | 18 | FAPELANNTFVVDGPETK | 124-141 | | 40 | 18 | SCGHRGPATRTTTESGKL | 290-307 |
| 18 | 18 | TFVVDGPETKECPTQNRA | 132-149 | | 41 | 17 | TRTTTESGKLITDWCCR | 298-314 |
| 19 | 16 | TKECPTQNRAWNSLEV | 140-155 | | 42 | 18 | GKLITDWCCRSCTLPPLR | 305-322 |
| 20 | 18 | TQNRAWNSLEVEDFGFGL | 145-162 | | 43 | 18 | CRSCTLPPLRYQTDSGCW | 313-330 |
| 21 | 18 | LEVEDFGFGLTSTRMFLK | 153-170 | | 44 | 16 | LRYQTDSGCWYGMEIR | 321-336 |
| 22 | 18 | GLTSTRMFLKVRESNTTE | 161-178 | | 45 | 17 | SGCWYGMEIRPQRHDEK | 327-343 |
| 23 | 16 | LKVRESNTTECDSKII | 169-184 | | 46 | 19 | EIRPQRHDEKTLVQSQVNA | 334-352 |

* Numbering from the beginning of the NS1 protein in WNV.

**Table 3.3.2. Amino acid sequences of the 67 peptides in the array of the West Nile envelope protein.**

| Peptide | Length | Aminoacid sequence | Location * | Region (aa) | Peptide | Length | Aminoacid sequence | Location | Region (aa) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 18 | FNCLGMSNRDFLEGVSGA | 1-18 | Domain I (1-51) | 35 | 17 | ALGSQEGALHQALAGAI | 254-270 | Domain II |
| 2 | 17 | RDFLEGVSGATWVDLVL | 9-25 | | 36 | 19 | ALHQALAGAIPVEFSSNTV | 261-279 | |
| 3 | 18 | SGATWVDLVLEGDSCVTI | 16-33 | | 37 | 18 | IPVEFSSNTVKLTSGHLK | 270-287 | |
| 4 | 18 | VLEGDSCVTIMSKDKPTI | 24-41 | | 38 | 18 | TVKLTSGHLKCRVKMEKL | 278-295 | Domain I (284-298) |
| 5 | 17 | TIMSKDKPTIDVKMMNM | 32-48 | | 39 | 17 | LKCRVKMEKLQLKGTTY | 286-302 | |
| 6 | 18 | PTIDVKMMNMEAANLAEV | 39-56 | Domain II (52-132) | 40 | 18 | EKLQLKGTTYGVCSKAFK | 293-310 | Domain III (299-501) |
| 7 | 17 | NMEAANLAEVRSYCYLA | 47-63 | | 41 | 16 | TYGVCSKAFKFLGTPA | 301-316 | |
| 8 | 18 | AEVRSYCYLATVSDLSTK | 54-71 | | 42 | 18 | KAFKFLGTPADTGHGTVV | 306-324 | |
| 9 | 16 | LATVSDLSTKAACPTM | 62-77 | | 43 | 15 | PADTGHGTVVLELQY | 315-329 | |
| 10 | 18 | LSTKAACPTMGEAHNDKR | 68-85 | | 44 | 18 | HGTVVLELQYTGTDGPCK | 320-337 | |
| 11 | 18 | TMGEAHNDKRADPAFVCR | 76-93 | | 45 | 18 | LQYTGTDGPCKVPISSVA | 327-345 | |
| 12 | 18 | KRADPAFVCRQGVVDRGW | 84-101 | | 46 | 18 | PCKVPISSVASLNDLTPV | 335-352 | |
| 13 | 17 | CRQGVVDRGWGNGCGLF | 92-108 | Fusion peptide (98-111) | 47 | 16 | VASLNDLTPVGRLVTV | 343-358 | |
| 14 | 15 | RGWGNGCGLFGKGSI | 99-113 | | 48 | 17 | LTPVGRLVTVNPFVSVA | 349-365 | |
| 15 | 17 | GCGLFGKGSIDTCAKFA | 104-120 | | 49 | 18 | VTVNPFVSVATANAKVLI | 356-373 | |
| 16 | 18 | GSIDTCAKFACSTKAIGR | 111-128 | Domain II | 50 | 16 | VATANAKVLIELEPPF | 364-379 | |
| 17 | 18 | FACSTKAIGRTILKENIK | 119-136 | | 51 | 17 | KVLIELEPPFGDSYIVV | 370-386 | |
| 18 | 18 | GRTILKENIKYEVAIFVH | 127-144 | Domain I (133-193) | 52 | 17 | FPFGDSYIVVGRGEQQI | 377-393 | |
| 19 | 18 | IKYEVAIFVHGPTTVESH | 135-152 | | 53 | 16 | IVVGRGEQQINHHWHK | 384-399 | |
| 20 | 17 | VHGPTTVESHGNYSTQV | 143-159 | | 54 | 18 | EQQINHHWHKSGSSIGKA | 390-407 | |
| 21 | 18 | ESHGNYSTQVGATQAGRF | 150-167 | | 55 | 18 | HKSGSSIGKAFTTTLKGA | 398-415 | Pre-anchor of sub-domain III |
| 22 | 17 | TQVGATQAGRFSITPAA | 157-173 | | 56 | 16 | KAFTTTLKGAQRLAAL | 406-420 | |
| 23 | 17 | AGRFSITPAAPSYTLKL | 164-180 | | 57 | 17 | LKGAQRLAALGDTAWDF | 412-428 | |
| 24 | 18 | PAAPSYTLKLGEYGEVTV | 171-188 | | 58 | 17 | AALGDTAWDFGSVGGVF | 418-435 | |
| 25 | 18 | KLGEYGEVTVDCEPRSGI | 179-196 | Domain II (194- | 59 | 18 | WDFGSVGGVFTSVGKAVH | 426-443 | |
| 26 | 18 | TVDCEPRSGIDTNAYYVM | 187-203 | | 60 | 18 | VFTSVGKAVHQVFGGAFR | 434-449 | |
| 27 | 18 | GIDTNAYYVMTVGTKTFL | 195-212 | | 61 | 18 | VHQVFGGAFRSLFGGMSW | 442-459 | Trans-membrane of sub-domain III |
| 28 | 17 | VMTVGTKTFLVHREWFM | 203-219 | | 62 | 18 | FRSLFGGMSWITQGLLGA | 450-467 | |
| 29 | 16 | TFLVHREWFMDLNLPW | 210-224 | | 63 | 17 | SWITQGLLGALLLWMGI | 458-474 | |
| 30 | 18 | EWFMDLNLPWSSAGSTVW | 216-233 | | 64 | 18 | LGALLLWMGINARDRSIA | 465-483 | |
| 31 | 17 | PWSSAGSTVWRNRETLM | 225-240 | | 65 | 16 | GINARDRSIALTFLAV | 473-488 | |
| 32 | 17 | TVWRNRETLMEFEEPHA | 231-247 | | 66 | 17 | RSIALTFLAVGGVLLFL | 479-495 | |
| 33 | 18 | TLMEFEEPHATKQSVIAL | 238-255 | | 67 | 16 | LAVGGVLLFLSVNVHA | 486-501 | |
| 34 | 18 | HATKQSVIALGSQEGALH | 246-263 | | - | - | - | - | |

* Numbering from the beginning of the envelope glycoprotein in WNV.

The synthetic peptide based diagnostic methods were characterized using individual antibody specimens as well as pooled specimens. For the individual antibody specimens, a panel of 14 samples was used to evaluate the immunoreactivity of the peptides in Dot Blot analyses. The panel of 14 samples was comprised of 2 monoclonal antibodies (3.112G MAb and 6B6C-1), 10 human serum specimens, and 2 horse serum samples. These samples were also characterized for immunological responses in WNV and flavivirus b-ELISA tests, and for neutralizing antibodies in DENV, SLEV, and WNV $PRNT_{90\%}$ tests (Table 3.3.3). The WNV epitope b-ELISA gave negative results for 10 of 11 samples (91%) that were expected to be negative for WNV antibody results. The WNV epitope b-ELISA gave positive results for 2 of 3 samples (67%) expected to be positive for WNV antibodies. The flavivirus epitope b-ELISA gave negative results for 6 of 6 samples (100%) expected to be negative for flavivirus antibodies. The flavivirus epitope b-ELISA gave positive results for 7 of 8 samples (91%) expected to be positive for flavivirus antibodies (Table 3.3.3).

The 3.1112G MAb gave negative $PRNT_{90\%}$ test results for DENV, SLEV, and WNV, and the 6B6C-1 MAb gave positive $PRNT_{90\%}$ test results for DENV, SLEV, and WNV as expected. All of the other $PRNT_{90\%}$ results correlated perfectly with all expected positive samples giving positive results, and all expected negative samples giving negative results. There were three exceptions; primary WNV infection samples for 1 horse and 1 human yielded 1:10 SLEV $PRNT_{90\%}$ titers, and 1 human WNV primary infection that yielded a 1:1:1280 SLEV $PRNT_{90\%}$ titer. These last results indicate some cross-reactivity between WNV and SLEV $PRNT_{90\%}$ tests, but the numbers of samples (n) are so small that they are not statistically significant.

**Table 3.3.3. Panel of individual specimens used to determine the diagnostic efficacy of the Dot Blot and synthetic peptide tests.** The mouse monoclonal antibodies, and human and horse serum samples were characterized by b-ELISA and PRNT.

| MAb or Serum ID | Characteristics Of the sample | Origin of the antibody | Epitope b-ELISA[a] | | PRNT 90% | | |
|---|---|---|---|---|---|---|---|
| | | | WNV | Flavi | DENV | SLEV | WNV |
| **3.1112G** | MAb specific for NS1 | Mouse | ND | ND | <1:10 | <1:10 | <1:10 |
| **6B6C-1** | MAb specific for E | Mouse | ND | ND | 1:400 | ≥1:800 | 1:100 |
| **538** | WNV-primary infection | Human | 76 | 66 | <1:10 | 1:10 | ≥1:320 |
| **34** | WNV-primary infection | Human | 17 | 74 | ND | 1:1,280 | 1:160 |
| **15738** | DENV-primary infection | Human | 4 | 42 | 1:320 | <1:10 | <1:10 |
| **SJL-1[C]** | DENV-previous infection | Human | 6 | 75 | 1:160 | <1:10 | <1:10 |
| **SJL-2** | DENV-previous infection | Human | 2 | 52 | 1:10 | <1:10 | <1:10 |
| **SJL-6** | DENV-previous infection | Human | 12 | 14 | 1:10 | <1:10 | <1:10 |
| **SJL-8** | DENV-previous infection | Human | 54 | 76 | 1:160 | <1:10 | <1:10 |
| **525** | Negative to flavivirus | Human | -6 | -9 | <1:10 | <1:10 | <1:10 |
| **527** | Negative to flavivirus | Human | 15 | 1 | <1:10 | <1:10 | <1:10 |
| **529** | Negative to flavivirus | Human | 24 | 5 | <1:10 | <1:10 | <1:10 |
| **WNV (+) Ctrl.** | WNV-primary infection | Horse | 60 | 82 | ND | 1:10 | ≥1:320 |
| **Flavi (-) Ctrl.** | Negative to flavivirus | Horse | 1 | 1 | ND | <1:10 | <1:10 |

[a] An epitope blocking ELISA value ≥ 30% indicates the presence of viral antibodies. This was the cut-off value used. The antigen used was WNV-cell lysate.

A second panel consisting of 185 human serum samples was used to further evaluate the utility of immunoblots, direct ELISA with Universal bind ELISA plates, and synthetic peptide-based ELISAs. These 185 samples were segregated into 6 pooled samples and 185 individual serum samples (Table 3.3.4). The samples were further grouped into 4 groups, based on their expected presence of WNV or DENV antibodies: Group 1: "Recent WNV Infections", Group 2: "Recent DENV Infections, Group 3: "Previous DENV Infections, and Group 4: "Flavivirus Negative Samples". Group 1 ("Recent WNV Infections") consisted of 55 individual samples and 5 pools of WNV infected human sera samples also created from the same 55 individuals (pooled as 20, 8, 9, 9, & 9 groups of individual samples) that had been infected with WNV and were highly positive in prior test results. Group 2 ("Recent DENV Infections") consisted of 20 individual convalescent serum samples from patients with recent DENV infections. Group 3 ("Previous DENV Infections") consisted of 9 individual samples from patients that had past DENV infections. Group 4 ("Flavivirus Negative Samples") consisted of 24 individual flavivirus-negative human sera samples from Colorado and one pool of 77 flavivirus-negative human sera samples, also collected from Colorado in 2003. The first three groups were used to evaluate the binding potential of synthetic peptides by peptide-based ELISA. The Group 4 sera from the flavivirus-antibody negative patients were selected to determine the background binding potential of human serum samples in peptide-based ELISAs. The tests that were characterized using these 4 groups are described in the following sections: 3.3.4 Immunoblots, 3.3.5 Direct ELISA with Universal bind ELISA plates, and 3.3.6 Peptide-based ELISAs. The results for the tests are provided in section 3.4.

**Table 3.3.4. Panel of 185 human serum samples confirmed by laboratory tests as WNV-infection, DENV-infection or without neutralizing antibodies to flavivirus infections.** The serum samples were prepared at AIDL and used for the evaluation of Imunoblots, Direct ELISAs, and synthetic peptide based ELISAs.

| Group | Number of pools prepared | Total number of serum samples in the pools | Total number of individual serum samples |
|---|---|---|---|
| **1: Recent WNV Infections** | 5 | 55 | 55 |
| **2: Recent DENV Infections** | 0 | 0 | 20 |
| **3: Previous DENV Infection** | 0 | 0 | 9 |
| **4: Flavivirus Negative Samples** | 1 | 77 | 24 |
| **Total** | 6 Pools | 185 Individual | |

### 3.3.4. Immunoblots.

Immunoblot analyses are used to detect and semi-quantitatively measure antibodies to proteins or peptides spotted on a membrane. A dot blot approach was used to screen the peptides for immunoreactivity. This is a simple technique that uses specific primary antibodies that bind to peptides or proteins attached to a membrane; this is followed by treatment with secondary antibodies conjugated with horse-radish peroxidase, which bind to the primary antibodies and act as substrates for the development of color (97).

Immunoblot analyses were performed after drying spots of 5 μL (of 1 μg/mL) of each peptide onto nitrocellulose membranes (Sigma, St. Louis, MO). Each membrane was incubated with blocking buffer (pH 7.4 saline phosphate buffer, 0.05% Tween 20 and 5% skim milk) for 1 hour at room temperature (RT). After washing the membrane 3 times, it was incubated overnight at 4°C on a rocking table with monoclonal antibodies or serum samples (diluted 1:200 in blocking buffer). This was followed by washing 3 times, then incubated for 1 hour at RT with antimouse IgG, anti-human IgG-IgM-IgA, or anti-horse IgG, all labeled with horse-radish peroxidase [HRP] and diluted in blocking buffer.

Excess unbound antibodies were eliminated by washing the membrane 3 times with washing buffer. Membranes were then incubated with 3'3'DAB, (Sigma, St. Louis, MO), and hydrogen peroxide to reveal the peptides that reacted with the antibodies. Finally, the reactions were stopped with the addition of water.

### 3.3.5 Direct ELISA with Universal bind ELISA plates.

ELISAs are commonly used to detect antibodies that bind viral components using antigens immobilized on polystyrene ELISA plates (173). This can alter the conformation of the proteins and therefore expose surfaces and epitopes that are not normally recognized on intact virions. These proteins may be denatured due to exposure to high pH during binding to the plastic surface and as a result, the epitopes are exposed and accessible to reaction with selected antibodies (88). The immobilization may also eliminate conformational/non-linear epitopes. Synthetic

peptides are linear epitopes, making direct ELISA an appropriate test for determining the potential usefulness of the respective peptides for diagnosis.

Direct ELISAs were used to assay the immunoreactivity of E and NS1 synthetic peptides immobilized on plates. All peptides were coupled covalently to 96-well microtitre Universal-bind ELISA plates (Corning Inc., Acton, MA), using the vendor's recommended procedure. Briefly, 1 μg of the peptide in 100 μL of bicarbonate-carbonate buffer, pH 9.6 was added to each well and incubated for 1 hour at room temperature (33). Sample solution was decanted, and the plate was exposed to UV light for 30 seconds. Wells were then blocked with 100 μL of blocking buffer (PBS containing 0.1% Tween 20 and 2% bovine serum albumin) and incubated for 1 hour at room temperature. The buffer was decanted and 100 μL of one of the human serum samples, diluted 1:5 in blocking buffer, was added and incubated for 1 hour at room temperature. The wells were washed 5 times with washing buffer (PBS containing 0.1% Tween 20). One hundred micro-liters of peroxidase labeled IgG-anti human IgM, IgG and IgA (KPL, Gaithersburg, MD) were added, and the test wells were then incubated for 1 hour at room temperature. The wells were washed 5 more times with washing buffer. Finally, the conjugates were detected using ABTS (2,2' azino-bis [3-ethyl-benzothiazoline-6-sulfonic acid]). Absorbances were measured at 415 nm using an ELISA reader (BIO-RAD Laboratories Inc., Hercules, CA). The cut-off values for the ELISAs were determined and selected using Receiver Operating Characteristics (ROC) analyses and were set at the differing optical densities (65, 72).

### 3.3.6. Peptide-based ELISAs with 96-well plates from different companies.

Peptides that were recognized by immunoblot analysis (NS1-1, NS1-2, NS1-26, NS1-27 and E-44) were dissolved at 1 μg in 100 μL carbonate/bicarbonate buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6) and used to coat high-binding ELISA plates from three different companies: Immulon 2HB plates from Dynatech Industries, Maxisorp plates from NUNC, and Costar 9018 plates from Corning. All were used following the same procedure. After coating with peptides overnight at 4°C, the plates were washed with phosphate buffered saline (containing 0.1% Tween 20), and blocking was performed using PBS buffer with 5 % skim milk for 40 minutes at 37°C. Monoclonal antibodies or serum samples were diluted in blocking buffer and incubated for 2 hours at 37°C. Horse-radish peroxidase-labeled goat anti-mouse IgG (Zymed Laboratories, South San Francisco, CA) or anti-human IgG, IgA, or IgM (KPL, Gaithersburg, MD, USA) were incubated for 1 hour at 37°C. Two different substrates were then evaluated. When using TMB (3,3',5,5'-tetramethylbenzidine, GIBCO, Gaithersburg, Maryland), the reactions were stopped after 10 minutes with 1 N sulfuric acid. Absorbances were then determined at 450 nm. When ABTS was used, readings were conducted after 30 minutes of incubation at 37°C, and absorbances were determined at 415 nm. Cut-off values were calculated using Receiver Operating Characteristics (ROC) analyses.

## 3.4. RESULTS.

Immunoblot (Dot Blot) analysis followed by peptide-based ELISAs were used to identify the NS1 and E synthetic peptides of WNV recognized by the two monoclonal antibodies and serum samples from humans and horses.

### 3.4.1. Immunoblot analysis of synthetic peptides and WNV infected cells.

A total of 46 peptides from NS1 and 67 peptides from E proteins were studied. Dot Blots were made by spotting 5 μg of each synthetic peptide (nine peptides per row) onto nitrocellulose membrane sheets. Tests were then conducted to determine which peptides were recognized by the MAbs and representative individual serum samples from 3 previous WNV or DENV infections (Groups 1,2, and 3), plus representative Group 4 serum samples which lacked flaviviruses antibodies.

The MAb 3.1112G, specific for NS1 protein of WNV, bound to the Triton X-100 WNV-lysate and also bound to peptide 27 of NS1 (aa 197-214) (Fig. 3.4.1). The peptide 27 was also recognized by both horse samples and all individual human serum samples representing Groups 1, 2, 3 , and 4. Representative results with samples from confirmed WNV infections, confirmed primary or secondary DENV infections, and flavivirus negative samples are presented in Fig. 3.4.1. The dot blot of WNV-lysate of infected cells was recognized by the individual human serum samples with WNV infections (Group 1) but not by the serum samples from DENV infections (Groups 2 and 3). Similarly, the DENV-lysate of infected cells was recognized by the human serum samples with DENV infections but not by the individual representative serum samples from WNV infections (Group 1). The peptide 1 of the WNV NS1 protein was recognized by eight serum specimens from patients with recent DENV infections (Group 2). These Group 2 samples also reacted to Triton X-100 DENV lysate, but not with the Triton X-100 WNV lysate. The dot blot results for 10 representative serum samples are shown in Fig. 3.4.1, and the other results are listed in Table 3.4.1. Immunoblot analyses were then used to determine the ability of serum samples from

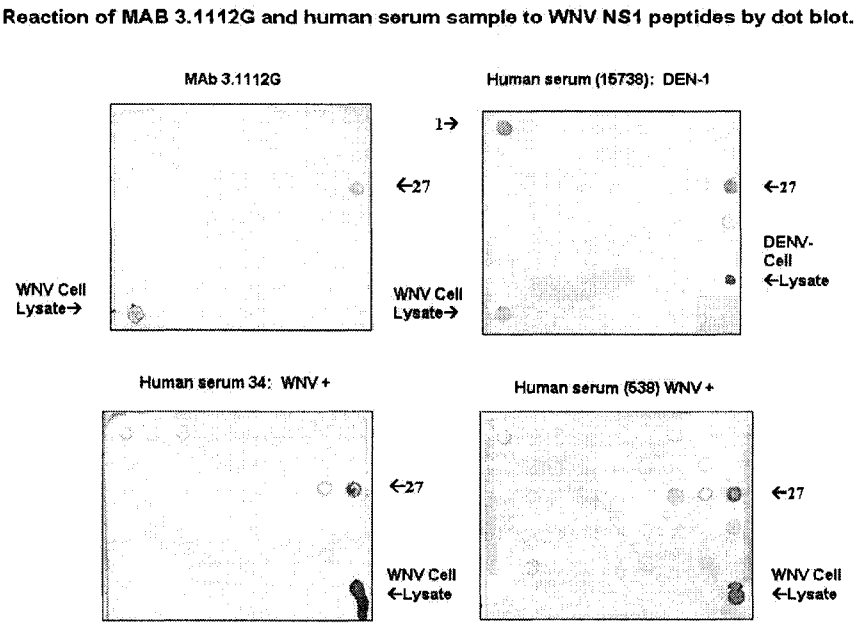



**Figure 3.4.1. Representative reactions of MAb 3.1112G and three individual human serum samples to WNV NS1 peptides.**

individuals with past DENV infections (Group 3) to detect the 46 peptides of NS1 protein. The four serum samples reacted strongly with NS1 peptides 2, 26, & 27 and with the lysates of cells infected with DENV (Figure 3.4.2). Sample SJL-1 also reacted with the lysate of cells infected with WNV.

The serum samples were from WNV and DENV infected humans. The 46 peptides from NS1 protein and lysates of cells infected with WNV or DEN viruses were spotted on the nitrocellulose membrane in rows of 9 peptides. The position of the spots with peptides and WNV or DENV cell lysates are marked with arrows designating the specific immune reactions.

**Reaction of human serum samples to WNV NS1 peptides and lysated of cells infected and non-infected with viruses by immunoblot analysis.**

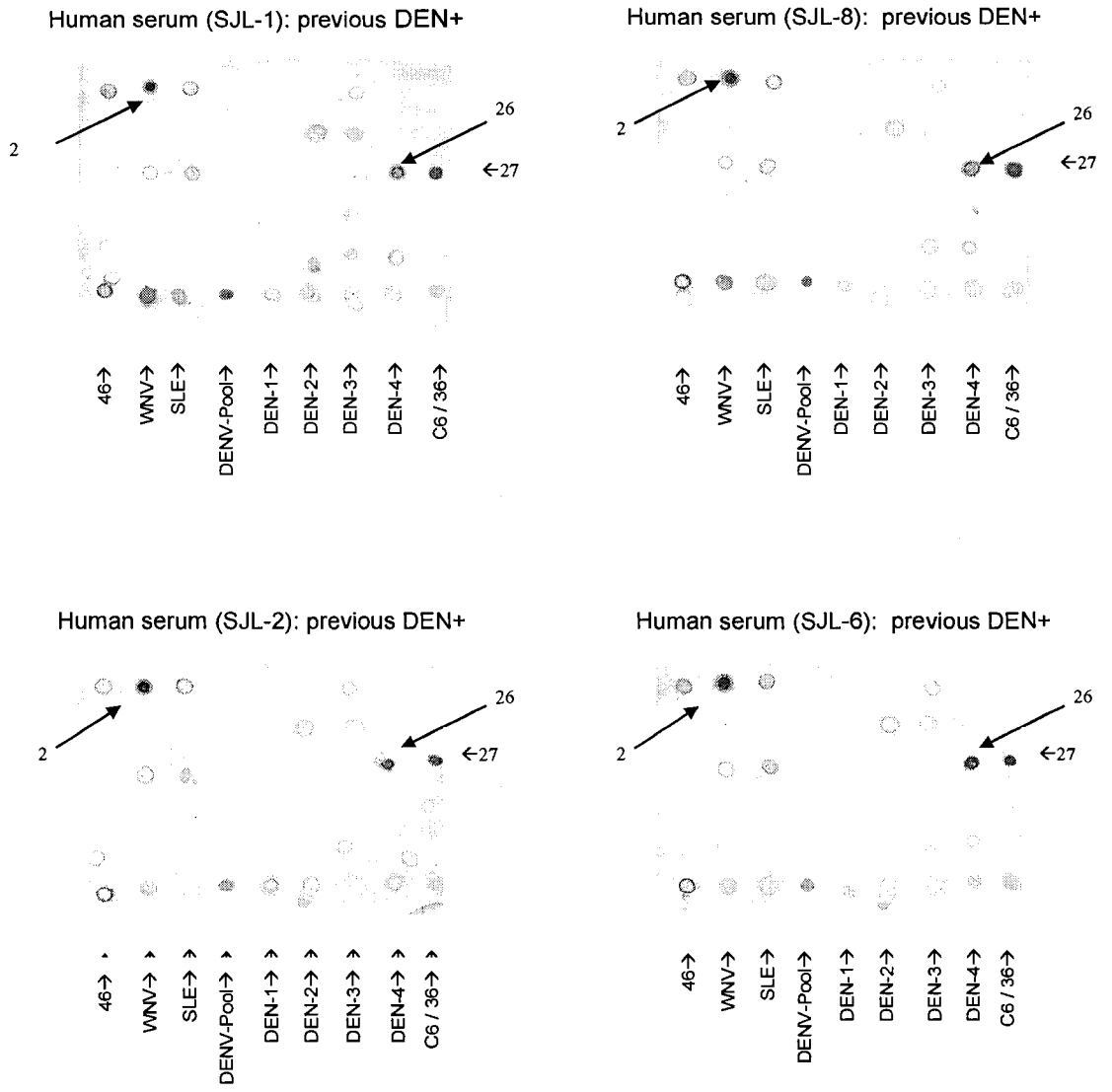



**Figure 3.4.2. Immnunoblot assays showing reactivity to 46 peptides of the NS1 protein using individual serum samples from persons with previous dengue infections.** The last lane contains spots of the peptide 46 and lysates of cells infected with WNV, SLEV, DENV-pool with all 4 DENV serotypes, DEN-1, DEN-2, DEN-3, DEN-4 or non-infected (C6/36) cells.

An immunoblot study was then conducted to determine the WNV envelope peptides that would be recognized by MAb 6B6C-1. Two human sera for WNV infections and one from a previous dengue infection were included in the test (Figure 3.4.3.). MAb 6B6C-1 did not react with any of the peptides of the E protein of WNV in the immunoblot analysis; it reacted only with WNV and SLEV lysates of infected cells (Figure 3.4.3). However, the E-44 synthetic peptide was recognized by all human serum samples tested: Group 1 (confirmed WNV infections), Groups 2 and 3 (DENV primary or previous infections), and Group 4 (samples without antibodies to flaviviruses).

Surprisingly, all human sera (including sera from Groups 1, 2, 3, & 4) and horse sera tested in this study contained antibodies that bound to the NS1-27 peptide and to the E-44 peptide. WNV patient samples did not bind to peptides 1 and 2 of the NS1 protein, whereas DEN patient samples had cross-reactive antibodies against one of the 2 peptides of the WNV-NS1. Convalescent serum samples from patients with recent dengue infections (Group 2 representatives) strongly reacted with peptide 1 of the WNV-NS1 and to Triton X-100 DENV lysate, but not to triton X-100 WNV lysate (Fig. 3.4.1).

Immunoblot results for the NS1 and E peptides and WNV-infected cell lysates that were recognized by the MAb and serum samples are summarized in Table 3.4.1. This table contains: the ID names of the samples tested, the characteristics of classification of the samples, and the numbers of the peptides that showed immunological reactions with the samples tested.

**Reaction of MAb 6B6C-1 and human serum samples to WNV E peptide by immunoblot analysis.**

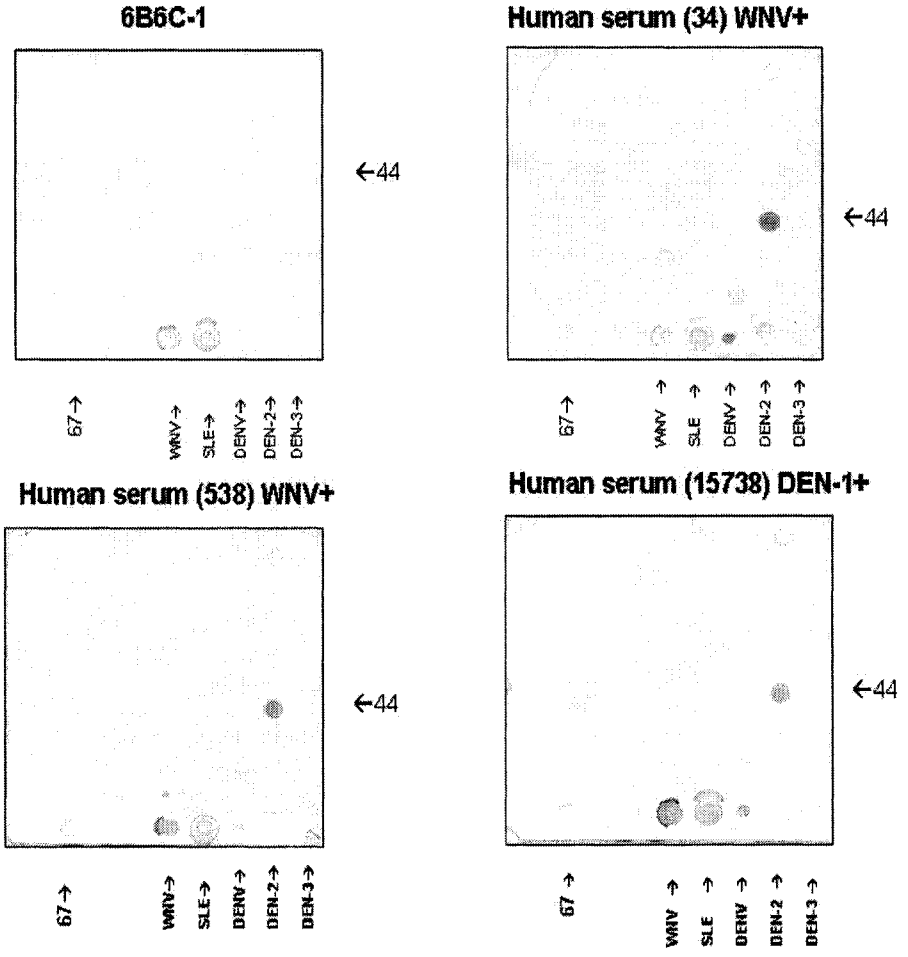



**Figure 3.4.3. Representative immunoblot reactions of MAb 6B6C-1 and three individual serum samples to the WNV envelope glycoprotein peptides and lysates of infected cells.** Dot Blots of the 67 peptides from the envelope protein of WNV and lysates of cells infected with WNV, SLE or DEN viruses were tested with the MAb 6B6C-1, and three human serum samples from patients with antibodies to DENV or WNV. The position of the peptide number and the lysates of infected cells are marked with arrows.

**Table 3.4.1. Peptides and WNV infected cell lysates that were recognized by the MAbs or individual serum samples by Immunoblot analyses.**

| MAb or serum sample | Characteristics of the sample | Immunoblot analysis[a] | | |
|---|---|---|---|---|
| | | Peptide No. of NS1 | Peptide No. of E | Infected cell lysate |
| **3.1112G** | MAb specific for NS1 | 27 | ND | WNV |
| **6B6C-1** | MAb specific for E | None | None | SLE, WNV, DENV |
| **538** | WNV-primary infection | 27 | 44 | WNV |
| **34** | WNV-secondary infection | 27 | 44 | SLE, WNV, DENV |
| **15738** | DENV-primary infection | 1, 27 | 44 | DENV |
| **SJL-1**[b] | DENV-previous infection | 2, 26, 27 | 44 | DENV |
| **SJL-2** | DENV-previous infection | 2, 26, 27 | 44 | DENV |
| **SJL-6** | DENV-previous infection | 2, 26, 27 | 44 | DENV |
| **SJL-8** | DENV-previous infection | 2, 26, 27 | 44 | DENV |
| **525** | Negative to flavivirus | 27 | 44 | None |
| **527** | Negative to flavivirus | 27 | 44 | None |
| **529** | Negative to flavivirus | 27 | 44 | None |
| **WNV (+) Ctrl.** | WNV-primary infection | 27 | 44 | WNV |
| **Flavi (-) Ctrl.** | Negative to flavivirus | 27 | 44 | None |

[a]All peptides from NS1 and E proteins of WNV were dot blotted onto nitrocellulose membranes and incubated with MAb or serum samples from humans or horses.

[b] San Juan Laboratories.

### 3.4.2 Peptide-based ELISA.

Four types of ELISA plates were tested to evaluate the immunoreactivity of the synthetic peptides. Only the Immulon 2HB from Dynatech Industries) gave results that agreed well with the results obtained with immunoblot analysis.

Based on the results obtained from dot blot analyses, 4 synthetic peptides were chosen to evaluate their utility for the diagnosis of WNV infections by peptide-based ELISA. The peptides chosen were NS1-1, NS1-2, NS1-27 and the E-44. The specimens used for the evaluation of the synthetic peptides were described in Table 3.3.4. Briefly, the serum samples were characterized and classified into 4 groups: Group 1: Recent WNV infections (55 individuals and 5 pools from 55 individual sera), Group 2: Recent DENV Infections (20 individuals), Group 3: Previous DENV Infections (9 individuals); and Group 4: Flavivirus Negative (24 individuals and 1 pool from 77 sera) (Table 3.3.4). The Group 4: Flavivirus Negative samples were chosen based upon previous results in IgM-capture ELISA, PRNT, epitope b-ELISA and peptide-based ELISA testing.

Each sample was then further characterized by the following tests: IgM-capture ELISA, PRNT for IgG, percentage of blocking by epitope blocking ELISA, and O.D. values by Peptide-based ELISA. The serological results are presented in Table 3.4.2.

Receiver Operating Characteristics (ROC) analysis was used to characterize the performance of the peptide-based ELISA for the detection of antibodies to WNV or DENV (Table 3.4.3.). For each group the optimal cut-off value, the area under the curve (AUC), the sensitivity, the specificity, and the significance level were determined. The positive controls were convalescent serum samples from patients confirmed as

**Table 3.4.2. Serologic results using individual and pooled human serum specimens with WNV or DENV infections. Group 1: (WNV positive pool #'s:1, 3, 4, 5, 6), Group 2: (5 recent DENV infected individuals), Group 3: (4 previously infected DENV individuals), and Group 4: (flavivirus negative samples).**

| Serum ID | No. samples | Diagnosis | Antibody response [a] | | Epitope b-ELISA [c] | | Peptide-based ELISA [f] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | WNV | DENV [b] | WNV [d] % | Flavi [e] % | O.D. NS1-1 | O.D. NS1-2 | O.D. NS1-27 | O.D. E-44 |
| WNV pool 1 | 20 | WNV infection | IgM+, IgG+ | ND | 95 | 83 | 0.179 | 0.223 | 0.104 | 0.320 |
| WNV-pool 3 | 8 | WNV infection | IgM+, IgG+ | ND | 76 | 75 | 0.149 | 0.226 | 0.078 | 0.364 |
| WNV-pool 4 | 9 | WNV infection | IgM+, IgG+ | ND | 78 | 80 | 0.142 | 0.201 | 0.077 | 0.484 |
| WNV-pool 5 | 9 | WNV infection | IgM+, IgG+ | ND | 85 | 88 | 0.207 | 0.265 | 0.104 | 0.305 |
| WNV-pool 6 | 9 | WNV infection | IgM+, IgG+ | ND | 34 | 90 | 0.120 | 0.189 | 0.094 | 0.306 |
| 15449 | 1 | DENV infection | IgG$^-$ | IgM+, IgG+ | 1 | 20 | 0.457 | 0.651 | 0.163 | 0.837 |
| 15454 | 1 | DENV infection | IgG$^-$ | IgM+, IgG+ | 2 | 41 | 0.931 | 0.118 | 0.035 | 0.241 |
| 15768 | 1 | DENV infection | IgG+ | IgM+, IgG+ | 73 | 97 | 0.385 | 0.515 | 0.231 | 0.625 |
| 15877 | 1 | DENV infection | IgG+ | IgM+, IgG+ | 54 | 94 | 0.462 | 0.660 | 0.150 | 1.033 |
| 15899 | 1 | DENV infection | IgG+ | IgM+, IgG+ | 60 | 94 | 0.408 | 0.588 | 0.174 | 1.083 |
| DEN Ctr. IgM+ | 1 | DENV infection | IgG$^-$ | IgM+, IgG+ | 22 | 93 | 0.399 | 0.483 | 0.121 | 0.536 |
| Flavi-negative [g] | 77 | Flavi neg | IgM$^-$, IgG$^-$ | IgG$^-$ | 0 | 0 | 0.216 | 0.222 | 0.080 | 0.443 |
| 525 | 1 | Flavi neg | IgM$^-$, IgG$^-$ | IgG$^-$ | -6 | -9 | 0.049 | 0.036 | 0.051 | 0.166 |
| 534 | 1 | Flavi neg | IgM$^-$, IgG$^-$ | IgG$^-$ | -1 | -13 | 0.056 | 0.054 | 0.052 | 0.230 |
| 535 | 1 | Flavi neg | IgM$^-$, IgG$^-$ | IgG$^-$ | -23 | -11 | 0.021 | 0.023 | 0.100 | 0.080 |
| 542 | 1 | Flavi neg | IgM$^-$, IgG$^-$ | IgG$^-$ | -7 | -12 | 0.080 | 0.040 | 0.093 | 0.159 |
| 544 | 1 | Flavi neg | IgM$^-$, IgG$^-$ | IgG$^-$ | -2 | -13 | 0.045 | 0.055 | 0.037 | 0.222 |
| SJL-2 | 1 | DENV - Previous | IgG$^-$ | IgM$^-$, IgG+ | 13 | 74 | 0.190 | 0.308 | 0.137 | 0.373 |
| SJL-3 | 1 | DENV - Previous | IgG$^-$ | IgM$^-$, IgG+ | 31 | 97 | 0.258 | 0.464 | 0.141 | 0.417 |
| SJL-4 | 1 | DENV - Previous | IgG$^-$ | IgM$^-$, IgG+ | 33 | 72 | 0.198 | 0.304 | 0.192 | 0.289 |
| SJL-7 | 1 | DENV - Previous | IgG$^-$ | IgM$^-$, IgG+ | 40 | 92 | 0.281 | 0.325 | 0.122 | 0.340 |
| DEN Ctr. IgM$^-$ | 1 | DENV - Previous | IgG$^-$ | IgM$^-$, IgG+ | 1 | 98 | 0.420 | 0.541 | 0.189 | 0.686 |

[a] Results based on IgM-capture ELISA (for IgM) and PRNT (for IgG); [b]Dengue virus type 2; [c] Cut-off value ≥ 30 is considered positive; [d] MAb 3.1112G is specific for NS1 for WNV; [e] MAb 6B6C-1 is flavivirus group-reactive; [f] the 96- well ELISA plate used was Immulon 2HB from Dynatech technologies; [g] Pool of 77 flavivirus-negative serum samples.

positive for WNV or DENV infections by IgM-ELISA and PRNT. The negative controls consisted of sera from patients confirmed as being negative for WNV and DENV specific antibodies by IgM-ELISA and PRNT.

The sensitivity and specificity of the peptide-based ELISA for the DENV-confirmed cases were determined by ROC analyses. The optimal cut-off value for the ELISA with the NS1-1 synthetic peptide was found to be 0.242 O.D (Table 3.4.3.). The sensitivity was 60%, (95% CI: 36.1%-80.8%, corresponding to to a 40% false negative rate), and the specificity was 95.8%, (95% CI: 78.8% - 99.3%, corresponding to a 4.2% false positive rate). The optimal cut-off value for the peptide-based ELISA with NS1-2 peptide was found to be 0.276 O.D. The sensitivity was 55%, (95% CI: 31.6%-76.9%), and the specificity was 100%, (95% CI: 85.6% -100%). The results obtained for all four groups of individual serum samples are described in Table 3.4.3. The confidence intervals are unavoidably wide, due to the small sample sizes. More precise estimates could be determined in future studies by simply using larger sample sizes. Specifically, the 100% sensitivity value obtained with the Group 1: Recent WNV Infections, with a wide confidence interval, is an artifact of having a very small data set.

The Optical Density (O.D.) cut-off values for the peptides NS1 and NS2 were twice the average of the O.D.s of the other peptides (Table 3.4.3). The O.D.'s obtained using the peptides NS1-1 and NS1-2 as antigens in a peptide-based ELISA were plotted against each other for 52 serum samples to determine their relative efficacies and to generally determine how the results using NS1-1 compared with the results using NS1-2 (Figure 3.4.4). The results from the two peptides correlated very well. For $n = 52$, the Spearmans Correlation Coefficient (R) was 0.903 (95% CI for R = 0.837 to 0.944).

There was however one obvious outlier, sample 4, which is clearly demonstrated in Figure 3.4.4. When sample 4 (the outlier) was excluded, the correlation values increased significantly (n = 51, R = 0.968 with a CI = 0.945 to 0.982) (Figure 3.4.5). It is not clear at this time why sample 4 deviates so significantly from the trend, but it is reasonable to exclude its results from the data set. The y intercept of this plot was effectively zero (b=0.014 ± 0.0143), and the slope of this plot was significantly smaller than 0.5 (m= 0.313 ± 0.022) using 95% Student T values. The intercept equaling zero further demonstrates correspondence between NS1-1 results and NS1-2 results across a broad range of responses (absorbances varied from 0.02 up to 0.66). When correlating results from different test methods, a slope equal to 0.5 demonstrates a 1:1 numerical ratio across the set. The slope of 0.313 (with a narrow 95% CI) shows that NS1-2 gave larger and very consistently more sensitive responses for every sample.

The results using only Group 2 (Recent Dengue Infection) samples also exhibited a good correlation (17 out of 20 samples: n=17, Spearmans R = 0.9855, 95% CI for R = 0.9593 to 0.9949) (Figure 3.4.6). Similar to the entire data set, the y intercept of this plot was effectively zero (b=0.017 ± 0.018), and the slope of this plot also was significantly smaller than 0.5 (m= 0.311 ± 0.019) using 95% Student T values. The intercept equaling zero demonstrated the excellent correspondence across a broad range of responses (absorbances varying from 0.116 up to 0.66) for samples with recent DENV infections. The slope of 0.311 (with a narrow 95% CI) shows that NS1-2 also gave larger and consistently a more sensitive response for the samples that were also positive for recent DENV infections.

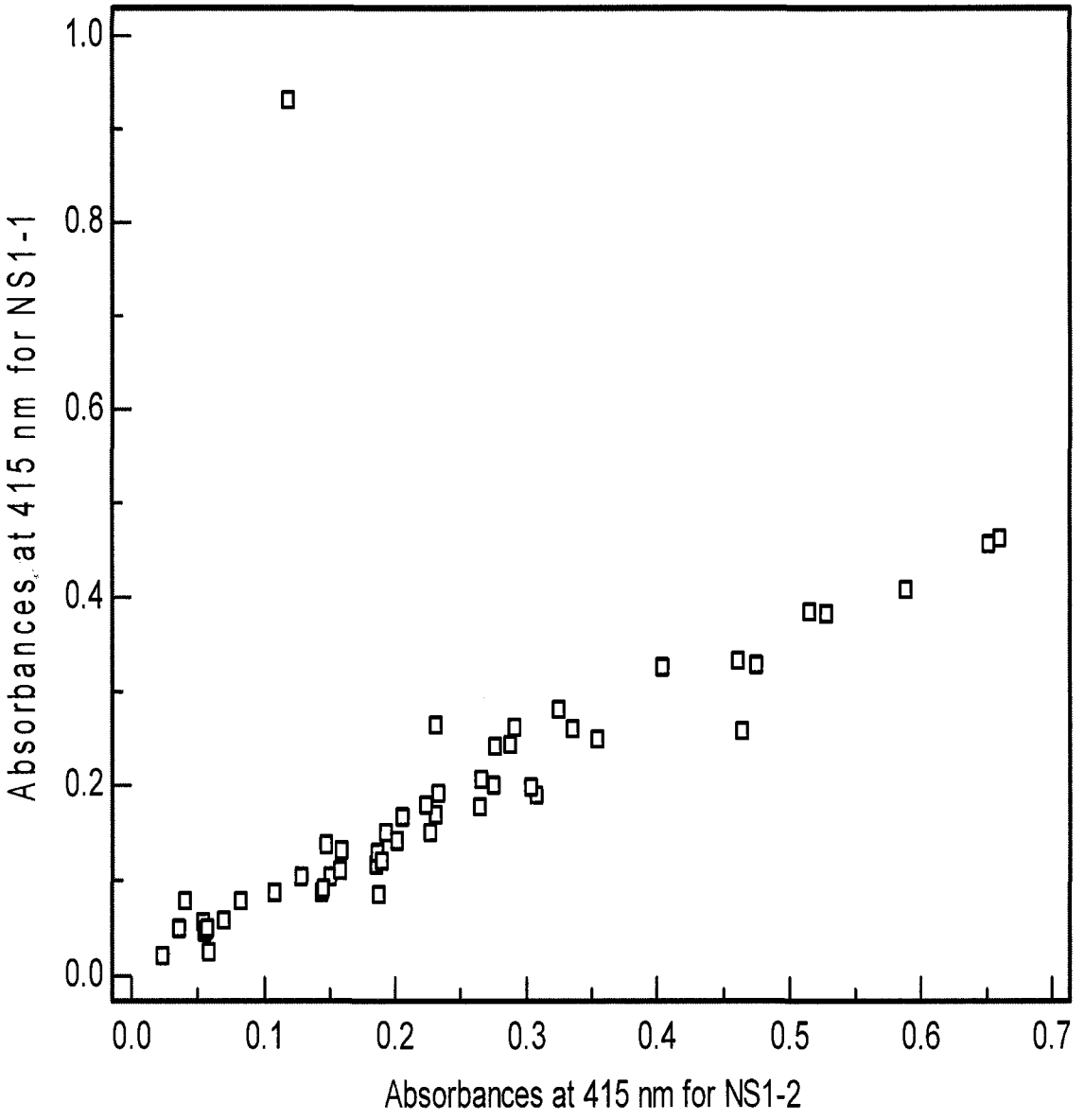



**Figure 3.4.4. Correlation of NS1 vs. NS2 peptide-ELISA tests, including an outlier.** The peptide-ELISA results obtained comparing peptides NS1-1 and NS1-2 for all samples, including those with previous DENV infections and current DENV infections. This data set is shown including an obvious outlier, Sample 4 (point x = 0.118, y = 0.931). With Sample 4 included, Spearmans R = 0.903 with a CI = 0.837 to 0.944.

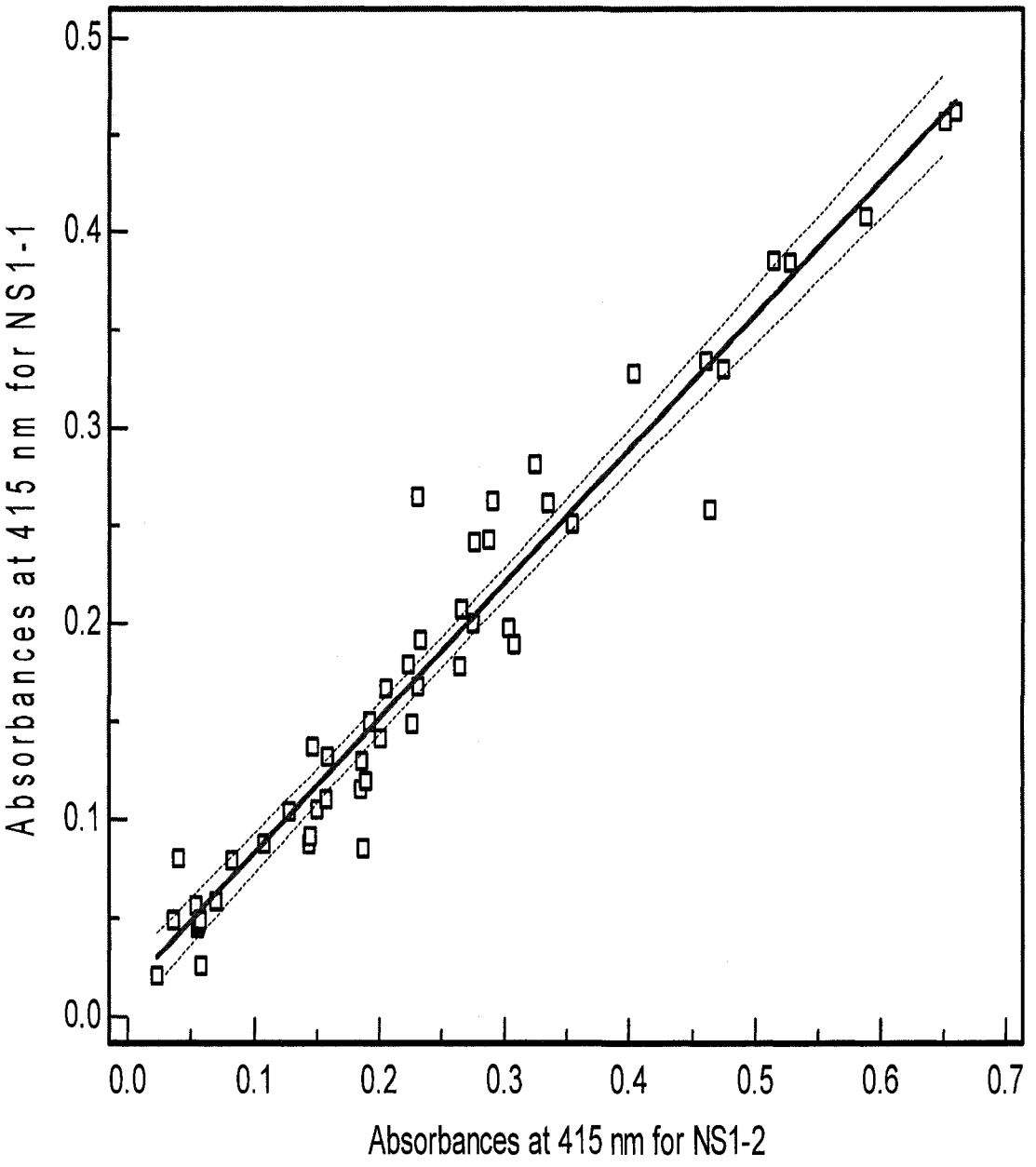



**Figure 3.4.5. Correlation of NS1 vs. NS2 peptide-ELISA tests, excluding an outlier.** The peptide-ELISA results obtained comparing peptides NS1-1 and NS1-2 for all samples, including those with previous DENV infections and current DENV infections. This data set is shown excluding an outlier, Sample 4. For n = 51, Spearmans R = 0.968, 95% CI for R = 0.945 to 0.982. For comparison purposes, when the outlier (Sample 4) was included in the calculation, R = 0.903 with a CI = 0.837 to 0.944.

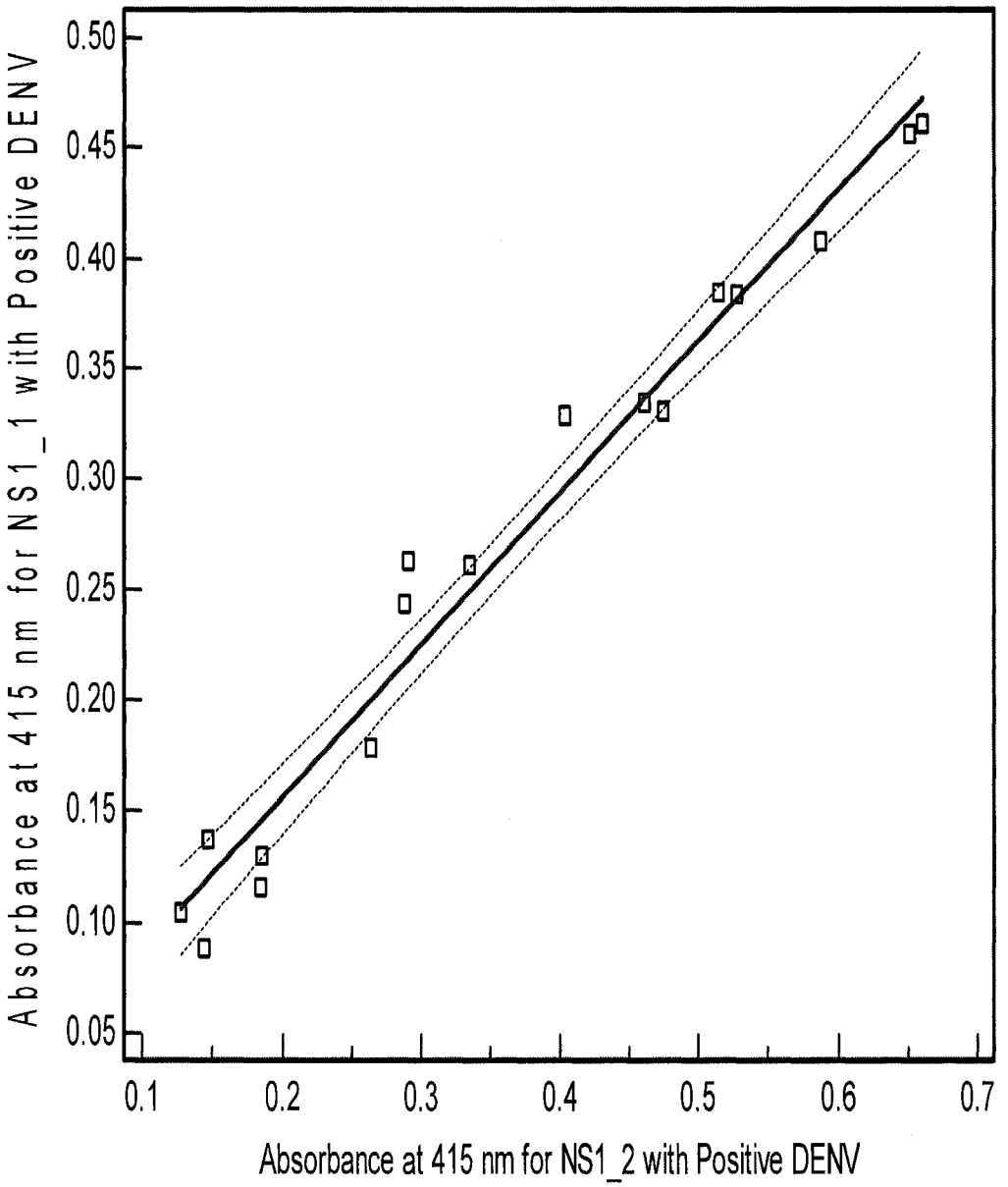



**Figure 3.4.6. Correlation of NS1 vs. NS2 peptide-ELISA test results in patients with recent dengue infections.** The peptide-ELISA results obtained comparing peptides NS1-1 and NS1-2 that were only positive for recent DENV Infections, plotted to show correlation and 95% CI values. For n=17, Spearmans R = 0.9855, 95% CI for R = 0.9593 to 0.9949. As described above, one outlier (Sample 4) was excluded from these calculations and is not shown. Note: when the outlier (Sample 4) was included, Spearmans R falls to 0.33.

**Table 3.4.3. Receiver Operating Curve analysis of the peptide-based ELISA.**

| Positive serum samples | Negative serum samples | Peptide | Cut-off value in D.O. | Sensitivity % | Specificity % | Area under the curve (classification) [e] | Significance level (p- value) |
|---|---|---|---|---|---|---|---|
| Recent WVN Infection [a] | Flavivirus-negative [d] | NS1-1 | 0.110 | 100 (95% CI, 48-100) | 57.9 (95% CI, 33.5-79.7) | 0.695 (poor) | 0.1769 |
| | | NS1-2 | 0.159 | 100 (95% CI, 48-100) | 63.2 (95% CI, 38.4-83.6) | 0.758 (fair) | 0.0575 |
| | | NS1-27 | 0.072 | 100 (95% CI, 48-100) | 42.1 (95% CI, 20.3-66.5) | 0.676 (poor) | 0.2346 |
| | | E-44 | 0.278 | 100 (95% CI, 48-100) | 57.9 (95% CI, 38.5-79.7) | 0.674 (poor) | 0.2346 |
| Recent DENV Infection [b] | Flavivirus-negative | NS1-1 | 0.242 | 60 (95% CI, 36.1-80.8) | 95.8 (95% CI, 78.8-99.3) | 0.749 (fair) | 0.0010 |
| | | NS1-2 | 0.276 | 55 (95% CI, 31.6-76.9) | 100 (95% CI, 85.6-100) | 0.740 (fair) | 0.0018 |
| | | NS1-27 | 0.124 | 45 (95% CI, 23.1-68.4) | 100 (95% CI, 85.6-100) | 0.579 (fail) | 0.3654 |
| | | E-44 | 0.494 | 55 (95% CI, 31.6-76.9) | 95.8 (95% CI, 78.8-99.3) | 0.707 (poor) | 0.0095 |
| Previous DENV Infection [c] | Flavivirus-negative | NS1-1 | 0.179 | 66.7 (95% CI, 30.1-92.1) | 79.2 (95% CI, 57.8-92.8) | 0.678 (poor) | 0.1090 |
| | | NS1-2 | 0.276 | 55.6 (95% CI, 21.4-86) | 100 (95% CI, 85.6-100) | 0.773 (poor) | 0.0067 |
| | | NS1-27 | 0.104 | 55.6 (95% CI, 21.4-86) | 91.7 (95% CI, 73-98.7) | 0.641 (poor) | 0.2135 |
| | | E-44 | 0.339 | 66.7 (95% CI, 30.1-92.1) | 66.7 (95% CI, 44.7-84.3) | 0.560 (fail) | 0.6024 |

[a] High titred WNV-positive convalescent serum samples obtained in Colorado, 2003; [b] High titred DENV-positive convalescent serum samples obtained in a DENV endemic area (Mérida, México); [c] Serum samples from patients with previous DENV infections obtained in Mérida, México; [d] Flavivirus-negative serum samples obtained in Colorado, 2003; [e] The value obtained in the area under the curve is an indication of the ability of the test to distinguish between the positive and negative groups. A value of 1 indicates that the test differentiate between positive and negative groups. When the test can not discriminate between the two groups, the area under the curve is 0.5.

The immunoblot results using synthetic peptides and the corresponding peptide-based ELISA results are presented in Table 3.4.4 to facilitate easy sample group by sample group comparisons of the two methods. The results for Dot Blot testing of synthetic peptides NS1-27, and E-44 show concordance with the corresponding peptide-based ELISA results for Group 1, 2, and 3 samples (Recent WNV Infections, Recent DENV Infections, and Previous DENV Infections respectively). The Group 4 Flavivirus Negative Samples show only slight concordance between Dot Blot and the peptide-based ELISA results, with all 6 samples inappropriately positive by Dot Blot (6/6). The NS1-27 peptide-based ELISA gave more appropriate results for the Flavivirus Negative Sample Group 4: only 2 out of 6 positive. The E-44 peptide based ELISA also more appropriately showed only 1 positive out of 6 from the Flavivirus Negative Samples Group 4.

The results for Dot Blot testing of NS1-1 and NS1-2 synthetic peptides did not agree at all with the peptide-based ELISA results for Groups 1, 2, and 3, with negative Dot Blot results for all samples except Dot Blot NS1-1 results for Group 2 (Recent DENV Infections) and Dot Blot NS1-2 results for Group 3 (Previous DENV Infections). In contrast the peptide-based ELISA results gave positive results for 35 out of 36 samples in Groups 1, 2, and 3, even though only the 12 test results for Recent WNV Infections were expected to be positive, since NS1-1 NS1-2 synthetic peptides are from the West Nile Virus amino acid sequence and were not expected to react with DENV infected samples. Finally, the results for Dot Blot testing of NS1-1 and NS1-2 synthetic peptides agreed perfectly with the peptide-based ELISA results for Groups 4,

Flavivirus Negative samples, with all Group 4 results being appropriately negative by Dot Blot and peptide-based ELISA.

**Table 3.4.4. Summary of the Dot-blot and peptide-based ELISA test results.**

| Samples | No. Individual samples | Test | Synthetic peptides | | | | |
|---|---|---|---|---|---|---|---|
| | | | NS1-1 | NS1-2 | NS1-26 | NS1-27 | E-44 |
| **Recent WNV Infections (Grp. 1)** | 6 | Dot Blot | - | - | - | + | + |
| | | Peptide-based ELISA | + (6/6) | + (6/6) | N.A.[a] | + (6/6) | + (6/6) |
| **Recent DENV Infections (Grp. 2)** | 8 | Dot Blot | + | - | - | + | + |
| | | Peptide-based ELISA | + (8/8) | + (7/8) | N.A. | + (7/8) | + (7/8) |
| **Previous DENV Infections (Grp. 3)** | 4 | Dot Blot | - | + | + | + | + |
| | | Peptide-based ELISA | + (4/4) | + (4/4) | N.A. | + (4/4) | + (3/4) |
| **Flavivirus Negative Samples (Grp. 4)** | 6 | Dot Blot | - | - | - | + | + |
| | | Peptide-based ELISA | - (0/6) | - (0/6) | N.A. | + (2/6) | + (1/6) |

[a]N.A., not analyzed.

## 3.5. DISCUSSION

Synthetic peptides of the E and NS1 proteins of WNV were used to identify immunogenic linear epitopes recognized by the two monoclonal antibodies and human and horse serum samples. After identifying the most promising peptides by immunoblot analyses, the peptides were used in peptide-based ELISA to confirm cases of WNV. To our surprise, all horse and human serum samples tested by immunoblot, whether they contained WNV or DENV antibodies or not, bound to two peptides of WNV, the NS1-27 (amino acids 197-214) and the E-44 (amino acids 320-337). This observation was confirmed by the peptide-based ELISA in 94% (17/18) of Groups 1, 2, and 3 WNV or DENV positive samples, and 33% (2/6) Group 4 flavivirus negative samples (Table 3.4.4). The MAb 3.1112G also bound the NS1-27 peptide, as demonstrated by immunoblot analyses.

Interestingly, in the immunoblot analysis results, all the serum samples from patients with recent DENV infections bound the NS1-1 peptide of WNV, but the samples from patients with recent WNV infections or samples with old dengue infections did not bind the peptide NS1-1 of WNV (Figures 3.4.1 and 3.4.2). When we attempted to validate the ability of the peptide-based ELISA to distinguish between recent dengue infections and sera classified as flavivirus-negative by ROC analysis (using a statistically significant p-value of 0.0001), the sensitivity was 60% and a specificity of 95.8%. These results demonstrate a reasonable ability of the test to distinguish between the groups tested, with a low 4.2% false positive rate, but with a correspondingly high false negative rate of 40% (Table 3.4.3).

IgM may be participating in the recognition of the NS1-1 peptide. Huang et al, (99) identified the NS1 of DENV (a.a. 1-15) as the immunodominant epitope that reacted with sera from dengue fever patients, but not with Japanese encephalitis (JE) patients. They also determined that the isotype of the antibody that reacted the peptide 1 was IgM (99). Their ELISA results determined a cut-off value of 0.392 O.D. In our study human serum samples with previous DENV infections were found to bind to peptide NS1-2 of WNV. It is possible that treatment of the infected cell lysates with ionic detergents and characteristics of attachment to the ELISA plates leads to the exposure of conserved cryptic sites, allowing the cross-reactive antibodies to bind (197).

Peptide 44 represents an antigenic region that was recognized by all the human and horse serum sample tested, but not by MAB 6B6C-1 (Figure 3.4.3). Peptide 44 of the envelope protein is part of the pre-anchor stem region following domain III. Domain III is a carboxyl terminal Ig-like domain, containing predominantly beta sheet folds (98). In our study, peptide 44 was not recognized by the MAb 6B6C-1. This MAb has been studied by many researchers (35, 174, 175, 197) to define the region that is recognized on the E protein. The MAb 6B6C-1 is sub-class IgG2A and is flavivirus group specific (175). This monoclonal antibody recognizes a cluster of epitopes at or around the the fusion peptide loop (amino acid position 98 – 111) and its reactivity is higher with disrupted virions and soluble forms of E than with native whole-virus preparations (197).

In future studies synthetic peptides of DENV NS1-1 and NS1-2 will need to be included in the tests in addition to the WNV peptides. Synthetic peptides from DENV

may yield better sensitivity and specificity in distinguishing between DENV and WNV infections than the results determined using the WNV peptide-based ELISA.

Our results showed that NS1-1 peptide shows promise in differentiating between DENV and WNV infections by Dot blot. The reason why NS1-1 reacts with sera from patients with dengue infections but not with sera from patients with WNV infections remains to be determined (Table 3.4.4). Clearly, more tests are necessary, using larger numbers of well characterized serum samples and synthetic peptides of both WNV and DENV to further characterize the diagnostic efficacy of the peptide-based ELISA to determine flavivirus infections.

# CHAPTER IV

# CHARACTERIZATION OF THE EPITOPE-BLOCKING ELISA FOR DIAGNOSIS OF WNV INFECTION IN HORSES SEQUENTIALLY INFECTED WITH FLAVIVIRUSES.

## 4.1. ABSTRACT.

An epitope blocking enzyme linked immunosorbent assay (b-ELISA) was evaluated for its diagnostic capabilities to detect antibodies to WNV in 14 horses sequentially infected with WNV and SLEV, or SLEV and WNV, or DENV and WNV. The sensitivity and specificity of the b-ELISA for detection of antibodies to WNV (using the standard inhibition cut-off value of 30%) were 90.9% (true positive rate), (95% CI: 81.2%-96.6%) and 91.7% (true negative rate), (95% CI: 85.9%-95.6%), respectively. When a cut-off value of 35% was used, the sensitivity remained the same, but the specificity increased to 94.4%, (95% CI: 89.3%-97.6%). The b-ELISA was specific for the detection of antibodies to WNV and did not cross react with DENV.

## 4.2. INTRODUCTION.

West Nile virus (WNV) is a flavivirus that was isolated for first time in Uganda from a febrile woman in 1937 (191). WNV is causing great concern around the world, because it has caused significant outbreaks of encephalitis in humans and horses in many geographic areas in Africa, the Middle East, parts of Europe, Asia, and recently the Americas (90, 100). WNV is classified as a member of the genus *Flavivirus* (family

Flaviviridae) and belongs to the Japanese encephalitis serocomplex. In addition to its public health impact in North America, WNV has caused extensive morbidity and mortality in equines (http://www.aphis.usda.gov/vs/nahss/wnv/wnv_distribution_maps.htm). Other important members of the JE serocomplex group that infect horses are Japanese encephalitis virus (JEV) and Saint Louis encephalitis virus (SLE). SLEV is widely distributed in the Americas. Both WNV and SLEV are transmitted mainly by *Culex spp.* mosquitoes and birds are the principal amplifying hosts. Since both viruses infect humans and horses, differential diagnosis of the two etiological agents is important (147).

The criteria for diagnosing WNV encephalitis in horses include the presence of neurological abnormalities, and positive laboratory tests like virus isolation in cell culture, genome identification by RT-PCR (159), IgM-ELISA, and plaque reduction neutralization test (PRNT) tests. Diagnoses of WNV infections in horses has principally been based on serological test results, mainly by ELISA detection of immunoglobulin M (IgM) antibodies to WNV, and confirmation of neutralizing antibodies to WNV by PRNT (14).

Current methods of diagnosing WNV infections in horses are complicated by at least three significant factors. First, because WNV is classified as a biosafety level 3 agent (171), few laboratories are equipped to safely isolate live WNV viruses or safely use the live virus to prepare antigens for virological and serological testing. Second, serological diagnoses are complex, due to several properties of the antibodies induced by WNV. The IgM antibodies to WNV become detectable 8-10 days after infection and persist less than 2 months (159), so the window for detection and confirmation is

relatively narrow. The test also requires species-specific anti-IgM antibodies for each type of mammal being assayed, limiting diagnoses to only the species for which there are currently available species-specific reagents. Neutralizing antibodies to WNV persist at least 15 months and cross-react with other flaviviruses. Thus, a single sample has not been sufficient for conclusive diagnosis. To diagnose an infection, a four fold or greater increase in PRNT antibody titer to WNV between acute and convalescent sera samples is required. Accurate diagnoses are also complicated by serological cross-reactions among closely-related members of the genus *Flavivirus* (87, 180). Current methods are prone to false positive WNV results due to serological cross-reactions, when the individual has been previously infected with other flaviviruses. Third, virus isolation is very difficult because the viremia titer of WNV in horses is low (153), thereby limiting ante-mortem diagnoses. Most isolates of WNV are obtained from horse CNS tissue taken post-mortem.

To address the problems in current WNV diagnoses, an epitope-blocking enzyme linked immunosorbent assay (b-ELISA) was developed and used to detect antibodies specific to WNV in surveillance studies of bird, reptile, and mammal species, including horses (17, 18). The b-ELISA method measures the ability of antibodies in serum specimens from any species to block the attachment of a monoclonal antibody against WNV-specific epitope on the non-structural 1 (NS1) protein (83). Thus, b-ELISA offers the potential to overcome a number of the problems previously listed. However, there is little information concerning the diagnostic efficacy, specificity, and selectivity of the b-ELISA for detecting WNV antibodies in animals sequentially infected with different flaviviruses. This is very important because in many parts of the

world, where different flaviviruses are sympatric, sequential infections greatly complicate diagnoses.

The main objectives of this study were to evaluate the diagnostic performance of the b-ELISA for the detection of antibodies to WNV in horses sequentially infected with heterologous flaviviruses, e.g. Saint Louis encephalitis and dengue viruses, and to characterize the performance of b-ELISA in sequentially infected horses, using different cut-off diagnostic values. Experiments investigating sequential infections of flaviviruses in animals and the diagnostic implications of sequential infections could informative. The results could potentially be extrapolated to human diagnosis, and the conclusions could be exploited to design more efficacious diagnostic tests for flavivirus infections in tropical regions of the world.

## 4.3. MATERIALS AND METHODS.

### 4.3.1. Infection of and serum sampling from horses.

Fourteen horses were sequentially inoculated with WNV, followed by SLEV, or SLEV and then WNV, or DENV and then WNV. All horses were bled prior to and every three days following the first inoculation with the selected virus. Blood was collected via jugular venipuncture into vacuum tubes (Becton and Dickinson vacutainer tube). The three groups of horses consisted of 1) a group of four 4 horses infected with WNV strain NY99 and subsequently with SLEV strain TBH-28; 2) a group of 8 horses infected with SLEV (4 with a single booster of SLEV and 4 with two SLEV boosters) and then infected with WNV after 3 weeks; and 3) a group of 2 horses infected with DENV-2 and then 3 weeks later with WNV. All three groups of horses were inoculated with the chosen flavivirus via subcutaneous injection. Each horse was infected with a

dose of 10,000 PFU/mL of the respective flavivirus. The schedule of the inoculations is provided in Table 4.3.1. All animal experiments were conducted according to the Guidelines for Animal Safety and Experimentation at the Department of Biomedical Sciences, Animal Reproduction and Biotechnology Laboratory for of Colorado State University, Fort Collins, Colorado.

**Table 4.3.1. Schedule for sequential virus infection of horses with WNV and SLEV, SLEV and WNV or DENV-2 and WNV [a].**

| Horse ID | First virus inoculated by day 0 (Strain) | Second virus inoculated by day 21th (Strain) | Third virus inoculatedby day 42th (Strain) |
|---|---|---|---|
| **EF-1** | WNV (NY99) | SLEV (TBH-28) | - |
| **EF-2** | WNV (NY99) | SLEV (TBH-28) | - |
| **EF-3** | WNV (NY99) | SLEV (TBH-28) | - |
| **EF-4** | WNV (NY99) | SLEV (TBH-28) | - |
| **EF-5** | SLEV (TBH-28) | SLEV (TBH-28) | WNV (NY99) |
| **EF-6** | SLEV (TBH-28) | SLEV (TBH-28) | WNV (NY99) |
| **EF-7** | SLEV (TVP9083) | SLEV (TVP9083) | WNV (NY99) |
| **EF-8** | SLEV (TVP9083) | SLEV (TVP9083) | WNV (NY99) |
| **EF-9** | SLEV (TVP9083) | WNV (NY99) | - |
| **EF-10** | SLEV (TVP9083) | WNV (NY99) | - |
| **EF-11** | SLEV (TBH-28) | WNV (NY99) | - |
| **EF-12** | SLEV (TBH-28) | WNV (NY99) | - |
| **EF-13** | DENV-2 (NGC) | WNV (NY99) | - |
| **EF-14** | DENV-2 (NGC) | WNV (NY99) | - |

[a]Vero cells were infected with the corresponding virus strain to prepare the inoculum. Each horse was inoculated with approximately 10,000 PFU of the respective virus.

### 4.3.2. b-ELISA for WNV and flavivirus diagnosis

Two monoclonal antibodies (MAb) were used to determine the ability of the b-ELISA test to detect antibodies specific for WNV and for flaviviruses in general. The first MAb 3.1112G binds to the WNV specific epitope on NS1 protein, and the second MAb 6B6C-1 binds to a flavivirus-specific epitope of the E protein (174). As previously described in Chapters 2 and 3 (sections 2.3.2 and 3.3.2, respectively), the b-ELISA was performed using a cell lysate antigen prepared from *Aedes albopictus* C6/36 cells that had been infected with WNV (NY-99 strain) at a multiplicity of infection of 0.1 (18). The b-ELISA was performed using either MAb 3.1112G or MAb 6B6C-1, following the protocol described by Blitvich et al (18). The optimal cut-off value for the determination of antibodies to WNV and flaviviruses by b-ELISA was found to be 30% for animals. This value was initially chosen from previous studies with horses (17), and later confirmed by our test results. All serum samples were tested by PRNT for antibodies to WNV, SLE or DEN-2 (14), capture IgG-ELISA (104), capture IgM-ELISA (144), and b- ELISA for WNV and flavivirus antibodies (18).

### 4.3.3. Statistical analysis.

To determine the optimal cut-off value, sensitivity and specificity of the b-ELISA, the Receiver Operating Characteristic (ROC) analyses were performed with MedCalc statistical software (Belgium) (72). The term "ROC" curve originated in describing performance characteristics of observers using mechanical devices. Different points on the ROC curve represent different choices of cut-off points, where each pairing balances maximizing sensitivity (true positive rates) and specificity (true negative rates), simultaneously minimizing Type I and Type II errors. The area under

the ROC curve (AUC) was used as a measure of the test accuracy (199). The ROC curve and Dot Plot analyses were described in Chapter 2, (section 2.3.8.). Probability levels of less than 0.05 were considered significant.

## 4.4. RESULTS.

PRNT was used to determine the neutralizing titers to WNV, SLEV, and DEN-2 for all fourteen (14) horses, and it was also used as the gold standard for identification of antibodies to each specific flavivirus used in this study. To characterize the type of antibody response each horse developed, several tests were performed on all collected samples, before and after primary, secondary, and tertiary challenges. Those tests were PRNT, capture IgG-ELISA, capture IgM-ELISA, and epitope-blocking ELISA for specific detection of WNV and for flavivirus antibodies.

### 4.4.1 Antibody responses of horses sequentially inoculated, first with WNV and later with SLEV.

In November 2004, four horses were experimentally infected with WNV (Table 4.4.1.). Before infection, serum samples from these horses were screened by PRNT for the presence of antibodies to WNV or SLEV. All were negative. These horses were then injected with WNV or SLEV (Table 4.4.1.). These experimental infections resulted in subclinical infections in all 4 horses. The horses were monitored for PRNT antibodies. On the $6^{th}$ day after inoculation with WNV (strain NY-99), the PRNT WNV antibody titers in the horses were: 1:640 (horse EF-1), 1:40 (horse EF-2), 1:320 (horse EF-3), and 1:320 (horse EF-4). After the inoculation with SLEV, the neutralizing antibodies against WNV decreased, until by day 30, antibody titers had declined to 1:20 and 1:40. After day 30, antibody titers increased until day 39, with all 4 horses

showing the same WNV neutralizing antibody titer of 1:160. PRNT antibody titers to SLE were very low, and disappeared by day 21 after SLEV inoculation (Table 4.4.1).

**Table 4.4.1 Neutralizing antibodies in 4 horses sequentially infected with WNV and SLEV.** The first virus inoculated in the horses was WNV (NY-99) and 21 days later the same horses were inoculated with SLEV (TBH-28).

| Date | Virus inoculated | Days after virus inoculation | PRNT (WNV) | | | | PRNT (SLEV) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | EF | | | | EF | | | |
| | | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| 11-04-04 | - | -9 | -[a] | - | - | - | - | - | - | - |
| 11-07-04 | - | -6 | - | - | - | - | - | - | - | - |
| 11-10-2004 | - | -3 | - | - | - | - | - | - | - | - |
| 11-12-2004 | WNV | 0 | - | - | - | - | - | - | - | - |
| 11-15-2004 | - | 3 | NT | NT | NT | NT | NT | NT | NT | NT |
| 11-18-2004 | - | 6 | 640[b] | 40 | 320 | 320 | - | - | - | - |
| 11-21-2004 | - | 9 | NT | NT | NT | NT | NT | NT | NT | NT |
| 11-24-2004 | - | 12 | NT | NT | NT | NT | NT | NT | NT | NT |
| 11-27-2004 | - | 15 | NT | NT | NT | NT | NT | NT | NT | NT |
| 30-11-2004 | - | 18 | NT | NT | NT | NT | NT | NT | NT | NT |
| 12-02-2004 | SLEV | 21 | 20 | 20 | 40 | 80 | 20 | 10 | 20 | 10 |
| 12-05-2004 | - | 24 | 20 | 20 | 10 | 80 | 20 | 10 | 10 | 10 |
| 12-08-2004 | - | 27 | 20 | 20 | 40 | 40 | 10 | 10 | 20 | 0 |
| 12-11-2004 | - | 30 | 20 | 20 | 160 | 40 | 10 | 0 | 10 | 10 |
| 12-14-2004 | - | 33 | 80 | 40 | 80 | 80 | 10 | 10 | 20 | 0 |
| 12-17-2004 | - | 36 | 160 | 80 | 80 | 80 | 20 | 10 | 20 | 10 |
| 12-20-2004 | - | 39 | 80 | 160 | 160 | 160 | 10 | 0 | 10 | 20 |
| 12-23-2004 | - | 41 | 160 | 160 | 160 | 160 | - | - | - | - |

[a] – denotates < 1:10 value; [b] reciprocal of 1:640 dilution.

The horses were also monitored for the presence of WNV and SLEV antibodies by ELISA (Table 4.4.2.). IgM antibodies were detected in only 2 horses (EF-2 and EF-3) corresponding to day 21th day after inoculation with WNV (NY-99), but IgG and blocking antibodies were detected in all four of them (Table 4.4.2).

**Table 4.4.2. ELISA analysis of antibody responses detected in 4 horses sequentially infected with WNV and SLEV.**

| Virus inocula-ted | Days alter virus inoculation | IgG-ELISA (WNV) | | | | IgM-ELISA (WNV) | | | | b-ELISA (WNV) | | | | b-ELISA (FLAVI) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | EF | | | | EF | | | | EF | | | | EF | | | |
| | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| - | -9 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | -6 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | -3 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **WNV** | 0 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 3 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 6 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 9 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 12 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 15 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 18 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **SLEV** | 21 | + | + | + | + | - | + | + | - | + | + | + | + | + | + | + | - |
| - | 24 | + | + | + | + | - | + | + | - | + | + | + | + | + | + | + | + |
| - | 27 | + | + | + | + | - | + | + | - | + | + | + | + | + | + | + | + |
| - | 30 | + | + | + | + | - | - | + | - | + | + | + | + | + | + | + | + |
| - | 33 | + | + | + | + | - | - | + | - | + | + | + | + | + | + | + | + |
| - | 36 | + | + | + | + | - | - | - | - | + | + | + | + | + | + | + | + |
| - | 39 | + | + | + | + | - | - | - | - | + | + | + | + | + | + | + | + |
| - | 41 | + | + | + | + | - | - | - | - | + | + | + | + | + | + | + | + |

### 4.4.2. Antibody responses of horses sequentially inoculated with SLEV and later with WNV.

In January 2006, eight horses (EF-5 – EF-12) were injected with SLEV to cause primary flavivirus infections (Table 4.4.3). Four of these horses (EF-5 – EF-8) were subsequently boosted with SLEV and then infected with WNV. Two horses (EF-6 and EF-7) that were inoculated with SLEV (one horse with strain TBH-28 and the other one with strain TVP 9083) developed neutralizing antibodies to SLEV after a subsequent booster with the same strain of SLEV used for the primary infection. Neutralizing antibodies to SLEV were detectable at day 9 in horse EF-6 and at day 33 in horse EF-7. Nine to thirteen days after inoculations with WNV, all these horses, except EF-6, were positive by PRNT (Table 4.4.3.), and capture IgG and IgM-ELISA, and b-ELISA specific for WNV and for flaviviruses (Table 4.4.4).

The remaining 4 horses (EF-9 to EF-12) that were inoculated first with SLEV but did not generate neutralizing antibodies to the flavivirus were subsequently injected with WNV (Table 4.4.5). Following challenge with WNV, 3 out of 4 horses evaluated were positive by PRNT (Table 4.4.5.), and IgG and IgM-capture ELISA and WNV and flavivirus blocking ELISA tests (Table 4.4.6). Interesting, no antibodies to WNV or to flaviviruses were detected in EF-10 (Table 4.4.6.), but the sample from this horse did yield a positive neutralization response to SLEV (Table 4.4.5.).

**Table 4.4.3 Neutralizing antibodies in 4 horses sequentially infected with SLE, SLEV, and then with WNV.**

| Date | Virus inocu-lated | Days after virus inocula-tion | PRNT (WNV) | | | | PRNT(SLEV) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | EF | | | | EF | | | |
| | | | 5 | 6 | 7 | 8 | 5 | 6 | 7 | 8 |
| **1-05-06** | SLE | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-08-06** | - | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-11-06** | - | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-14-06** | - | 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-17-06** | - | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-20-06** | - | 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-23-06** | - | 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-26-06** | SLE | 21 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 0 |
| **1-29-06** | - | 24 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 0 |
| **2-01-06** | - | 27 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 0 |
| **2-04-06** | - | 30 | 0 | 0 | 0 | 0 | 0 | 40 | 0 | 0 |
| **2-07-06** | - | 33 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 |
| **2-10-06** | - | 36 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 |
| **2-13-06** | - | 39 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 |
| **2-16-06** | WNV | 41 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 |
| **2-19-06** | - | 44 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 |
| **2-22-03** | - | 47 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 |
| **2-25-06** | - | 50 | 160 | 0 | 0 | 0 | 20 | 0 | 20 | 0 |
| **2-28-06** | - | 53 | 160 | 0 | 20 | 80 | 80 | 0 | 40 | 0 |
| **3-03-06** | - | 56 | 160 | 0 | 40 | ≥320 | 80 | 0 | 160 | 20 |
| **3-06-06** | - | 59 | 320 | 0 | 40 | ≥320 | 80 | 0 | 80 | 0 |
| **3-09-06** | - | 61 | 320 | 0 | 20 | 160 | 80 | 0 | 40 | 10 |

**Table 4.4.4. ELISA analysis of antibody responses detected in 4 horses sequentially infected with SLEV, SLEV and WNV.**

| Virus inoculated | Days after virus inoculation | IgG-ELISA (WNV) | | | | IgM-ELISA (WNV) | | | | b-ELISA (WNV) | | | | b-ELISA (FLAVI) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | EF | | | | EF | | | | EF | | | | EF | | | |
| | | 5 | 6 | 7 | 8 | 5 | 6 | 7 | 8 | 5 | 6 | 7 | 8 | 5 | 6 | 7 | 8 |
| SLE | 0 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 3 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 6 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 9 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 12 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 15 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 18 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| SLE | 21 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 24 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 27 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 30 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 33 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 36 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 39 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| WNV | 41 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 44 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 47 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 50 | - | - | - | - | +/- | - | - | - | - | - | - | - | + | - | + | - |
| - | 53 | + | - | + | - | + | - | +/- | + | - | - | + | - | + | - | + | + |
| - | 56 | + | - | + | - | + | - | +/- | + | + | - | + | + | + | - | + | + |
| - | 59 | + | - | + | - | + | - | - | + | + | - | + | + | + | - | + | + |
| - | 61 | + | - | + | - | + | - | - | + | + | - | + | + | + | - | + | + |

**Table 4.4.5 Neutralizing antibodies in 4 horses sequentially injected with SLE and WNV.**

| Date | Virus inocula-ted | Days after virus inocula-tion | PRNT (WNV) | | | | PRNT (SLEV) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | EF | | | | EF | | | |
| | | | 9 | 10 | 11 | 12 | 9 | 10 | 11 | 12 |
| **1-05-06** | SLE | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-08-06** | - | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-11-06** | - | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-14-06** | - | 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-17-06** | - | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-20-06** | - | 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-23-06** | - | 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-26-06** | WNV | 21 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1-29-06** | - | 24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **2-01-06** | - | 27 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **2-04-06** | - | 30 | 10 | 0 | 0 | 0 | 20 | 0 | 0 | 20 |
| **2-07-06** | - | 33 | 80 | 0 | 80 | 40 | 40 | 0 | 40 | 80 |
| **2-10-06** | - | 36 | 160 | 0 | 80 | 40 | 40 | 0 | 40 | 0 |
| **2-13-06** | - | 39 | 80 | 0 | 160 | 40 | 40 | 20 | 20 | 0 |
| **2-16-06** | - | 41 | 80 | 0 | 80 | 20 | 80 | 20 | 20 | 0 |

**Table 4.4.6. ELISA antibody responses in 4 horses sequentially infected with SLEV and then with WNV.**

| Virus inoculated | Days after virus inocula-Tion | IgG-ELISA (WNV) | | | | IgM-ELISA (WNV) | | | | b-ELISA (WNV) | | | | b-ELISA (FLAVI) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | EF | | | | EF | | | | EF | | | | EF | | | |
| | | 9 | 10 | 11 | 12 | 9 | 10 | 11 | 12 | 9 | 10 | 11 | 12 | 9 | 10 | 11 | 12 |
| SLE | 0 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 3 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 6 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 9 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 12 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 15 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 18 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| WNV | 21 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 24 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 27 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | 30 | - | - | - | - | + | - | + | - | + | - | - | - | - | - | - | + |
| - | 33 | - | - | + | + | + | - | + | + | + | - | + | + | - | - | + | + |
| - | 36 | + | - | + | + | + | - | + | + | + | - | + | + | + | - | + | + |
| - | 39 | + | - | + | + | + | - | + | + | + | - | + | + | + | - | + | + |
| - | 41 | + | - | + | + | + | - | + | - | + | - | + | + | + | - | + | + |

### 4.4.3. Antibody responses of horses sequentially inoculated with DENV-2 and then WNV.

In January 2006, two horses (EF-13 and EF-14) were inoculated with DENV-2 as the primary infecting flavivirus (Table 4.4.7). By 6 days post-inoculation, neutralizing antibodies to DENV-2 were detected in both horses. Neutralizing antibody titers of 1:40 and 1:20 (for horses EF-13 & EF-14) to WNV appeared day 6 post WNV infection (21 days post DENV-2 infection) and then spiked to 1:≥ 320 and 1:1280 for horses EF 13 and 14, respectively, by day 9 post WNV infection. The titers of

neutralizing antibodies to WNV remained high throughout 41 days post DENV infection and 21 days post WNV infection (Table 4.4.7). Sera from these horses also contained significant neutralizing titers for SLEV.

**Table 4.4.7. Neutralizing antibodies in 2 horses sequentially infected with DENV-2 and then WNV.**

| Date | Virus inocu-lated | Days after virus inoculation | PRNT (WNV) | | PRNT (SLEV) | | PRNT (DENV-2) | |
|---|---|---|---|---|---|---|---|---|
| | | | EF | | EF | | EF | |
| | | | 13 | 14 | 13 | 14 | 13 | 14 |
| **5-26-06** | DEN | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **5-29-06** | - | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| **6-01-06** | - | 6 | 0 | 0 | 0 | 0 | 10 | 160 |
| **6-04-06** | - | 9 | 0 | 0 | 0 | 0 | 80 | ≥320 |
| **6-07-06** | - | 12 | 0 | 0 | 0 | 0 | 80 | ≥320 |
| **6-10-06** | - | 15 | 0 | 0 | 0 | 0 | 40 | ≥320 |
| **6-13-06** | - | 18 | 0 | 0 | 0 | 0 | 20 | ≥320 |
| **6-16-06** | WNV | 21 | 0 | 0 | 0 | 0 | 40 | 160 |
| **6-19-06** | - | 24 | 0 | 0 | 0 | 0 | 40 | 160 |
| **6-22-06** | - | 27 | 40 | 20 | 0 | 40 | 40 | ≥320 |
| **6-25-06** | - | 30 | ≥320 | 1280 | 20 | 640 | 40 | 320 |
| **6-28-06** | - | 33 | ≥2560 | 1280 | 40 | 640 | 320 | 160 |
| **7-01-06** | - | 36 | 2560 | 1280 | 40 | 640 | 160 | 80 |
| **7-04-06** | - | 39 | 1280 | 640 | 80 | 320 | 160 | 80 |
| **7-07-06** | - | 41 | 1280 | 640 | 40 | 320 | 160 | 80 |

The horses were also assayed by ELISA for antibodies to WNV (Table 4.4.8.). Following DENV infection, the IgG and IgM-capture ELISA results were negative. The b-ELISA reaults using the WNV antigen and the two MAbs were also negative.

After inoculation with WNV, all serological test results were positive by day 6 or 9 post challenge with WNV, which corresponded to days 27 and 30 after challenge with DENV-2 (Table 4.4.8).

**Table 4.4.8. ELISA analysis of antibody responses detected in 2 horses sequentially infected with DENV and then with WNV.**

| Virus inoculated | Days after virus inoculation | IgG-ELISA (WNV) | | IgM-ELISA (WNV) | | b-ELISA (WNV) | | b-ELISA (FLAVI) | |
|---|---|---|---|---|---|---|---|---|---|
| | | EF | | EF | | EF | | EF | |
| | | 13 | 14 | 13 | 14 | 13 | 14 | 13 | 14 |
| DEN | 0 | - | - | - | - | - | - | - | - |
| - | 3 | - | - | - | - | - | - | - | - |
| - | 6 | - | - | - | - | - | - | - | - |
| - | 9 | - | - | - | - | - | - | - | - |
| - | 12 | - | - | - | - | - | - | - | - |
| - | 15 | - | - | - | - | - | - | - | - |
| - | 18 | - | - | - | - | - | - | - | - |
| WNV | 21 | - | - | - | - | - | - | - | - |
| - | 24 | - | - | - | - | - | - | - | - |
| - | 27 | - | - | - | - | - | - | - | - |
| - | 30 | + | + | + | + | - | + | - | - |
| - | 33 | + | + | + | + | + | + | + | + |
| - | 36 | + | + | + | + | + | + | + | + |
| - | 39 | + | + | + | + | + | + | + | + |
| - | 41 | + | + | + | + | + | + | + | +/- |

### 4.4.4. Sensitivity and specificity of the b-ELISA for WNV antibody detection.

The sensitivity and specificity of the b-ELISA for WNV antibody detection, using a cut-off value of 30% and calculated by ROC analyses, were 90.9% sensitivity (true positive rate), (95% CI, 81.2%-96.6%), and 91.7% specificity (true negative rate), (95% CI, 85.9%-95.6%), respectively. When a cut-off value of 35% was applied, the

sensitivity was maintained, but the specificity increased to 94.4%, (with a tighter 95% CI: 89.3%-97.6%), and the positive predictive value (PPV) was 88.3% (Table 4.4.9). The Spearman's coefficient of rank correlation (rho) between neutralizing antibodies to WNV by PRNT and the blocking antibodies to WNV by b-ELISA was 0.732, (95% CI: 0.663 - 0.790), with a p value $< 0.0001$.

### 4.4.5. Performance of the b-ELISA for the detection of antibodies to flaviviruses in sequential infections.

The results obtained with b-ELISA to detect WNV or flaviviruses antibodies were concordant with the PRNT results in 100% of the cases (Table 4.4.9). The sensitivity and specificity of the b-ELISA for the detection of antibodies to flaviviruses, using a cut-off value of 30% and calculated by ROC analyses, were 95.5% true positive rates, (95% CI: 87.3%-99.0%), and 92.4% true negative rates, (95% CI: 86.7%-96.1%), respectively. When a cut-off value of 26% was applied, the sensitivity increased to a 98.5% true positive rate, (95% CI: 91.8%-99.7%), but the specificity decreased to a 91.7% true negative rate, (95% CI: 85.9%-95.6%), (Table 4.4.9).

### 4.4.6. Dot plots.

Dot plots of b-ELISA results for 66 WNV-positive and 144 WNV-negative serum samples from horses using the MAb 6B6C-1 and the MAb 3.1112G are shown in Figures 4.4.1 and 4.4.2. Each dot plot presents the values obtained for positive (right) samples and the negative (left) samples, with their percentage blocking b-ELISA values plotted on the y axis. The horizontal line indicates the best cut-off values as determined by ROC analyses. The plots clearly show that the cut-off values determined by ROC analyses fit the data, by accurately identifying which samples were

Table 4.4.9. Receiver Operating Curve analyses results from 210 serum samples of 14 horses by blocking–ELISA using MAb 3.1112G and MAb 6B6C-1.

| MAb | Cut- off value (%) | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive Value (%) | Area under the curve * | Significance level |
|---|---|---|---|---|---|---|---|
| 3.1112G for WNV | 30 | 90.9 (95% CI ; 81.2-96.6) | 91.7 (95% CI, 85.9-95.6) | 83.3 | 95.7 | 0.970 | 0.0001 |
| | 35 | 90.9 (95% CI ; 81.2-96.6) | 94.4 (95% CI, 89.3-97.6) | 88.2 | 95.8 | | |
| 6B6C-1for flavivirus | 30 | 95.5 (95% CI, 87.3- 99.0) | 92.4 (95% CI,86.7-96.1) | 85.1 | 97.8 | 0.962 | 0.0001 |
| | 26 | 98.5 (95%Ci, 91.8- 99.7) | 91.7 (95% CI, 85.9-95.6) | 84.4 | 99.2 | | |

*The value of the area under the curve indicates the ability of the test to distinguish between the positive and negative groups. A value of 1 indicates that the blocking ELISA test perfectly differentiated between positive and negative groups. If the value under the curve is 0.5, it indicates that the test cannot distinguish between the two groups.

positive and which were negative. The data are nicely grouped into clusters that clearly fall well above or well below the cut-off lines, with a few outliers.

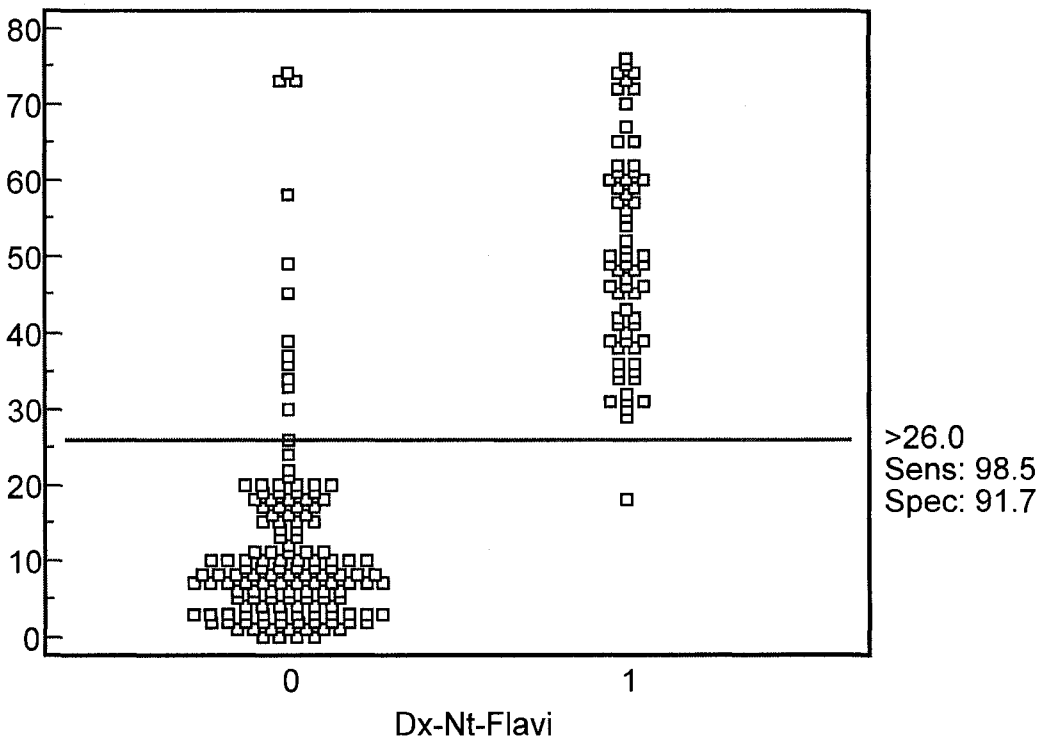



**Figure 4.4.1. b-ELISA using Mab 6B6C-1: Dot plots of 66 WNV-positive and 144 WNV-negative serum samples from horses.** The determination of the best cut-off values in the b-ELISA for detection of antibodies to flaviviruses was performed by ROC analysis. The positive (1) and the negative (0) states of tested specimens are indicated in x-axis. The percent blocking results obtained with b-ELISA for each specimen are indicated in the scale of the y-axis. The horizontal line indicates the best ROC cut-off values.

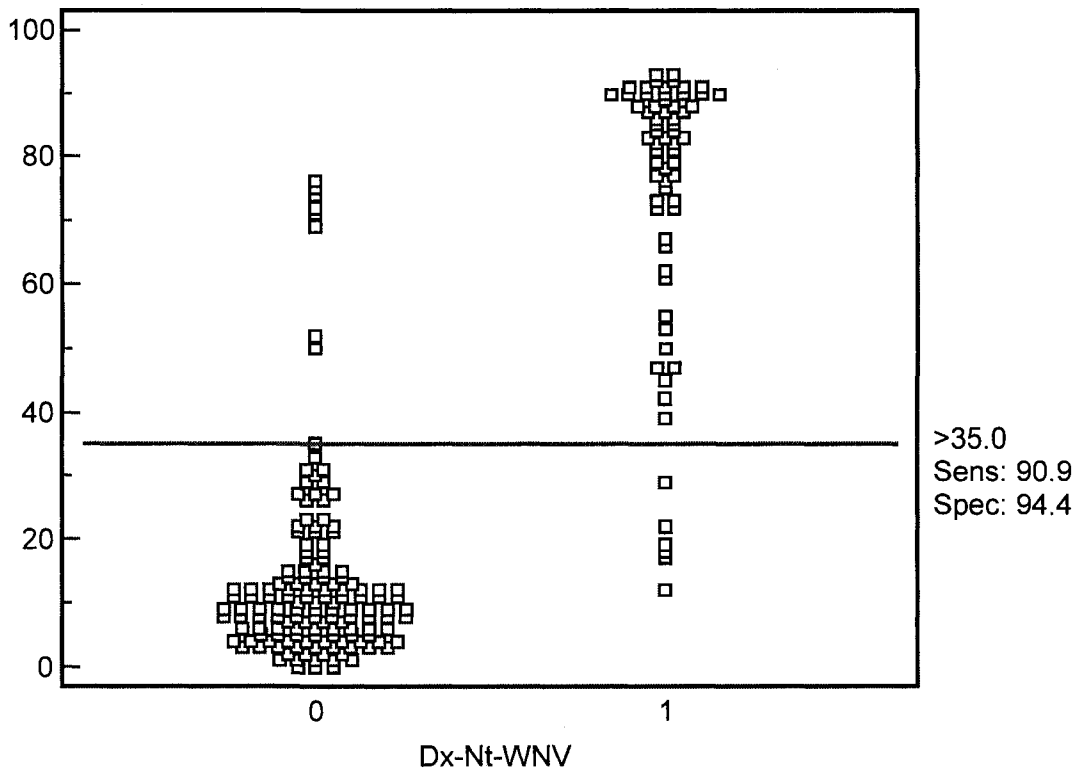



**Figure 4.4.2. b-ELISA using MAb 3.1112G: Dot plots of 66 WNV-positive and 144 WNV-negative serum samples from horses.** Scale of the y-axis is in percentages obtained with b-ELISA. The positive (1) and the negative (0) states of tested specimens are indicated in x-axis. The percent blocking results obtained with b-ELISA for each specimen are indicated in the scale of the y-axis. The horizontal line indicates the best ROC cut-off values.

## 4.5. DISCUSSION.

The b-ELISA is a robust technique that is useful in surveillance of WNV infections in many several different animals, including horses (17, 18). Its reagents are easily available and relatively inexpensive. The monoclonal antibodies used in the b-ELISA for WNV and flavivirus antibody detection are the MAb 3.1112G (specific for the binding to NS1 of the WNV), and the MAb 6B6C-1 (specific for the binding of the E protein of the flaviviruses). These MAbs compete with the antibodies from the serum sample for binding to the limited number of antibody binding epitopes present in the WNV antigens. This test has proven to remarkably useful for detection of antibodies to WNV in many species, and notably in equines (17). However, the issues of WNV diagnosis in equines which had experience a previous flavivirus infection had not been addressed.

Before the arrival of WNV in the Western Hemisphere, SLEV was the main flavivirus causing neurological diseases in horses. Thus, pre-existing antibodies to SLEV were considered as a potential confounder of WNV diagnosis in equines by b-ELISA. The experimental design was to infect horses with SLEV and then to challenge them with WNV. Serum from these horses would then be tested by b-ELISA to determine its ability to accurately diagnosis WNV in horses previously infected with an alternate flavivirus. Unfortunately the two strains of SLEV used in this study were poor inducers of an immune response in the horses. Only two (2) out of eight (8) horses challenged first with SLEV developed neutralizing antibodies (Tables 4.4.3 and 4.4.5). The titers were low (1:20 and 1:40) and lasted for relatively short periods (9 days duration in one case and not determined in the other). The reason for this lack of

infectivity is unknown. Perhaps increasing the virus dose and/or inclusion of more horses would result in more SLEV infected horses. Clearly, horses should be assayed for the presence of SLEV infection by RT-PCR or for antibody production using rapid diagnostic tests before WNV challenge.

Because of the issues with SLEV infection, an alternate flavivirus (dengue virus) was used to infect the horses for the primary flavivirus infection and WNV was used for the secondary infection. Although horses are not infected with DENV in nature, this sequence would have the advantage for more closely resembling the sequence of human flavivirus infections in the Americas (Chapter 2). Two horses were challenged first with DENV-2 and 21 days later with WNV (Table 4.4.7). The DENV-2 inoculation induced a good neutralizing antibody response to DENV-2 at day 6 post-infection. This is in sharp contrast to the horses infected with SLE, which showed no immunological responses (Tables 4.4.3, 4.4.4, and 4.4.5). Titers of neutralizing antibodies to DENV-2 were 1:10 and 1:160, which subsequently increased to maxima of 1:80 and 1: $\geq$ 320 at days 9 and 12 for horses EF-13 and EF-14, respectively (Table 4.4.7). After infection with WNV at day 21, the titers of neutralizing antibodies to DENV-2 significantly declined to 1:40 and 1:80 for horses EF-13 and EF-14, respectively. These results confirm the decline in the titer of neutralizing antibodies to a previous infection with a first virus, (DENV-2), after an infection with a second flavivirus (119, 120).

The main purpose of these trials was to determine whether the WNV b-ELISA could differentiate WNV infections from SLEV or DENV infections. Since the horses inoculated with SLEV and then WNV did not produce either significant or sustained antibody responses to the SLEV injections, the results of the WNV b-ELISA trials were

inconclusive as to whether WNV b-ELISA can diagnose WNV infections in the presence of SLEV (Tables 4.4.3, 4.4.4, 4.4.5, and 4.4.6). The horses inoculated with DENV and then WNV did produce sustained levels of antibodies to DENV (Tables 4.4.7 and 4.4.8) . WNV b-ELISA tests conducted on samples collected throughout the whole 41 day DENV/WNV trials were negative until 9 - 11 days after the WNV inoculations. All subsequent WNV b-ELISA results were WNV positive, demonstrating the WNV b-ELISA's ability to differentially diagnose WNV in the presence of prior DENV infections. The presence of neutralizing antibodies for WNV in both horses confirmed these results (Table 4.4.7.)

Overall these studies confirm that the b-ELISA, using WNV antigens and MAb specific for NS1, effectively detects antibodies specific to WNV infection, providing accurate WNV diagnoses in horses (Tables 4.4.2, 4.4.4, 4.4.6 and 4.4.8). The analyses of serum samples from sequentially infected horses revealed that the specificity (true negative rate) was substantial in primary infections with WNV. Due to the lack of the horse immune responses to SLEV inoculations, it was not possible to determine what the diagnostic implications when WNV occurs as a secondary infection, in the presence of a prior SLEV infection.

One might reasonably expect that humans respond differently, producing both higher viremias and greater antibody titers, resulting in convolved non-conclusive b-ELISA results. The analysis of serum samples from sequential infected horses revealed that the sensitivities obtained with the use of both monoclonal antibodies (specific for WNV and broad-reactive for flaviviruses) were high enough compared to PRNT to determine that the b-ELISA positive test results were due solely to a WNV infection in

the presence of a prior DENV infection (Table 4.4.9). As described earlier, the absence of the horse immune response to SLEV inoculations precluded any conclusions about the ability of the WNV b-ELISA to differentially diagnose WNV infections in the presence of prior SLEV infections.

The b-ELISA for WNV performed well when the standard cut-off value of 30% was used; however when a cut-off value of 35% was applied, the specificity (true positive rate) increased without sacrificing sensitivity (Table 4.4.9). It should be noted that there were no false negatives, because all the samples that tested negative for WNV antibodies by PRNT were also found to be negative by b-ELISA. The b-ELISA did not yield any false positive results, because it did not detect antibodies to DENV-2, and it only gave positive results after the horses were infected with WNV. These preliminary results suggest that b-ELISA can even be used to diagnose secondary WNV flavivirus infections in horses, as was demonstrated with sequential infections first with DENV and later with WNV infections. Additional studies using horses with positive immune responses to SLEV infections will be needed to determine if the WNV b-ELISA can be used to differentiate between SLEV and WNV infections. Several flaviviruses that infect horses are now endemic in North, Central, and South America. With the dissemination of WNV throughout the hemisphere and the world, b-ELISA testing can make significant contributions to investigations of WNV epidemiology in horses and other animals.

# CHAPTER V

## OVERALL SUMMARY AND CONCLUSIONS

West Nile virus (WNV) and Dengue virus (DENV) infections have impacted human and animal health around the world. The negative effects of circulating flavivirus require that communities and public health officials get accurate test results, so they can implement appropriate treatment and preventative measures. Due to the closely related nature of flaviviruses, avian and mammalian species generate antibodies that serologically cross-react with other flaviviruses in current diagnostic tests. These serologic cross-reactions among members of the genus Flavivirus have made differential serologic diagnoses nearly impossible in the presence of secondary flavivirus infections. As a result, current diagnostic methods produce an unacceptable number of false positive results for individuals living in areas where multiple flaviviruses circulate. The current DENV and WNV diagnostic methods also have the significant drawbacks of high cost or using hazardous procedures that limit their use to only a small number of qualified biosafety level 3 or 4 laboratories.

Our primary goal was to develop and evaluate tests that detect specific antibodies to WNV in the presence of secondary flavirus infections. The research also sought tests that do not require exotic, expensive, fragile, dangerous, or short-lived reagents. Finally, we desired test methods that are robust and easily adopted by a broad range of laboratories and easily implemented by typical laboratory personnel. We

compared results from traditional and new techniques for diagnosing of WNV and dengue infections in serum samples from humans and horses.

In Chapter 2, the blocking ELISA proved to be efficacious in detecting antibodies to WNV in humans experiencing primary flavivirus infection. Hundreds of human serum samples were characterized for the presence or absence of IgG and IgM antibodies to flaviviruses using traditional and new techniques. After characterization, the sera were used to evaluate the b-ELISA for WNV diagnosis. The results of this study found that the sensitivity and specificity with WNV b-ELISA were similar to other serological techniques when primary infections were evaluated. However, the 80% false positive rate for the detection of antibodies to WNV in samples from areas where DENV circulates, presumably due to cross reactivity with existing DENV and/or JEV antibodies, was unacceptably high. Thus, we conclude that the b-ELISA can be used for diagnosis of WNV infections in humans in areas where othere flaviviruses, eg, dengue, Japanese encephalitis, yellow fever, etc,, are not circulating, but its use is contraindicated for human diagnosis in the flavivirus endemic regions of the world.

In Chapter 3, WNV synthetic peptides of E and NS1 glycoproteins were evaluated by dot blot and ELISA methods to determine their potential use for flavivirus differential diagnosis. None of the peptides analysed were found to be useful for WNV diagnosis. Interestingly, antibodies to the NS1-1 peptide of WNV were demonstrated to be indicative of recent dengue infections in human serum samples with primary or secondary antibody responses. In contrast, patients infected with WNV did not have antibodies that reacted with the NS1-1 peptide of WNV. The reasons for these confusing results remain to be determined.

In Chapter 4, the capability of WNV b-ELISA to detect specific antibodies to WNV in serum from 14 horses sequentially infected with SLEV and WNV or SLEV and WNV or DENV-2 and WNV was determined. The b-ELISA for WNV was capable of diangnosing secondary WN flavivirus infections. The sensitivity and specificity obtained detecting antibodies for WNV by b-ELISA were equivalent to the WNV-PRNT results. These results are most promising, but whether or not they can be extrapolated to diagnosis of WNV infection of people who have had prior dengue or prior secondary dengue infections remains to be determined.

In conclusion, these efforts in flavivirus diagnosis were partially successful. The WNV b-ELISA could play a role as a screening test for diagnosing human WNV infection in regions where other flaviviruses are not circulating. However, it should not be used for WNV diagnosis in dengue-endemic areas because of the high false positive rates. The b-ELISA shows promise for diagnosing WNV secondary flavivirus infections in horses, but these results will need to be confirmed in a study involving more horses and with other flaviviruses, especially SLEV and JEV, being used as the primary infecting agent. The need for an inexpensive safe WNV-specific realiable test method still exists for routine use in laboratories around the world. With the continuing advances in science and technology, it is imperative to continue searching for better tools for specific diagnoses of WNV and DENV.

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## APPENDIX 6.1
## PROTOCOL FOR THE EPITOPE-BLOCKING ELISA

### 6.1.1 Materials

1. ELISA plates (Corning, Cat. No. 9118).
2. WNV cell lysate antigen.
3. Coating buffer (Carbonate-bicarbonate buffer, pH 9.6 containing 50 mM sodium carbonate, 50 mM sodium bicarbonate).
4. Washing buffer (PBS + 0.1 % Tween 20).
5. Blocking buffer (Washing buffer + 5% non-fat dry milk).
6. MAb 3.1112G (Chemicon, Cat. No. MAB8152)
7. Horse radish peroxidase labeled MAb 6B6C-1 (CDC)
8. Horse radish peroxidase-labeled rabbit anti-mouse IgG (Zymed Laboratories, Cat. No. 61-6520).
9. ABTS (KPL Laboratories, Cat. No. 50-62-00),

### 6.1.2 Procedure

1. Coat the internal 60 wells of an ELISA plate (96-well plate) with 100 μL of diluted WNV antigen. Add 100 μL of coating buffer to the external wells. Incubate overnight at 4°C.
2. Wash plate 5 times with washing buffer.

3. Add 200 µL of blocking buffer to each well. Incubate the plate at 37°C for 40 minutes.
4. Wash the plate 5 times with washing buffer.
5. Add 50 µL of diluted serum to each well. The serum test, positive, and negative serum controls are diluted 1:10 in blocking buffer. Incubate plate at 37°C for 2 hours.
6. Wash the plate 5 times.
7. Add 50 µL of diluted monoclonal antibody to each well. MAb is diluted in blocking buffer. Incubate at 37°C for 1 hour.
8. Wash the plate 5 times.
9. Add 50 µL of diluted conjugate to each well. Dilute the horse radish peroxidase-labeled rabbit anti-mouse IgG in blocking buffer. Incubate plate at 37°C for 1 hour. (If using HRP-labeled MAb 6B6C-1 in step 7, delete this step).
10. Wash the plate 5 times.
11. Add 75 µL of a mix of the developing solution of ABTS. Incubate at 37°C and measure the optical density (O.D.) at 415 nm at regular intervals until the average O.D. for the negative control serum exceeds 0.3.
12. Calculate the % inhibition value of the test serum using the following formula: 1-[(mean optical density of the test serum – mean optical dentity of the background)/(mean optical density of the control serum – mean optical density of the background)] x 100.

## APPENDIX 6.2

## PROTOCOL TO PREPARE VIRAL ANTIGENS FOR ELISA

### 6.2.1 Materials

1. *Aedes albopictus* cell line, clone C6/36
2. WNV strain NY99-35261-11
3. DMEM containing 10% FBS, antibiotics and anti-mycotics for cell culture
4. DMEM containing 2% FBS, antibiotics and anti-mycotics for virus propagation
5. Borate saline buffer, pH 9.0 (1.5 M sodium chloride, 0.5 M boric acid, 1.0 M sodium hydroxide).
6. 1% Sodium Dodecyl Sulfate
7. Triton X-100

### 6.2.2 Procedure

1. For the preparation of monolayer of C6/36 cell cultures for propagation of WNV, seed 2 x $10^6$ cells into 150 $cm^2$ flask containing 25 ml of DMEM (10% FBS, antibiotics and anti-mycotics).
2. Incubate at 28°C for 3 days and infect cells, when there is at least 90% confluency.

3. Prepare a virus inoculum for each flask of a final MOI of 0.1 PFU/cell with DMEM (2% FBS). Incubate the flasks at 28°C for 1 hour, and rock flasks occasionally to avoid drying the monolayer of cells.

4. After viral adsorption add 20 ml. of DMEM (2% FBS).

5. When most of cells are detached (usually 5 days after infection), transfer the cell suspension to a 50 ml centrifuge tube.

6. Centrifuge 10 minutes at 4,000 rpm, 4°C. Decant the supernatant and wash the cell pellet four times with cold borate saline buffer (1.5 M sodium chloride, 0.5 M boric acid, 1.0 M sodium hydroxide, pH 9.0). Centrifuge at 4,000 rpm for 20 minutes at 4°C.

7. Resuspend the cell pellet in 2 ml. cold borate saline buffer and add 0.9 ml of 1% SDS and 0.1 ml. of Triton X-100. Resuspend the pellet by pippetting up and down.

8. Sonicate on ice (20% output for 30 seconds).

9. Clarify by centrifuging at 8,000 rpm for 10 minutes at 4°C and store the suspension at -70°C until ready to use.

# APPENDIX 6.3

# MICROSPHERE IMMUNOFLUORESCENCE ASSAY FOR DETECTION OF ANTIBODIES TO WNV AND DENGUE VIRUS IN HUMANS

## 6.3.1. Tables.

**Table 6.3.1. Cross reactivity in serum samples (1A-22A) with recombinant proteins of WNV and DENV bya MIA.**

| | | | | Cutoff = 1246 | | | Cutoff = 2464 | | | Cutoff = 1340 | | | Cutoff = 679 | | | Cutoff = 717 | | | Cutoff = 764 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MAC ELISA | | WN E | | | WN IIS5 | | | WN IIS3 | | | DEN1 IIS5 | | | DEN2 IIS5 | | | WN IIS1 | | |
| DI # | ID # | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result |
| 1 | 1A | 0.390 | NR | 112.0 | 1.78 | NR | 93.5 | 1.24 | NR | 116.0 | 0.44 | NR | 301.0 | 1.85 | NR | 307.5 | 1.56 | NR | 226.0 | 1.37 | NR |
| 2 | 2A | 0.702 | NR | 1271.0 | 20.17 | R | 53.0 | 0.70 | NR | 273.5 | 1.03 | NR | 230.5 | 1.41 | NR | 233.0 | 1.18 | NR | 183.5 | 1.11 | NR |
| 3 | 3A | 0.392 | NR | 487.5 | 7.74 | IND | 26.0 | 0.34 | NR | 155.5 | 0.58 | NR | 226.5 | 1.40 | NR | 214.5 | 1.09 | NR | 181.0 | 1.10 | NR |
| 4 | 4A | 0.543 | NR | 1220.0 | 19.37 | IND | 143.0 | 1.89 | NR | 121.0 | 0.45 | NR | 103.0 | 0.63 | NR | 120.0 | 0.61 | NR | 320.0 | 1.94 | NR |
| 5 | 4B | 0.588 | NR | 986.5 | 15.66 | IND | 96.0 | 1.27 | NR | 77.0 | 0.29 | NR | 93.5 | 0.57 | NR | 139.0 | 0.71 | NR | 243.0 | 1.47 | NR |
| 6 | 5A | 0.434 | NR | 28.0 | 0.44 | NR | 66.0 | 0.87 | NR | 118.5 | 0.45 | NR | 113.0 | 0.69 | NR | 120.0 | 0.61 | NR | 107.0 | 0.65 | NR |
| 7 | 5B | 0.445 | NR | 13.0 | 0.21 | NR | 48.0 | 0.64 | NR | 63.0 | 0.24 | NR | 65.0 | 0.40 | NR | 102.5 | 0.52 | NR | 65.0 | 0.39 | NR |
| 8 | 6A | 0.464 | NR | 2293.0 | 36.40 | R | 54.0 | 0.72 | NR | 189.0 | 0.71 | NR | 70.0 | 0.43 | NR | 86.5 | 0.44 | NR | 167.0 | 1.01 | NR |
| 9 | 6B | 0.529 | NR | 1738.0 | 27.59 | R | 30.0 | 0.40 | NR | 88.0 | 0.33 | NR | 69.0 | 0.42 | NR | 129.5 | 0.66 | NR | 97.5 | 0.59 | NR |
| 10 | 7A | 0.319 | NR | 5.0 | 0.08 | NR | 10.0 | 0.13 | NR | 58.0 | 0.25 | NR | 24.5 | 0.15 | NR | 28.0 | 0.15 | NR | 21.0 | 0.13 | NR |
| 11 | 8A | 0.425 | NR | 19.0 | 0.30 | NR | 96.5 | 1.28 | NR | 291.5 | 1.10 | NR | 91.0 | 0.56 | NR | 95.0 | 0.48 | NR | 147.5 | 0.89 | NR |
| 12 | 8B | 0.433 | NR | 38.0 | 0.60 | NR | 145.0 | 1.92 | NR | 424.0 | 1.59 | NR | 150.0 | 0.92 | NR | 124.0 | 0.63 | NR | 253.0 | 1.53 | NR |
| 13 | 9A | 0.254 | NR | 967.5 | 15.36 | IND | 115.0 | 1.52 | NR | 225.5 | 0.85 | NR | 444.5 | 2.73 | NR | 497.5 | 2.53 | NR | 1360.0 | 8.24 | R |
| 14 | 10A | 0.523 | NR | 323.0 | 5.13 | IND | 64.0 | 0.85 | NR | 122.0 | 0.46 | NR | 250.5 | 1.54 | NR | 374.0 | 1.90 | NR | 292.0 | 1.77 | NR |
| 15 | 11A | 0.347 | NR | 147.0 | 2.33 | NR | 37.0 | 0.49 | NR | 214.0 | 0.80 | NR | 196.5 | 1.21 | NR | 133.0 | 0.68 | NR | 85.0 | 0.52 | NR |
| 16 | 11B | 0.293 | NR | 162.5 | 2.58 | NR | 55.0 | 0.73 | NR | 189.0 | 0.71 | NR | 266.0 | 1.63 | NR | 215.0 | 1.09 | NR | 105.0 | 0.64 | NR |
| 17 | 12A | 1.564 | NR | 789.0 | 12.52 | IND | 329.0 | 4.36 | IND | 316.0 | 1.19 | NR | 535.5 | 3.29 | NR | 550.0 | 2.79 | NR | 1024.0 | 6.21 | R |
| 18 | 13A | 0.602 | NR | 86.0 | 0.89 | NR | 196.0 | 2.60 | NR | 150.0 | 0.56 | NR | 186.0 | 1.14 | NR | 176.0 | 0.89 | NR | 176.0 | 1.07 | NR |
| 19 | 14A | 0.706 | NR | 2421.0 | 38.43 | R | 47.0 | 0.62 | NR | 313.0 | 1.18 | NR | 98.0 | 0.60 | NR | 109.0 | 0.55 | NR | 2237.0 | 13.56 | R |
| 20 | 15A | 0.556 | NR | 2251.0 | 35.73 | R | 584.0 | 7.74 | IND | 417.5 | 1.57 | NR | 1205.0 | 7.39 | R | 1221.5 | 6.20 | R | 1003.0 | 6.08 | R |
| 21 | 15B | 0.791 | NR | 738.5 | 11.72 | IND | 66.0 | 0.87 | NR | 165.0 | 0.62 | NR | 338.5 | 2.06 | NR | 291.0 | 1.48 | NR | 567.5 | 3.44 | NR |
| 22 | 16A | 0.625 | NR | 2994.0 | 47.52 | R | 903.5 | 11.97 | IND | 576.5 | 2.17 | NR | 1875.5 | 11.51 | R | 1775.0 | 9.01 | R | 1594.0 | 9.66 | R |
| 23 | 17A | 0.466 | NR | 721.5 | 11.45 | IND | 646.0 | 8.56 | IND | 408.5 | 1.54 | NR | 1231.0 | 7.55 | R | 1210.0 | 6.14 | R | 1088.0 | 6.59 | R |
| 24 | 18A | 0.423 | NR | 641.5 | 10.18 | IND | 617.0 | 8.17 | IND | 388.0 | 1.46 | NR | 1310.0 | 8.04 | R | 1323.0 | 6.72 | R | 993.5 | 6.02 | R |
| 25 | 19A | 0.603 | NR | 1748.0 | 27.75 | R | 125.5 | 1.66 | NR | 109.5 | 0.41 | NR | 98.0 | 0.60 | NR | 155.0 | 0.79 | NR | 674.5 | 4.09 | IND |
| 26 | 20A | 2.671 | NR | 511.0 | 8.11 | IND | 114.0 | 1.51 | NR | 395.5 | 1.49 | NR | 214.0 | 1.31 | NR | 204.0 | 1.04 | NR | 730.0 | 4.42 | IND |
| 27 | 21A | 0.398 | NR | 429.0 | 6.81 | IND | 96.0 | 1.27 | NR | 259.0 | 0.97 | NR | 116.0 | 0.71 | NR | 138.5 | 0.70 | NR | 1555.0 | 9.42 | R |
| 28 | 22A | 0.445 | NR | 401.0 | 6.37 | IND | 89.0 | 1.18 | NR | 68.0 | 0.26 | NR | 100.0 | 0.61 | NR | 125.5 | 0.64 | NR | 879.0 | 5.33 | R |

Abreviations: MFI = microsphere fluorescence intensity, NR = non reactive, IND = indeterminate, R = reactive and EQ = equivocal.

**Table 6.3.1. Cross reactivity in serum samples (23A-42A) with recombinant proteins of WNV and DENV bya MIA.**

| | | | | Cutoff = 1246 | | | Cutoff = 2464 | | | Cutoff = 1340 | | | Cutoff = 679 | | | Cutoff = 717 | | | Cutoff = 764 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MAC ELISA | | WNE | | | WNIIS5 | | | WNIIS3 | | | DEN1IIS5 | | | DEN2IIS5 | | | WNIIS1 | | |
| DI # | ID # | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result |
| 29 | 23A | 0.967 | NR | 2999.0 | 47.60 | R | 47.0 | 0.62 | NR | 126.5 | 0.48 | NR | 75.0 | 0.46 | NR | 87.0 | 0.44 | NR | 3127.5 | 18.95 | R |
| 30 | 24A | 1.399 | NR | 943.0 | 14.97 | IND | 107.0 | 1.42 | NR | 150.0 | 0.56 | NR | 113.5 | 0.70 | NR | 99.0 | 0.50 | NR | 308.0 | 1.87 | NR |
| 31 | 25A | 0.908 | NR | 941.0 | 14.94 | IND | 65.0 | 0.86 | NR | 246.5 | 0.93 | NR | 135.5 | 0.83 | NR | 210.0 | 1.07 | NR | 623.0 | 3.78 | NR |
| 32 | 26A | 0.774 | NR | 2780.0 | 44.13 | R | 173.5 | 2.30 | NR | 138.5 | 0.52 | NR | 60.0 | 0.37 | NR | 81.0 | 0.41 | NR | 1379.0 | 8.36 | R |
| 33 | 27A | 0.834 | NR | 2461.5 | 39.07 | R | 160.0 | 2.12 | NR | 184.5 | 0.69 | NR | 107.0 | 0.66 | NR | 175.5 | 0.89 | NR | 583.0 | 3.53 | NR |
| 34 | 28A | 0.808 | NR | 778.0 | 12.35 | IND | 75.0 | 0.99 | NR | 163.5 | 0.61 | NR | 123.5 | 0.76 | NR | 158.0 | 0.80 | NR | 209.0 | 1.27 | NR |
| 35 | 29A | 0.695 | NR | 116.5 | 1.85 | NR | 150.0 | 1.99 | NR | 404.0 | 1.52 | NR | 280.0 | 1.72 | NR | 284.5 | 1.44 | NR | 666.5 | 4.04 | IND |
| 36 | 30A | 0.352 | NR | 495.0 | 7.86 | IND | 201.0 | 2.66 | NR | 303.0 | 1.14 | NR | 155.0 | 0.95 | NR | 149.0 | 0.76 | NR | 338.0 | 2.05 | NR |
| 37 | 31A | 0.492 | NR | 156.0 | 2.48 | NR | 87.5 | 1.16 | NR | 138.0 | 0.52 | NR | 94.0 | 0.58 | NR | 120.5 | 0.61 | NR | 613.5 | 3.72 | NR |
| 38 | 32A | 0.990 | NR | 986.0 | 15.65 | IND | 95.5 | 1.26 | NR | 242.5 | 0.91 | NR | 122.5 | 0.75 | NR | 243.5 | 1.24 | NR | 634.5 | 3.85 | NR |
| 39 | 33A | 1.326 | NR | 3324.5 | 52.77 | R | 65.5 | 0.87 | NR | 178.5 | 0.67 | NR | 97.0 | 0.60 | NR | 149.5 | 0.76 | NR | 4097.0 | 24.83 | R |
| 40 | 33B | 1.177 | NR | 3509.5 | 55.71 | R | 79.0 | 1.05 | NR | 236.0 | 0.89 | NR | 89.0 | 0.55 | NR | 114.5 | 0.58 | NR | 4340.0 | 26.30 | R |
| 41 | 34A | 0.539 | NR | 1639.0 | 26.02 | R | 110.0 | 1.46 | NR | 357.0 | 1.34 | NR | 198.5 | 1.22 | NR | 237.5 | 1.21 | NR | 685.0 | 4.15 | IND |
| 42 | 34B | 0.685 | NR | 1865.0 | 29.60 | R | 143.0 | 1.89 | NR | 414.5 | 1.56 | NR | 234.0 | 1.44 | NR | 264.0 | 1.34 | NR | 844.0 | 5.12 | R |
| 43 | 35A | 0.345 | NR | 845.0 | 13.41 | IND | 243.0 | 3.22 | NR | 230.5 | 0.87 | NR | 112.0 | 0.69 | NR | 153.0 | 0.78 | NR | 181.5 | 1.10 | NR |
| 44 | 35B | 0.376 | NR | 757.0 | 12.02 | IND | 215.5 | 2.85 | NR | 226.0 | 0.85 | NR | 71.0 | 0.44 | NR | 93.0 | 0.47 | NR | 164.0 | 0.99 | NR |
| 45 | 36A | 0.422 | NR | 367.0 | 5.83 | IND | 89.0 | 1.18 | NR | 209.5 | 0.79 | NR | 91.5 | 0.56 | NR | 116.0 | 0.59 | NR | 1383.0 | 8.38 | R |
| 46 | 36B | 0.418 | NR | 275.0 | 4.37 | IND | 66.0 | 0.87 | NR | 188.0 | 0.71 | NR | 96.0 | 0.59 | NR | 122.0 | 0.62 | NR | 1031.5 | 6.25 | R |
| 47 | 37A | 0.554 | NR | 536.0 | 8.51 | IND | 94.0 | 1.25 | NR | 182.5 | 0.69 | NR | 265.0 | 1.63 | NR | 220.0 | 1.12 | NR | 139.0 | 0.84 | NR |
| 48 | 37B | 0.694 | NR | 1270.0 | 20.16 | R | 173.0 | 2.29 | NR | 280.0 | 1.05 | NR | 385.0 | 2.36 | NR | 189 | 0.96 | NR | 214.0 | 1.30 | NR |
| 49 | 38A | 0.448 | NR | 146.0 | 2.32 | NR | 108.0 | 1.43 | NR | 144.0 | 0.54 | NR | 257.5 | 1.58 | NR | 253.5 | 1.29 | NR | 219.5 | 1.33 | NR |
| 50 | 38B | 0.515 | NR | 126.0 | 2.00 | NR | 135.5 | 1.79 | NR | 138.5 | 0.52 | NR | 242.5 | 1.49 | NR | 251.0 | 1.27 | NR | 233.0 | 1.41 | NR |
| 51 | 39A | 2.059 | NR | 766.0 | 12.48 | IND | 240.0 | 3.18 | NR | 201.5 | 0.76 | NR | 265.5 | 1.63 | NR | 348.0 | 1.77 | NR | 1098.5 | 6.66 | R |
| 52 | 40A | 0.590 | NR | 879.0 | 13.95 | IND | 48.0 | 0.64 | NR | 98.5 | 0.37 | NR | 70.0 | 0.43 | NR | 71.5 | 0.36 | NR | 580.0 | 3.52 | NR |
| 53 | 40B | 0.619 | NR | 878.0 | 13.94 | IND | 45.0 | 0.60 | NR | 94.5 | 0.36 | NR | 88.0 | 0.54 | NR | 111.0 | 0.56 | NR | 561.0 | 3.40 | NR |
| 54 | 41A | 0.437 | NR | 286.0 | 4.54 | IND | 32.0 | 0.42 | NR | 126.5 | 0.48 | NR | 48.5 | 0.30 | NR | 74.5 | 0.38 | NR | 26.5 | 0.16 | NR |
| 55 | 41B | 0.653 | NR | 1225.0 | 19.44 | IND | 91.0 | 1.21 | NR | 399.0 | 1.50 | NR | 180.0 | 1.10 | NR | 192.5 | 0.98 | NR | 145.5 | 0.88 | NR |
| 56 | 42A | 0.429 | NR | 529.0 | 8.40 | IND | 30.0 | 0.40 | NR | 133.5 | 0.50 | NR | 122.0 | 0.75 | NR | 139.5 | 0.71 | NR | 211.0 | 1.28 | NR |

Abreviations: MFI = microsphere fluorescence intensity, NR = non reactive, IND = indeterminate, R = reactive and EQ = equivocal.

**Table 6.3.1. Cross reactivity in serum samples (43A-63A) with recombinant proteins of WNV and DENV bya MIA.**

| | | | | Cutoff = 1246 | | | Cutoff = 2464 | | | Cutoff = 1340 | | | Cutoff = 679 | | | Cutoff = 717 | | | Cutoff = 764 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MAC ELISA | | WN E | | | WN NS5 | | | WN NS3 | | | DEN1 NS5 | | | DEN2 NS5 | | | WN NS1 | | |
| DI # | ID # | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result |
| 57 | 43A | 0.380 | NR | 339.0 | 5.36 | IND | 25.0 | 0.33 | NR | 128.0 | 0.48 | NR | 102.0 | 0.63 | NR | 182.0 | 0.92 | NR | 120.0 | 0.73 | NR |
| 58 | 44A | 1.938 | NR | 997.5 | 15.83 | IND | 48.0 | 0.64 | NR | 43.5 | 0.16 | NR | 46.0 | 0.28 | NR | 67.0 | 0.34 | NR | 63.0 | 0.38 | NR |
| 59 | 44B | 2.111 | NR | 1980.0 | 31.43 | R | 114.5 | 1.52 | NR | 134.5 | 0.51 | NR | 111.0 | 0.68 | NR | 186.5 | 0.95 | NR | 205.0 | 1.24 | NR |
| 60 | 45A | 0.414 | NR | 71.0 | 1.13 | NR | 105.0 | 1.39 | NR | 158.0 | 0.59 | NR | 158.5 | 0.98 | NR | 193.5 | 0.98 | NR | 135.0 | 0.82 | NR |
| 61 | 45B | 0.482 | NR | 15.0 | 0.24 | NR | 40.0 | 0.53 | NR | 82.0 | 0.31 | NR | 41.0 | 0.25 | NR | 52.0 | 0.26 | NR | 31.0 | 0.19 | NR |
| 62 | 46A | 1.570 | NR | 87.0 | 1.38 | NR | 89.5 | 1.19 | NR | 147.5 | 0.55 | NR | 68.0 | 0.42 | NR | 161.5 | 0.82 | NR | 180.0 | 1.09 | NR |
| 63 | 46B | 1.083 | NR | 153.0 | 2.43 | NR | 135.5 | 1.79 | NR | 269.5 | 1.01 | NR | 112.0 | 0.69 | NR | 242.0 | 1.23 | NR | 334.0 | 2.02 | NR |
| 64 | 47A | 0.590 | NR | 215.0 | 3.41 | NR | 205.5 | 2.72 | NR | 349.5 | 1.31 | NR | 339.0 | 2.08 | NR | 395.0 | 2.01 | NR | 272.5 | 1.65 | NR |
| 65 | 47B | 0.266 | NR | 91.0 | 1.44 | NR | 140.0 | 1.85 | NR | 268.0 | 1.01 | NR | 132.5 | 0.81 | NR | 152.5 | 0.77 | NR | 89.0 | 0.54 | NR |
| 66 | 48A | 0.423 | NR | 1538.0 | 24.41 | R | 89.0 | 1.18 | NR | 260.5 | 0.98 | NR | 325.5 | 2.00 | NR | 405.0 | 2.06 | NR | 796.0 | 4.82 | R |
| 67 | 49A | 0.454 | NR | 455.0 | 7.22 | IND | 91.0 | 1.21 | NR | 244.0 | 0.92 | NR | 85.5 | 0.52 | NR | 98.0 | 0.50 | NR | 125.0 | 0.76 | NR |
| 68 | 50A | 0.561 | NR | 947.0 | 15.03 | IND | 141.0 | 1.87 | NR | 503.0 | 1.89 | NR | 341.0 | 2.09 | NR | 381.5 | 1.94 | NR | 678.0 | 4.11 | IND |
| 69 | 51A | 0.398 | NR | 799.0 | 12.68 | IND | 81.0 | 1.07 | NR | 339.0 | 1.27 | NR | 242.5 | 1.49 | NR | 363.5 | 1.85 | NR | 371.0 | 2.25 | NR |
| 70 | 52A | 0.918 | NR | 2506.0 | 39.78 | R | 199.0 | 2.64 | NR | 128.0 | 0.48 | NR | 274.0 | 1.68 | NR | 334.5 | 1.70 | NR | 2374.0 | 14.39 | R |
| 71 | 53A | 0.648 | NR | 1771.0 | 28.11 | R | 114.0 | 1.51 | NR | 360.0 | 1.35 | NR | 74.0 | 0.45 | NR | 136.0 | 0.69 | NR | 711.5 | 4.31 | IND |
| 72 | 54A | 0.447 | NR | 68.0 | 1.08 | NR | 19.0 | 0.25 | NR | 20.0 | 0.08 | NR | 20.0 | 0.12 | NR | 24.0 | 0.12 | NR | 285.5 | 1.73 | NR |
| 73 | 55A | 1.800 | NR | 57.0 | 0.90 | NR | 187.0 | 2.48 | NR | 54.0 | 0.20 | NR | 108.0 | 0.66 | NR | 155.0 | 0.79 | NR | 81.0 | 0.49 | NR |
| 74 | 55B | 2.025 | NR | 40.0 | 0.63 | NR | 336.0 | 4.45 | IND | 96.0 | 0.36 | NR | 81.0 | 0.50 | NR | 182.0 | 0.92 | NR | 57.5 | 0.35 | NR |
| 75 | 56A | 2.142 | NR | 196.0 | 3.11 | NR | 264.0 | 3.50 | NR | 378.0 | 1.42 | NR | 247.0 | 1.52 | NR | 311.0 | 1.58 | NR | 150.0 | 0.91 | NR |
| 76 | 57A | 0.665 | NR | 1294.0 | 20.54 | R | 96.0 | 1.27 | NR | 92.0 | 0.35 | NR | 324.0 | 1.99 | NR | 353.0 | 1.79 | NR | 2329.0 | 14.12 | R |
| 77 | 58A | 8.976 | R | 4137.5 | 65.67 | R | 58.5 | 0.77 | NR | 620.5 | 2.33 | NR | 227.0 | 1.39 | NR | 370.5 | 1.88 | NR | 1507.0 | 9.13 | R |
| 78 | 58B | 12.962 | R | 4686.5 | 74.39 | R | 88.0 | 1.17 | NR | 361.5 | 1.36 | NR | 141.5 | 0.87 | NR | 253.0 | 1.28 | NR | 4681.0 | 28.37 | R |
| 79 | 59A | 0.456 | NR | 10.0 | 0.16 | NR | 33.0 | 0.44 | NR | 131.5 | 0.49 | NR | 69.0 | 0.42 | NR | 107.5 | 0.55 | NR | 89.0 | 0.54 | NR |
| 80 | 59B | 6.180 | EQ | 320.0 | 5.08 | IND | 24.0 | 0.32 | NR | 68.5 | 0.26 | NR | 48.5 | 0.30 | NR | 65.5 | 0.33 | NR | 52.0 | 0.32 | NR |
| 81 | 60A | 0.754 | NR | 1226.5 | 19.47 | IND | 62.0 | 0.82 | NR | 122.5 | 0.46 | NR | 125.0 | 0.77 | NR | 175.0 | 0.89 | NR | 471.5 | 2.86 | NR |
| 82 | 61A | 1.005 | NR | 2147.0 | 34.08 | R | 605.0 | 8.01 | IND | 495.5 | 1.86 | NR | 938.5 | 5.76 | R | 1073.0 | 5.45 | R | 2282.0 | 13.83 | R |
| 83 | 62A | 0.229 | NR | 164.0 | 2.78 | NR | 73.0 | 0.95 | NR | 99.5 | 0.43 | NR | 65.0 | 0.41 | NR | 74.5 | 0.40 | NR | 140.0 | 0.79 | NR |
| 84 | 63A | 0.908 | NR | 635.5 | 10.09 | IND | 397.0 | 5.26 | IND | 198.5 | 0.75 | NR | 730.5 | 4.48 | R | 740.0 | 3.76 | IND | 724.0 | 4.39 | IND |

Abreviations: MFI = microsphere fluorescence intensity, NR = non reactive, IND = indeterminate, R = reactive and EQ = equivocal.

**Table 6.3.1. Cross reactivity in serum samples (64A-82B) with recombinant proteins of WNV and DENV bya MIA.**

| | | MAC ELISA | | WII E | | | WII NS5 | | | WII NS3 | | | DEN NS5 | | | DEN2 NS5 | | | WII NS1 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DI # | ID # | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result |
| 85 | 64A | 0.382 | NR | 107.0 | 1.70 | NR | 44.0 | 0.58 | NR | 81.5 | 0.31 | NR | 117.0 | 0.72 | NR | 140.0 | 0.71 | NR | 87.0 | 0.53 | NR |
| 86 | 64B | 0.317 | NR | 170.5 | 2.71 | NR | 78.0 | 1.03 | NR | 93.5 | 0.35 | NR | 205.0 | 1.26 | NR | 229.0 | 1.16 | NR | 154.0 | 0.93 | NR |
| 87 | 65A | 0.220 | NR | 4.0 | 0.07 | NR | 11.0 | 0.14 | NR | 62.5 | 0.27 | NR | 41.0 | 0.26 | NR | 45.5 | 0.24 | NR | 38.0 | 0.21 | NR |
| 88 | 66A | 0.311 | NR | 237.0 | 3.76 | NR | 29.5 | 0.39 | NR | 70.5 | 0.27 | NR | 76.5 | 0.47 | NR | 94.5 | 0.48 | NR | 137.0 | 0.83 | NR |
| 89 | 67A | 0.318 | NR | 26.5 | 0.42 | NR | 93.5 | 1.24 | NR | 124.5 | 0.47 | NR | 110.0 | 0.67 | NR | 111.5 | 0.57 | NR | 72.0 | 0.44 | NR |
| 90 | 68A | 1.029 | NR | 1996.0 | 31.68 | R | 186.0 | 2.46 | NR | 106.5 | 0.40 | NR | 397.0 | 2.44 | NR | 519.0 | 2.63 | NR | 612.0 | 3.71 | NR |
| 91 | 69A | 0.258 | NR | 14.0 | 0.22 | NR | 95.0 | 1.26 | NR | 79.0 | 0.30 | NR | 76.5 | 0.47 | NR | 92.0 | 0.47 | NR | 214.0 | 1.30 | NR |
| 92 | 70A | 0.620 | NR | 1822.0 | 28.92 | R | 73.0 | 0.97 | NR | 109.0 | 0.41 | NR | 75.0 | 0.46 | NR | 110.0 | 0.56 | NR | 3165.0 | 19.18 | R |
| 93 | 70B | 0.402 | NR | 1032.0 | 16.38 | IND | 55.0 | 0.73 | NR | 48.0 | 0.18 | NR | 47.0 | 0.29 | NR | 91.5 | 0.46 | NR | 1775.5 | 10.76 | R |
| 94 | 71A | 0.469 | NR | 753.0 | 11.95 | IND | 54.0 | 0.72 | NR | 57.0 | 0.21 | NR | 38.5 | 0.24 | NR | 51.0 | 0.26 | NR | 1571.0 | 9.52 | R |
| 95 | 71B | 0.971 | NR | 2013.0 | 34.41 | R | 127.0 | 2.02 | NR | 182.5 | 0.89 | NR | 62.0 | 0.41 | NR | 93.0 | 0.08 | NR | 3904.0 | 21.33 | R |
| 96 | 72A | 0.235 | NR | 37.0 | 0.63 | NR | 41.0 | 0.65 | NR | 138.0 | 0.68 | NR | 168.5 | 1.12 | NR | 225.5 | 0.19 | NR | 117.0 | 0.64 | NR |
| 97 | 72B | 0.289 | NR | 58.0 | 0.99 | NR | 70.0 | 1.11 | NR | 204.5 | 1.00 | NR | 215.0 | 1.42 | NR | 331.0 | 0.28 | NR | 135.0 | 0.74 | NR |
| 98 | 73A | 0.307 | NR | 48.0 | 0.82 | NR | 27.0 | 0.43 | NR | 59.0 | 0.29 | NR | 49.0 | 0.32 | NR | 69.0 | 0.06 | NR | 42.0 | 0.23 | NR |
| 99 | 73B | 0.307 | NR | 55.0 | 0.94 | NR | 29.0 | 0.46 | NR | 34.0 | 0.17 | NR | 48.0 | 0.32 | NR | 55.0 | 0.05 | NR | 39.5 | 0.22 | NR |
| 100 | 74A | 0.616 | NR | 592.0 | 10.12 | IND | 51.0 | 0.81 | NR | 141.0 | 0.69 | NR | 86.5 | 0.57 | NR | 169.0 | 0.14 | NR | 394.5 | 2.16 | NR |
| 101 | 75A | 0.325 | NR | 202.5 | 3.46 | NR | 59.5 | 0.94 | NR | 230.5 | 1.13 | NR | 92.0 | 0.61 | NR | 101.0 | 0.09 | NR | 119.5 | 0.65 | NR |
| 102 | 76A | 0.263 | NR | 25.5 | 0.44 | NR | 103.0 | 1.63 | NR | 196.5 | 0.96 | NR | 73.0 | 0.48 | NR | 91.5 | 0.08 | NR | 71.0 | 0.39 | NR |
| 103 | 76B | 0.285 | NR | 14.0 | 0.24 | NR | 148.0 | 2.35 | NR | 123.5 | 0.61 | NR | 37.0 | 0.25 | NR | 45.0 | 0.04 | NR | 45.0 | 0.25 | NR |
| 104 | 77A | 0.575 | NR | 195.5 | 3.34 | NR | 76.0 | 1.21 | NR | 190.5 | 0.93 | NR | 168.0 | 1.11 | NR | 250.5 | 0.21 | NR | 543.5 | 2.97 | NR |
| 105 | 78A | 1.316 | NR | 1941.0 | 33.18 | R | 112.0 | 1.78 | NR | 106.5 | 0.52 | NR | 214.5 | 1.42 | NR | 264.5 | 0.23 | NR | 3216.5 | 17.58 | R |
| 106 | 79A | 0.713 | NR | 1378.0 | 23.56 | R | 39.0 | 0.62 | NR | 108.5 | 0.53 | NR | 86.5 | 0.57 | NR | 110.0 | 0.65 | NR | 770.0 | 4.21 | R |
| 107 | 79B | 0.684 | NR | 1350.0 | 23.08 | R | 39.0 | 0.62 | NR | 107.0 | 0.52 | NR | 102.0 | 0.68 | NR | 132.0 | 0.78 | NR | 788.0 | 4.31 | R |
| 108 | 80A | 0.674 | NR | 27.0 | 0.46 | NR | 75.0 | 1.19 | NR | 119.5 | 0.59 | NR | 84.5 | 0.56 | NR | 133.0 | 0.78 | NR | 47.5 | 0.26 | NR |
| 109 | 80B | 0.611 | NR | 44.0 | 0.75 | NR | 83.0 | 1.32 | NR | 239.5 | 1.17 | NR | 91.0 | 0.60 | NR | 175.0 | 1.03 | NR | 77.0 | 0.42 | NR |
| 110 | 81A | 0.572 | NR | 15.0 | 0.26 | NR | 129.0 | 2.05 | NR | 206.5 | 1.01 | NR | 77.0 | 0.51 | NR | 109.0 | 0.64 | NR | 63.0 | 0.34 | NR |
| 111 | 82A | 0.273 | NR | 46.0 | 0.79 | NR | 123.0 | 1.95 | NR | 160.5 | 0.79 | NR | 137.5 | 0.91 | NR | 184.0 | 1.08 | NR | 103.5 | 0.57 | NR |
| 112 | 82B | 0.291 | NR | 31.0 | 0.53 | NR | 91.0 | 1.44 | NR | 119.5 | 0.59 | NR | 92.0 | 0.61 | NR | 112.0 | 0.66 | NR | 147.0 | 0.80 | NR |

Abreviations: MFI = microsphere fluorescence intensity, NR = non reactive, IND = indeterminate, R = reactive and EQ = equivocal.

**Table 6.3.1. Cross reactivity in serum samples (83A-102B) with recombinant proteins of WNV and DENV bya MIA.**

| | | | | Cutoff = 1246 | | | Cutoff = 2464 | | | Cutoff = 1340 | | | Cutoff = 679 | | | Cutoff = 717 | | | Cutoff = 764 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MAC ELISA | | WN E | | | WN NS5 | | | WN NS3 | | | DEN1 NS5 | | | DEN2 NS5 | | | WN NS1 | | |
| DI # | ID # | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result | MFI | P/N | Result |
| 113 | 83A | 0.256 | NR | 1604.0 | 27.42 | R | 364.0 | 5.78 | IND | 958.0 | 4.70 | IND | 1321.5 | 8.75 | R | 1395.0 | 8.21 | R | 1424.0 | 7.78 | R |
| 114 | 84A | 0.326 | NR | 13.0 | 0.22 | NR | 135.5 | 2.15 | NR | 169.5 | 0.83 | NR | 80.5 | 0.53 | NR | 105.5 | 0.62 | NR | 62.0 | 0.34 | NR |
| 115 | 84B | 0.469 | NR | 9.0 | 0.15 | NR | 114.0 | 1.81 | NR | 186.5 | 0.92 | NR | 57.0 | 0.38 | NR | 94.5 | 0.56 | NR | 44.0 | 0.24 | NR |
| 116 | 85A | 1.004 | NR | 2403.0 | 41.08 | R | 760.5 | 12.07 | IND | 261.0 | 1.28 | NR | 925.0 | 6.13 | R | 906.5 | 5.33 | R | 2454.0 | 13.41 | R |
| 117 | 86A | 0.451 | NR | 214.0 | 3.66 | NR | 150.0 | 2.38 | NR | 122.0 | 0.60 | NR | 179.0 | 1.19 | NR | 220.0 | 1.29 | NR | 181.0 | 0.99 | NR |
| 118 | 87A | 0.725 | NR | 763.5 | 13.05 | IND | 379.0 | 6.02 | IND | 236.5 | 1.16 | NR | 558.0 | 3.70 | NR | 586.5 | 3.45 | NR | 606.0 | 3.31 | NR |
| 119 | 88A | 0.212 | NR | 20.0 | 0.34 | NR | 43.0 | 0.68 | NR | 79.0 | 0.39 | NR | 88.5 | 0.59 | NR | 115.5 | 0.68 | NR | 81.0 | 0.44 | NR |
| 120 | 89A | 0.551 | NR | 1239.0 | 21.18 | IND | 52.5 | 0.83 | NR | 263.0 | 1.29 | NR | 245.5 | 1.63 | NR | 271.5 | 1.60 | NR | 5120.0 | 27.98 | R |
| 121 | 90A | 0.153 | NR | 311.0 | 5.32 | IND | 287.0 | 4.56 | IND | 409.5 | 2.01 | NR | 483.0 | 3.20 | NR | 465.5 | 2.74 | NR | 481.0 | 2.63 | NR |
| 122 | 91A | 0.220 | NR | 12.0 | 0.20 | NR | 15.0 | 0.19 | NR | 55.5 | 0.24 | NR | 39.5 | 0.25 | NR | 39.0 | 0.21 | NR | 29.0 | 0.16 | NR |
| 123 | 92A | 0.664 | NR | 590.0 | 10.09 | IND | 203.0 | 3.22 | NR | 209.0 | 1.02 | NR | 538.5 | 3.57 | NR | 603.0 | 3.55 | NR | 566.0 | 3.09 | NR |
| 124 | 93A | 0.232 | NR | 1275.0 | 21.79 | R | 118.0 | 1.87 | NR | 133.5 | 0.65 | NR | 180.0 | 1.19 | NR | 224.0 | 1.32 | NR | 901.0 | 4.92 | R |
| 125 | 94A | 0.192 | NR | 20.0 | 0.34 | NR | 68.0 | 1.08 | NR | 109.0 | 0.53 | NR | 79.0 | 0.52 | NR | 83.0 | 0.49 | NR | 68.0 | 0.37 | NR |
| 126 | 95A | 1.166 | NR | 6.0 | 0.10 | NR | 40.0 | 0.63 | NR | 186.0 | 0.91 | NR | 48.0 | 0.32 | NR | 94.0 | 0.55 | NR | 385.0 | 2.10 | NR |
| 127 | 96A | 0.926 | NR | 1185.5 | 20.26 | IND | 130.5 | 2.07 | NR | 150.5 | 0.74 | NR | 147.0 | 0.97 | NR | 129.0 | 0.76 | NR | 1178.0 | 6.44 | R |
| 128 | 97A | 0.451 | NR | 128.0 | 2.19 | NR | 98.5 | 1.56 | NR | 66.0 | 0.32 | NR | 274.0 | 1.81 | NR | 286.5 | 1.69 | NR | 237.5 | 1.30 | NR |
| 129 | 97B | 0.378 | NR | 215.0 | 3.64 | NR | 127.0 | 1.65 | NR | 46.0 | 0.20 | NR | 469.5 | 2.93 | NR | 445.0 | 2.39 | NR | 339.0 | 1.92 | NR |
| 130 | 98A | 0.421 | NR | 301.0 | 5.15 | IND | 185.0 | 2.94 | NR | 282.0 | 1.38 | NR | 667.0 | 4.42 | IND | 646.5 | 3.80 | NR | 587.0 | 3.21 | NR |
| 131 | 98B | 0.255 | NR | 1074.0 | 18.36 | IND | 288.0 | 4.57 | IND | 127.5 | 0.63 | NR | 258.5 | 1.71 | NR | 154.0 | 0.91 | NR | 2637.0 | 14.41 | R |
| 132 | 99A | 0.324 | NR | 321.0 | 5.49 | IND | 161.0 | 2.56 | NR | 54.0 | 0.26 | NR | 93.5 | 0.62 | NR | 78.0 | 0.46 | NR | 703.0 | 3.84 | NR |
| 133 | 99B | 0.720 | NR | 389.5 | 6.66 | IND | 223.0 | 3.54 | NR | 224.5 | 1.10 | NR | 821.5 | 5.44 | R | 846.0 | 4.98 | R | 722.0 | 3.95 | NR |
| 134 | 100A | 0.303 | NR | 604.0 | 10.32 | IND | 142.0 | 2.25 | NR | 188.0 | 0.92 | NR | 319.5 | 2.12 | NR | 242.5 | 1.43 | NR | 457.0 | 2.50 | NR |
| 135 | 100B | 0.099 | NR | 48.0 | 0.82 | NR | 16.0 | 0.25 | NR | 41.5 | 0.20 | NR | 53.0 | 0.35 | NR | 47.0 | 0.28 | NR | 44.0 | 0.24 | NR |
| 136 | 101A | 0.381 | NR | 1842.0 | 31.49 | R | 254.5 | 4.04 | IND | 187.5 | 0.92 | NR | 404.5 | 2.68 | NR | 412.0 | 2.42 | NR | 474.0 | 2.59 | NR |
| 137 | 101B | 0.387 | NR | 1634.0 | 27.93 | R | 273.0 | 4.33 | IND | 158.0 | 0.77 | NR | 313.0 | 2.07 | NR | 352.0 | 2.07 | NR | 422.5 | 2.31 | NR |
| 138 | 102A | 3.408 | EQ | 2502.0 | 42.77 | R | 43.0 | 0.68 | NR | 181.0 | 0.89 | NR | 137.0 | 0.91 | NR | 173.0 | 1.02 | NR | 144.0 | 0.79 | NR |
| 139 | 102B | 9.111 | R | 5489.0 | 93.83 | R | 33.5 | 0.53 | NR | 163.0 | 0.80 | NR | 114.0 | 0.75 | NR | 144.0 | 0.85 | NR | 3917.0 | 21.40 | R |

**Abreviations: MFI = microsphere fluorescence intensity, NR = non reactive, IND = indeterminate, R = reactive and EQ = equivocal.**

### 6.3.2. Materials

1 A total of 139 serum samples from Yucatán, México from 102 dengue patients: 37 patients had paired acute and convalescent serum samples, and 65 patients had single convalescent samples.

2 Four recombinant proteins for WNV (E, NS1, NS3 and NS5).

3 One recombinant protein for DENV-1 (NS5).

4 One recombinant protein for DENV-2 (NS5).

5 Microsphere beads (Luminex Corporation, Austin, TX).

6 N-hydroxysuccinimide (Pierce, Rockford, IL).

7 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide-HCl (Pierce, Rockford, IL).

8 96-well plates.

9 PBN buffer (PBS with 1% bovine serum albumin and 0.05% sodium azide, pH 7.4).

10 PBS-Tween buffer (PBS with 0.05% Tween-20).

11 Polyvalent goat anti-human immunoglobulins (IgG, IgA and IgM) conjugated with red-phycoerythrin (a fluorescent conjugate).

### 6.3.3 Procedure

1. For coupling the recombinant proteins to the microspheres a two-step carbodiimide process was performed: First, activate the multinanalyte microspheres with 50 µL N-hydroxysuccinimide and 50 µL of carbodiimide-HCl. Incubate 20 minutes at room temperature.

2. Second, add 50 µg of purified recombinant protein to 6.25 x $10^6$ microspheres. Incubate 2 hours at room temperature in the dark on a rotator.

3. Wash twice the microspheres by centrifugation and resuspend in 1 ml of PBN buffer. Store the protein-conjugated microspheres at 4°C until use.
4. Block a 96-well filter plate for 2 minutes with PBN buffer.
5. Add serum samples diluted 1:100 in PBN and recombinant protein-conjugated beads. Incubate the plate for 30 minutes, using a shaker at 37°C.
6. Wash the wells three times with PBS-Tween.
7. Add polyvalent goat anti-human immunoglobulins (IgG, IgA and IgM) conjugated with red-phycoerythrin. Incubate at 37°C for 30 minutes.
8. Wash the wells three times using PBS-T.
9. Resuspend the microspheres and transfer them to 96-well plate.
10. Quantify the intensity of the fluorescence of 100 microsphers with Luminex 100 flow analyzer (Luminex Corporation, Austin, TX). The cut-off value for each assay was considered as the mean value obtained for 20 normal serum samples plus 3 standard deviations.

## APPENDIX 6.4

## CDC PROTOCOL FOR THE DETECTION OF IgM IN PATIENTS WITH FLAVIVIRUS INFECTIONS

### 6.4.1. Tables

**Table 6.4.1. Cross reactivity in serum samples (1-16) by b-ELISA, IgM- ELISA, HI and CF tests.**

| No. | Blocking-ELISA | | IgM-ELISA | | | Hemagglutination Inhibition | | | | Complement Fixation | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | WNV | Flavi | DEN 1,2,3,4 | SLE | WNV | DEN-1 | DEN-2 | SLE | WN | DEN-1 | DEN-2 | SLE | WNV |
| 1 | 42 | 91 | 5.8 | 9.1 | 7.5 | ≥1:2560 | ≥1:2560 | ≥1:2560 | 1:1280 | 1:640 | 1:320 | 1:640 | 1:320 |
| 2 | 14 | 87 | 2.0 | 2.8 | 1.4 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | - | - | - | - |
| 3 | 38 | 94 | 1.7 | 3.3 | 1.5 | 1:160 | 1:160 | 1:160 | 1:160 | 1:80 | 1:80 | 1:80 | 1:20 |
| 4 | -11 | 95 | 1.2 | 4.2 | 3.7 | 1:320 | 1:320 | 1:320 | 1:320 | 1:20 | 1:20 | 1:20 | 0 |
| 5 | 17 | 14 | 32.5 | 3.8 | 3.5 | 1:160 | 1:20 | 1:10 | 1:10 | 0 | 0 | 0 | 0 |
| 6 | 90 | 94 | 6.7 | 12.4 | 8.4 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | 1:640 | ≥1:1280 | ≥1:1280 | ≥1:1280 |
| 7 | 33 | 97 | 7.8 | 11.1 | 11.4 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:1280 | ≥1:1280 | ≥1:1280 | 1:640 |
| 8 | 17 | -7 | 9.5 | 2.0 | 1.5 | 1:40 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | 3 | 30 | 30.7 | 3.9 | 2.8 | 1:80 | 1:80 | 1:40 | 1:40 | 0 | 1:20 | 1:10 | 0 |
| 10 | 52 | 89 | 4.1 | 3.7 | 5.0 | ≥1:2560 | ≥1:2560 | ≥1:2560 | 1:1280 | ≥1:1280 | ≥1:1280 | ≥1:1280 | 1:640 |
| 11 | 73 | 97 | 29.2 | 4.1 | 3.4 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:1280 | ≥1:1280 | ≥1:1280 | ≥1:1280 |
| 12 | 61 | 100 | 8.0 | 12.9 | 9.2 | ≥1:2560 | ≥1:2560 | ≥1:2560 | 1:1280 | ≥1:1280 | ≥1:1280 | ≥1:1280 | ≥1:1280 |
| 13 | 82 | 93 | 8.8 | 12.5 | 6.8 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:1280 | ≥1:1280 | ≥1:1280 | ≥1:1280 |
| 14 | 16 | 79 | 36.2 | 5.5 | 3.0 | 1:160 | 1:20 | 1:20 | 1:20 | 0 | 0 | 0 | 0 |
| 15 | 54 | 94 | 29.6 | 2.1 | 1.8 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | 1:320 | 1:640 | 1:640 | 1:320 |
| 16 | 60 | 94 | 26.4 | 1.7 | 1.6 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | 1:640 | ≥1:1280 | ≥1:1280 | 1:640 |

**Table 6.4.1. Cross reactivity in serum samples (17-32) by b-ELISA, IgM-ELISA, HI and CF tests.**

| No. | Blocking-ELISA | | IgM-ELISA | | | Hemagglutination Inhibition | | | | Complement Fixation | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | WNV | Flavi | DEN 1,2,3,4 | SLE | WNV | DEN-1 | DEN-2 | SLE | WN | DEN-1 | DEN-2 | SLE | WNV |
| 17 | 75 | 95 | 5.0 | 8.5 | 3.9 | ≥1.2560 | ≥1.2560 | ≥1.2560 | ≥1.2560 | 1.640 | ≥1.1280 | ≥1.1280 | ≥1.1280 |
| 18 | 12 | 34 | 6.6 | 15.8 | 15.8 | 1:1280 | 1:1280 | 1:1280 | 1:1280 | 0 | 1:40 | 1:80 | 0 |
| 19 | 80 | 96 | 6.1 | 6.3 | 3.3 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:1280 | ≥1:1280 | ≥1:1280 | ≥1:1280 |
| 20 | 8 | 26 | 12.7 | 4.2 | 2.8 | 1:20 | 1:160 | 1:40 | 1:40 | 1:40 | 1:80 | 1:40 | 1:20 |
| 21 | 38 | 96 | 40.3 | 6.0 | 4.1 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:1280 | ≥1:1280 | ≥1:1280 | ≥1:1280 |
| 22 | 25 | 52 | 25.3 | 7.8 | 5.2 | 1:160 | 1:160 | 1:160 | 1:160 | 1:20 | 1:20 | 1:20 | 0 |
| 23 | 81 | 93 | 29.1 | 2.7 | 2.1 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:1280 | ≥1:1280 | ≥1:1280 | ≥1:1280 |
| 24 | 58 | 96 | 11.3 | 10.3 | 9.0 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:1280 | ≥1:1280 | ≥1:1280 | ≥1:1280 |
| 25 | 69 | 97 | 6.9 | 7.3 | 4.8 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:1280 | ≥1:1280 | ≥1:1280 | 1:640 |
| 26 | 50 | 94 | 7.8 | 7.3 | 5.4 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:1280 | ≥1:1280 | ≥1:1280 | 1:640 |
| 27 | 14 | 96 | 27.0 | 1.4 | 1.6 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | 1:320 | 1:640 | 1:640 | 1:160 |
| 28 | 28 | - | 7.9 | 4.2 | 1.6 | 1:20 | 1:20 | 1:10 | 1:10 | 0 | 0 | 0 | 0 |
| 29 | 21 | - | 7.6 | 10.8 | 5.9 | 1:320 | 1:320 | 1:320 | 1:320 | 0 | 1:10 | 1:10 | 0 |
| 30 | 36 | - | 4.2 | 4.5 | 1.6 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:1280 | ≥1:1280 | ≥1:1280 | 1:320 |
| 31 | 32 | - | 11.9 | 16 | 5.8 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:2560 | ≥1:1280 | ≥1:1280 | ≥1:1280 | 1:640 |
| 32 | 29 | - | 1.0 | 1.0 | 1.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Abreviations - = undetermined.

**Table 6.4.2. Additional data and final diagnosis of the 32 human serum samples fromYucatán, México.**

| No. | ID | SEX | AGE | DAYS AFTER ONSET | YEAR | IMMUNE RESPONSE | CLINIC DX | LAB DX |
|---|---|---|---|---|---|---|---|---|
| 1 | 9158 | M | 18 | 14 | 1997 | Secondary | DF | FLAVI |
| 2 | 9178 | M | 54 | 9 | 1997 | Secondary | DF | NEG |
| 3 | 15412 | F | 60 | 36 | 2003 | Primary | DFHM | FLAVI |
| 4 | 15421 | F | 48 | 19 | 2003 | Primary | DF | FLAVI |
| 5 | 15548 | M | 42 | 7 | 2004 | Primary | DF | DEN |
| 6 | 15581 | M | 43 | 11 | 2004 | Secondary | DF | FLAVI |
| 7 | 15604 | M | 16 | 9 | 2004 | Secondary | DF | FLAVI |
| 8 | 15729 | M | 17 | 5 | 2005 | Primary | DF | DEN-1 |
| 9 | 15738 | M | 17 | 14 | 2005 | Primary | DF | DEN-1 |
| 10 | 15739 | M | 49 | 13 | 2005 | Secondary | DF | FLAVI |
| 11 | 15768 | M | 11 | 14 | 2005 | Secondary | DHF | DEN |
| 12 | 15800 | F | 24 | 8 | 2005 | Secondary | DF | FLAVI |
| 13 | 15868 | F | 21 | 9 | 2005 | Secondary | DF | FLAVI |
| 14 | 15876 | F | 64 | 9 | 2005 | Primary | DF | DEN |
| 15 | 15877 | F | 34 | 7 | 2005 | Secondary | DF | DEN |
| 16 | 15899 | F | 34 | 11 | 2005 | Secondary | DF | DEN |
| 17 | 15902 | M | 21 | 14 | 2005 | Secondary | DF | FLAVI |
| 18 | 15903 | F | 8 | 13 | 2005 | Secondary | DF | FLAVI |
| 19 | 15908 | F | 21 | 8 | 2005 | Secondary | DF | FLAVI |
| 20 | 15912 | M | 13 | 16 | 2005 | Primary | DF | DEN |
| 21 | 15927 | M | 37 | 11 | 2005 | Secondary | DFHM | DEN |
| 22 | 15931 | F | 10 | 14 | 2005 | Primary | DFHM | DEN |
| 23 | 15940 | M | 37 | 8 | 2005 | Secondary | DF | DEN |
| 24 | 15945 | F | 26 | 8 | 2005 | Secondary | DFHM | FLAVI |
| 25 | 15946 | F | 9 | 8 | 2005 | Secondary | DF | FLAVI |
| 26 | 15957 | M | 17 | 10 | 2005 | Secondary | DF | FLAVI |
| 27 | 15967 | F | 31 | 5 | 2005 | Secondary | DFHM | DEN |
| 28 | 15994 | M | 9 | 23 | 2005 | Primary | DF | DEN |
| 29 | 15997 | M | 9 | 30 | 2005 | Primary | DF | FLAVI |
| 30 | 16002 | F | 36 | 11 | 2006 | Secondary | DHF | FLAVI |
| 31 | 16018 | M | 17 | 13 | 2006 | Secondary | DHF | FLAVI |
| 32 | 16033 | M | 2 | 13 | 2006 | Negative | DF | NEG |

Abreviations: M = male, F = female, CLINIC DX = clinical diagnosis, LAB DX = laboratory diagnosis, DF = dengue fever, DFHM = dengue fever with hemorrhagic manifestations, DHF = dengue hemorrhagic fever, FLAVI = flaviviruses, DEN-1 = virus serotype isolated or identified by RT-PCR, NEG = negative.

### 6.4.2. Materials.

1. Coating buffer: Carbonate/bicarbonate buffer pH 9.6, (0.915 M sodium carbonate, 0.035 M sodium bicarbonate).
2. Washing buffer: PBS + 0.05% Tween 20, pH 7.2.
3. Blocking buffer: Washing buffer + 5% dry skim milk.
4. Coating antibody: Goat anti-human IgM (KPL, Cat. 01-10-03).
5. Substrate: 3,3'5, 5' tetramethylbenzidine base (Enhanced K-blue TMB substrate, Neogen Corp. Cat. 308175).
6. Detecting antibody conjugate: Horseradish peroxidase labeled monoclonal antibody 6B6C-1.
7. Stoping solution: 1 N sulfuric acid.
8. ELISA plates: Immulon II HB flat-bottomed 96 well plates (Dynatech Laboratories, Cat. 3455).
9. Viral antigens: WNV COS-1 recombinant tissue culture antigen, SLE mouse brain antigen, DEN antigens from supernatant of infected C6/36 cells (pool of all 4 dengue serotypes).
10. Control antigens: COS-1 negative tissue culture antigen; Mouse brain negative antigen and Supernatant of non-infected C6/36 cells.
11. IgM positive serum controls of WNV, SLE and DENV infections and IgM negative pool of serum control of flavivirus infections.

### 6.4.3. Procedure.

1 Coat inner 60 wells with goat anti-human IgM (diluted with coating buffer) overnight at 4°C.

2 Dump out buffer. Add 200 μL of blocking buffer to wells and incubate for 30 minutes at room temperature.

3 Dilute serum 1:400 in washing buffer. Add 50 μL of diluted serum to each well and incubate the plate in a humidified chamber for 1 hour at 37°C.

4 Wash wells 5 times with washing buffer.

5 Add 50 μL of diluted antigen to each well and incubate the plate in a humidified chamber overnight at 4°C.

6 Wash wells 5 times with washing buffer.

7 Dilute the HRP-labeled MAb 6B6C-1 in blocking buffer. Add 50 μL of diluted MAb to wells and incubate plate in a humidified chamber for 1 hour at 37°C.

8 Wash wells 10 times with washing buffer, to reduce background.

9 Add 75 μL of TMB. Cover plates to block out light and incubate 10 minutes at room temperature.

10 Add 50 μL of Stop solution.

11 Read plates at 450 nm.

### 6.4.4. Interpretation.

This test is considered positive when: the mean of the optical densities (OD) of the positive control serums reacting on viral antigen (P) divided with the mean OD of the negative control serums reacting on viral antigen (N) is greater than or equal to 2.0. This is called the P/N ratio of the positive control. In addition, the P for the test specimen must be $\geq$ to twice the mean OD of the test specimen reacted on normal antigen. A sample is considered positive when the P/N ratio of the test serum is > 3.0.

All positive results were confirmed by titration, using 6 x 2-fold dilutions of the serum specimen.