

DEVELOPMENT OF A COMPETITIVE INHIBITION ENZYME-LINKED  
IMMUNOSORBENT ASSAY (CI ELISA) FOR SEROSURVEY OF WILDLIFE  
SPECIES FOR WEST NILE VIRUS EMPHASIZING MARINE MAMMALS

By

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by

Martha Keller

This work is dedicated to my mother, for instilling me with the tenacity I needed, to my father for infusing me with a love of learning, and to my wonderful loving husband who has always been there to support me in every way possible. And lastly, a special dedication to my daughter Emily, who makes every day a joy.

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Abstract of Thesis Presented to the Graduate School  
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DEVELOPMENT OF A COMPETITIVE INHIBITION ENZYME-LINKED  
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SPECIES FOR WEST NILE VIRUS EMPHASIZING MARINE MAMMALS

By

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Chair: Maureen T. Long  
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West Nile virus (WNV), an arthropod-borne virus introduced to the New World in 1999, causes neurological disease primarily in birds, horses, and humans. This virus is maintained in a life cycle involving birds and mosquitoes. West Nile virus has clinically affected many wildlife and domestic species including harbor seals, monk seals, alligators, gray squirrels, alpacas, llamas, a wolf, and one dog. Many more mammals have seroconverted to WNV without any signs of clinical disease. Studies of actual disease status in wildlife species are lacking.

As of 2005, WNV has been detected in all of the continental United States and has expanded into Canada, Mexico, and the Caribbean. Currently, several serological methods are used to identify WNV virus exposure in animals. These assays are either labor intensive or require the use of species-specific reagents. Our goal was to develop and validate a WNV-specific ELISA using monoclonal antibodies in a competitive inhibition format (CI ELISA). Samples were screened using the plaque reduction

neutralization test (PRNT) and compared against the CI ELISA. A total of 262 serum samples were tested representing 28 different species. Seven species were positive for WNV including alpacas, camels, cougars, elephants, lions, tigers, and a box turtle. Four species were positive against SLEV including camels, elephants, a lion, and a tiger.

WNV ChimeriVax® antigen was selected over mouse-brain antigen for the CI ELISA. The use of a single monoclonal (7H2) was determined to be as efficient as using a combination of three. Inter-assay variation based on a calculated coefficient of variance (CV) was 7.9% with a standard deviation of 4.9%.

Alligators and horses samples were analyzed to determine a proper cut-off. Using a receiver operating characteristic (ROC) curve analysis, a cut-off of 28% inhibition was identified, resulting in an assay sensitivity of 92.6% and a specificity of 100.0%. The alligator data resulted in 100% specificity and sensitivity. The best cut-off for elephants was 0% inhibition, which essentially renders the CI ELISA inappropriate for this species. Sample numbers were inadequate to validate this assay for any marine mammal, cat, or bat samples.

Results showed that this assay has higher specificity than sensitivity. Using the ROC curve, this value can be increased by changing the cut-off value; however, the specificity of the test would decrease. As the PRNT is the gold standard, confirmatory testing would be used to validate the CI ELISA results in a diagnostic laboratory setting. Results indicate that this ELISA can accurately identify WNV exposure in alligators and horses. Our assay would need to be validated for each individual species and cannot be used as a broad-based screening assay.

## CHAPTER 1 INTRODUCTION

West Nile virus (WNV), an arthropod-borne virus introduced to the New World in 1999, causes neurological disease primarily in birds, horses, and humans.<sup>1</sup> Since its initial appearance, this virus has spread rapidly across the North American continent.<sup>2</sup> In 2002 alone, over 15,000 equine cases were reported by the U.S. Department of Agriculture (USDA).<sup>2</sup> Thus far, harbor seals, monk seals, alligators, gray squirrels, an alpaca, a llama, a wolf, and one dog have all been clinically affected by natural infections.<sup>3-10</sup> A wide range of other animals have seroconverted, with unknown clinical status. In the late summer and fall of 2001, WNV became endemic in Florida. During 2002, WNV was established in much of the U.S. affecting over 2000 people with WNV encephalitis, and 2000 with WNV fever in 43 states. In addition, 14,717 horses and 137 species of birds were affected. The actual infection/disease status has not been completely assessed for many species. Because of the presence of this virus in many species of birds and mammals, assessment of multiple species of free-ranging and captive wildlife species is needed to determine the danger of WNV infection for Florida's valuable wildlife resources.

Florida marine mammals may be at particular risk for arboviruses, since these viruses have been described in several aquatic species. Alphaviruses have recently appeared in aquatic fish species such as Atlantic salmon and rainbow trout, indicating the possibility of aquatic transmission.<sup>11,12</sup> Pollution and habitat destruction are thought to make certain species of marine mammals susceptible to acquisition of new viruses and

increased outbreaks of marine mammal-specific viruses.<sup>13</sup> Recently, an alphavirus, similar in morphology to Eastern equine encephalomyelitis (EEE) virus was detected in southern elephant seals (*Mirounga leonina*) in Australia.<sup>14</sup> Transmission is thought to occur through blood sucking lice. In 2002, a captive harbor seal (*Phoca vitulina*) in New Jersey was diagnosed with WNV, making it the first marine mammal reported to die from this disease.<sup>5</sup> Since then, there have been several other reports of *P. vitulina* and Hawaiian monk seals (*Monachus schauinslandi*) clinically affected by WNV.<sup>3,6</sup>

St. Louis encephalitis virus (SLEV) is a flavivirus like WNV, and is endemic in the state of Florida.<sup>15</sup> Any serosurvey that involves WNV must also include SLEV, since these have the potential to cross-react on testing.<sup>16</sup> Because of the multiplicity of syndromes, infection of arboviruses could easily be overlooked.

The gold standards for serologic testing for encephalitis viruses are neutralization tests. These tests are applicable to multiple species, but are expensive as screening tools, requiring viral expertise, special facilities, and often special permits. The availability of WNV monoclonal antibodies allowed development of an ELISA in a competitive inhibition (CI ELISA) format to test multiple species. This CI ELISA was developed as a rapid screening assay for use in multiple wildlife species.

The purpose of this study was to examine WNV and SLEV seroprevalence in Florida mammals (with an emphasis on marine mammal species), and to develop a CI ELISA for broad-based species testing. Several specific aims were identified.

- **Specific aim 1:** Perform a serosurvey of wildlife species in the state of Florida using the plaque reduction neutralization test (PRNT).
- **Specific aim 2:** Develop a CI ELISA using known positive and known negative samples.

- **Specific aim 3:** Use the CI ELISA to perform screening of blinded samples.
- **Hypothesis:** Wildlife species in the state of Florida are being exposed to WNV. Using the CI ELISA will allow broad-based cross-species screening for WNV.

## CHAPTER 2 LITERATURE REVIEW

### **General Causes of Neurological Disease in Marine Mammals**

Three orders were emphasized in our review of captive and free-ranging marine mammals:

- **Cetacea:** whales, dolphins, and porpoises
- **Carnivora:** with suborder Pinnipedia including seals, sea lions, and walruses; and the families *Mustelidae* and *Ursidae* consisting of the sea otter and the polar bear respectively
- **Sirenia:** manatees and dugongs

Encephalitic diseases in marine mammals are caused by numerous etiologic agents. These include parasites, bacteria, viruses, neoplasias, and toxins. As clinical and diagnostic marine mammal medicine and research progresses, new etiologic agents are being discovered. Many of these agents have been around for some time; others appear to be emerging. Historically, neurological diseases in marine mammals are not uncommon. One study on stranded cetaceans off the coast of Italy from 1990 to 1997 found that encephalitis was present in nearly 11 of 17 (64%) of the striped dolphins (*Stenella coeruleoalba*) in which the brain was examined.<sup>17</sup> However, in another study in Belgium and France, only 6 of 55 stranded harbor porpoises (*Phocoena phocoena*) examined had evidence of an encephalitis.<sup>18</sup> This may be partly due to the fact that certain species are more susceptible to certain diseases than others. Thus far, a variety of viruses, bacteria, and protozoa have been identified as causative agents for neurological diseases in marine mammals. These are discussed next.

## Bacterial and Viral Encephalitides

### Brucella

*Brucella* is a Gram-negative, facultative, intracellular bacteria that affects a wide range of species, including humans. Infection in marine mammals was first reported in 1994 in free-ranging seals and cetaceans.<sup>19,20</sup> Recent PCR studies of the *Brucella* species affecting marine mammals indicate that there are two (newly discovered) species; one type affecting cetaceans and the other affecting pinnipeds.<sup>21</sup> The proposed new naming scheme is *B. cetaceae* and *B. pinnipediae* respectively. To date, there have been no reports of *Brucella* in Sirenia.

A large number of serosurveys have been performed to detect the presence of *Brucella* antibodies in free-ranging marine mammals. A large number of members of all families of cetacean and pinniped species have tested serologically positive for *Brucella* exposure.<sup>19,22-28</sup> Polar bears (*Ursus maritimus*) have also tested serologically positive for *Brucella* although there has been no report of clinical disease.<sup>29</sup>

Although serological evidence of exposure has been found in many species, clinical disease has been rarely described. Two captive bottlenose dolphins (*Tursiops truncatus*) experienced a placentitis and abortions and the fetuses had evidence of a *Brucella* infection.<sup>30</sup> Neurological disease associated with *Brucella* in marine mammals was described when a chronic, non-suppurative meningoencephalitis was found in three young *S. coeruleoalba*.<sup>31</sup> As these animals were found dead, no description of the clinical syndrome has yet been reported.

The exact means by which *Brucella* is transmitted among marine mammals remains unclear. There are several hypotheses including casual contact, sexual contact, maternal transmission, trauma, ingestion, and parasites.<sup>27</sup> There is a zoonotic concern

since many live marine mammals can become beached and infected carcasses could wash up on shore. Two cases of human neurobrucellosis have been reported with *B. pinnipediae*. The method of transmission remains unclear, as both subjects denied contact with any marine mammals.<sup>32</sup>

Thus far, the need has not arisen to treat captive animals. The marine mammal *B. pinnipediae* form has been treated successfully in a human using a six-week course of rifampin and doxycycline.<sup>33</sup>

### **Morbillivirus**

Morbillivirus is a single-stranded RNA virus in the family Paramyxoviridae. Until recently, there were four genus members: measles virus (MV), canine distemper virus (CDV), rinderpest virus (RPV) and peste-de-petits ruminants virus (PPRV).<sup>34,35</sup> In 1988, a series of epizootics occurred that led to the discovery of several previously undescribed morbilliviruses specific to marine mammals.<sup>36-38</sup> These include phocine distemper virus (PDV), dolphin morbillivirus (DMV), and porpoise morbillivirus (PMV). Marine mammals are currently affected by four members of the Paramyxoviridae family: CDV in seals<sup>39</sup> and polar bears,<sup>40,41</sup> PDV in seals,<sup>42</sup> DMV in dolphins and whales,<sup>43,44</sup> and PMV in porpoises.<sup>45</sup> DMV and PMV are closely related antigenically whereas PDV is closely related to CDV.<sup>43</sup>

There have been at least 8 recognized epidemics attributed to morbillivirus since 1987 encompassing North America, Europe, Asia and Africa and including numerous cetacean and pinniped species.<sup>34,35,38,42,46,46-54</sup> Although bronchial pneumonia and alveolitis are the most common findings, there can be CNS disease present with morbillivirus infections. The cerebrum is most commonly affected and histopathologically, a neuronal necrosis, gliosis, perivascular cuffing, and demyelination

with astrocytosis and syncytia occurs.<sup>34,35</sup> Clinical signs reported in pinnipeds include depression, muscle twitching, abnormal posture, head tremors, convulsions, seizures, and increased tolerance to humans.<sup>34,35,55</sup> Clinical signs in cetaceans have been observed less frequently but have included respiratory difficulty and abnormal swimming.<sup>34,55</sup> Diagnosis can be based on characteristic histopathological lesions.<sup>35</sup> Other methods such as neutralization tests, paired rising titers, and ELISA tests are also used.<sup>34,35,56,57</sup>

Treatment for morbillivirus-infected marine mammals is primarily supportive and the mortality rate is high. Vaccination has been performed in Europe using various commercially available canine distemper vaccines including a modified-live canine distemper, a killed, and a subunit vaccine. These vaccines appear to elicit a protective antibody response, but there have been no studies to verify efficacy in preventing disease or degree of virus shedding in the vaccinated animals. Most North American zoos and aquaria have strict quarantine procedures to prevent morbillivirus from entering their facilities. As such, vaccination is not performed in North America.<sup>58</sup>

## **Rabies**

Rabies virus, a fatal cause of encephalitis, is a member of the family *Rhabdoviridae*. Inoculation by bite is the primary mode of transmission and many mammalian species are susceptible to development of disease. Although all marine mammals are assumed susceptible to the rabies virus, there have been relatively few reports of marine mammals contracting the disease. In 1981, a ringed seal (*Phoca hispida*) in Norway was found disoriented and wounded.<sup>59</sup> The seal's condition progressively worsened and it became aggressive. Rabies was later confirmed using immunofluorescence. The seal likely may have been infected by contact with rabid foxes.

Hunters reported a polar bear with unilateral hind limb paresis.<sup>60,61</sup> Following its death, histopathology of the lumbar spinal cord revealed moderate to severe mononuclear inflammatory cell cuffing and gliosis in the gray matter. Rabies was later confirmed via immunoperoxidase testing of the spinal cord and a positive mouse inoculation test. Negri bodies were not detected in the spinal cord and there were no lesions in the brain. The bear was likely infected through contact with an infected canid in the area.

### **Arboviruses**

Arboviruses stand for arthropod borne viruses and are maintained by a cycle between vertebrate hosts and hematophagous arthropods such as ticks or mosquitoes. Arbovirus is not a taxonomic term, but rather, refers to four virus families that rely on arthropod transmission as part of their life cycle.<sup>62</sup> The four virus families include Togaviridae (including the alphaviruses), Reoviridae, Bunyaviridae, and Flaviviridae. Historically, no arboviruses had been described in any aquatic species. Recently however, there have been reports of alphaviruses in rainbow trout (*Oncorhynchus mykiss*),<sup>63</sup> Atlantic salmon (*Salmo salar*),<sup>64</sup> and southern elephant seals (*Mirounga leonina*).<sup>14</sup> This latter finding is of particular interest since the virus was isolated from the elephant seal louse (*Lepidophthirus macrorhini*) which routinely infests *M. leonina*.<sup>14</sup> It has been suggested that this new virus is transmitted by the louse, as the island on which infected seals were found has no mosquitoes and the seals are only rarely affected with ticks.<sup>14</sup> There is also a report of a captive killer whale (*Orcinus orca*) clinically affected with SLEV which later died of the disease.<sup>65</sup> Confirmation was made using virus isolation. More recently, several harbor seals (*Phoca vitulina*) and a Hawaiian monk seal (*Monachus schauinslandi*) were fatally affected with WNV.<sup>3,5,6</sup>

## Parasitic Encephalomyelitis

### Toxoplasmosis

*Toxoplasma gondii* is a commonly reported infection in marine mammals. Various syndromes have been reported in *T. truncatus*,<sup>66,67</sup> sea otters (*Enhydra lutris nereis*),<sup>68,69</sup> California sea lions (*Zalophus californianus*),<sup>70</sup> beluga whales (*Delphinapterus leucas*),<sup>71</sup> harbor seals (*P. vitulina richardsi*),<sup>72</sup> and a Florida manatee (*Trichechus manatus latirostris*).<sup>73</sup> Transplacental transmission is well-documented in terrestrial animals and *T. gondii* has been isolated from the tissues of a stillborn late-term fetal Indo-Pacific bottlenose dolphin (*Tursiops aduncus*).<sup>74</sup> A dual *Sarcocystis neurona* and *T. gondii* infection was reported in *E. lutris*.<sup>75</sup> This sea otter had a severe meningoencephalitis and malacia with hemorrhages, inflammation, and perivascular cuffing. Tissue cysts were visible at the periphery of the lesions, with central areas of necrosis. Diagnosis for protozoal diseases is based on rising titers. Treatment is primarily supportive although clindamycin has been used successfully to treat *M. schauinslandi*.<sup>76</sup>

### Sarcocystis

*S. neurona* is the causative agent of equine protozoal myeloencephalitis and is transmitted in the feces of opossums (*Didelphis virginiana*).<sup>77</sup> Despite its terrestrial nature, there have been several reports of marine mammals being clinically affected by *S. neurona* including harbor seals (*P. vitulina richardsi*)<sup>72,78</sup> and northern and southern sea otters (*E. lutris kenyoni*, *E. lutris nereis*).<sup>75,79-81</sup> In sea otters, isolation of the agent has been associated with lesions in the CNS resulting in a disseminated nonsuppurative meningoencephalomyelitis.<sup>79</sup> Studies have demonstrated that otters sampled near areas of maximal freshwater run-off in California have a higher seroprevalence to

*Toxoplasma*.<sup>82</sup> This has led to a hypothesis that marine mammals are becoming exposed to these protozoa due to storm run-off.<sup>68,82</sup>

### **Nasitrema**

*Nasitrema* is a trematode that is often found in the nasal sinuses of cetaceans. Trematode brain lesions have been implicated as the cause of encephalitis in *S. coeruleoalba*,<sup>83</sup> common dolphins (*Delphinus delphis*),<sup>84</sup> Risso's dolphins (*Grampus griseus*), and Pacific whitesided dolphins (*Lagenorhynchus obliquidens*).<sup>83-85</sup> It has been speculated that aberrant migration of the trematodes through nerves may result in loss of auditory function of in dolphins and resultant strandings due to the inability to catch food.

### **Neurotoxicosis**

#### **Paralytic shellfish poisoning**

Paralytic shellfish poisoning (PSP) is most commonly associated with saxitoxin but can be caused by other related biotoxins. The toxin accumulates within shellfish that have consumed dinoflagellates. Consumption of the shellfish results in PSP. Deaths from PSP have been documented in *E. lutris nereis*,<sup>86,87</sup> humpback whales (*Megaptera novaeangliae*),<sup>88</sup> and Mediterranean monk seals (*Monachus monachus*).<sup>89</sup> Clinical signs reported were lethargy, paralysis, and incoordination.<sup>90</sup>

#### **Brevetoxin**

Like shellfish poisoning, brevetoxins also accumulate in shellfish and seagrasses and can cause poisonings in mammals that eat contaminated food items. Ingestion can result in gastrointestinal and neurological signs although inhalation of the toxin has been implicated as another route of exposure.<sup>91</sup> Manatees (*T. manatus latirostris*) in Southwest Florida have frequently been affected by brevetoxin, with severe congestion of the nasopharynx, lungs, liver, and kidney noted.<sup>91,92</sup> The neurological signs reported in

manatees included incoordination, muscle fasciculations, and a loss of the righting reflex.<sup>91</sup> Death often follows. Supportive treatment has resulted in recovery once animals were removed from the brevetoxin contaminated environment.

### **Domoic acid**

Marine diatoms such as *Pseudonitzschia* are responsible for the production of domoic acid.<sup>93</sup> Domoic acid, when ingested, can have deleterious effects on marine mammals as well as humans. Experimentally, it has been associated with the development of neuronal degeneration in rats,<sup>94</sup> and has been implicated as the cause of a severe mortality event in California sea lions in 1998.<sup>95</sup> The affected sea lions exhibited neurological signs which included ataxia, depression, and seizures. The brain lesions observed in the sea lions were characterized by zonal vacuolization of the hippocampal neurophile, and were most severe in the ventral hippocampus.<sup>95,96</sup> Affected animals had high hematocrit levels, an eosinophilia and high creatine kinase levels.<sup>97</sup>

## **Neurological Evaluation**

### **Diagnostics**

The basic work-up for any neurological patient includes a complete blood panel including hematology and a serum chemistry profile. This identifies any electrolyte imbalances, identifies liver disease if present, and indicates inflammatory processes. Radiography or computed tomography (CT) is possible on marine mammals, depending on their size, to identify spinal or brain lesions. There have been no reports of intervertebral disc disease in any marine mammal species although diskospondylitis has been reported in *T. truncatus*.<sup>98</sup> The dolphin did not demonstrate any neurological signs. Cerebrospinal fluid (CSF), for cytological examination, can be obtained from pinnipeds (both phocids and otariids) from the atlanto-occipital joint in a method similar to that of

dogs.<sup>76</sup> Although unpublished, CSF can also be collected from otters and polar bears in a similar manner. No method has been published concerning CSF collection in cetaceans or sirenians.

### **Neurological Exam**

There has been very little published concerning the clinical neurological system of marine mammals. Work has been done examining the brains of several species, however, a thorough description of a proper neurological exam in these animals has not been published.<sup>99-102</sup>

The “classic” neurological exam, as performed in small animals, is rarely possible when dealing with large non-domestic species because very few animals can be safely handled without sedation. Although some reflexes remain in sedated animals, a proper workup is simply not possible. When dealing with marine mammals, this situation is made more difficult because these animals spend a good portion of their time in a weightless environment that is, for the most part, inaccessible to us. However, a good understanding of the nervous system of marine mammals can aid a clinician in assessing the neurological status of animals in their environment. This section of the thesis will discuss the similarities and differences of the neurological system of marine mammals, and how this information can be used to assess a patient. The marine mammals discussed will include polar bears, otters, manatees, cetacea (both mysticete and odontocete), and pinnipedia (both phocids and otariids).

Neurological disease is suspected when abnormalities of mentation, posture, and gait are noted. In marine mammals this would include incoordination, lethargy, paresis, paralysis, depression, muscle twitching, abnormal posture, abnormal swimming or ataxia, loss of the righting reflex, etc. For cetaceans and sirenians, all neurological observations

must take place with the animals swimming in the water, therefore access to an underwater viewing area is helpful. Depressed or lethargic animals may be easier to examine as they will linger near the surface. Certain non-neurological diseases, such as a one-sided pneumothorax, can result in an animal listing to one side. Although this can be a neurological sign, it should not be automatically assumed without further investigation. Depressed or stuporous animals often indicate cerebral cortical disease or brainstem inflammation.<sup>103</sup>

Pinnipeds, otters, and polar bears spend part of their time on land where posture and gait can be easily assessed. This would include such signs as a head tilt, ataxia, and/or circling. In small animals, postural reactions such as wheelbarrowing and conscious proprioception are normally tested; however, this cannot be accomplished with marine mammals due to either the absence of limbs or the presence of teeth. Therefore, it is imperative that the animal be observed moving in their environment over a period of time. Reliance on well trained animal keepers can aid in the observation of captive animals. Loss of muscle tone, if severe enough, can be evaluated visually in non-sedated animals. When examining an animal under sedation, muscle tone should be evaluated. Although the polar bears, otters, and pinnipeds likely possess the same spinal reflexes as small animals, handling prohibits their complete evaluation. In those animals with paresis and/or paralysis, deep pain should be assessed if at all possible to determine the severity.

### **Cranial Nerves**

Cranial nerve function should be assessed in marine mammals. Although there are many similarities between terrestrial and marine mammal species, there are several significant differences which should be noted. Many cranial nerves can be partially

assessed by observation alone. On animals that can be handled, a more accurate assessment can be made.

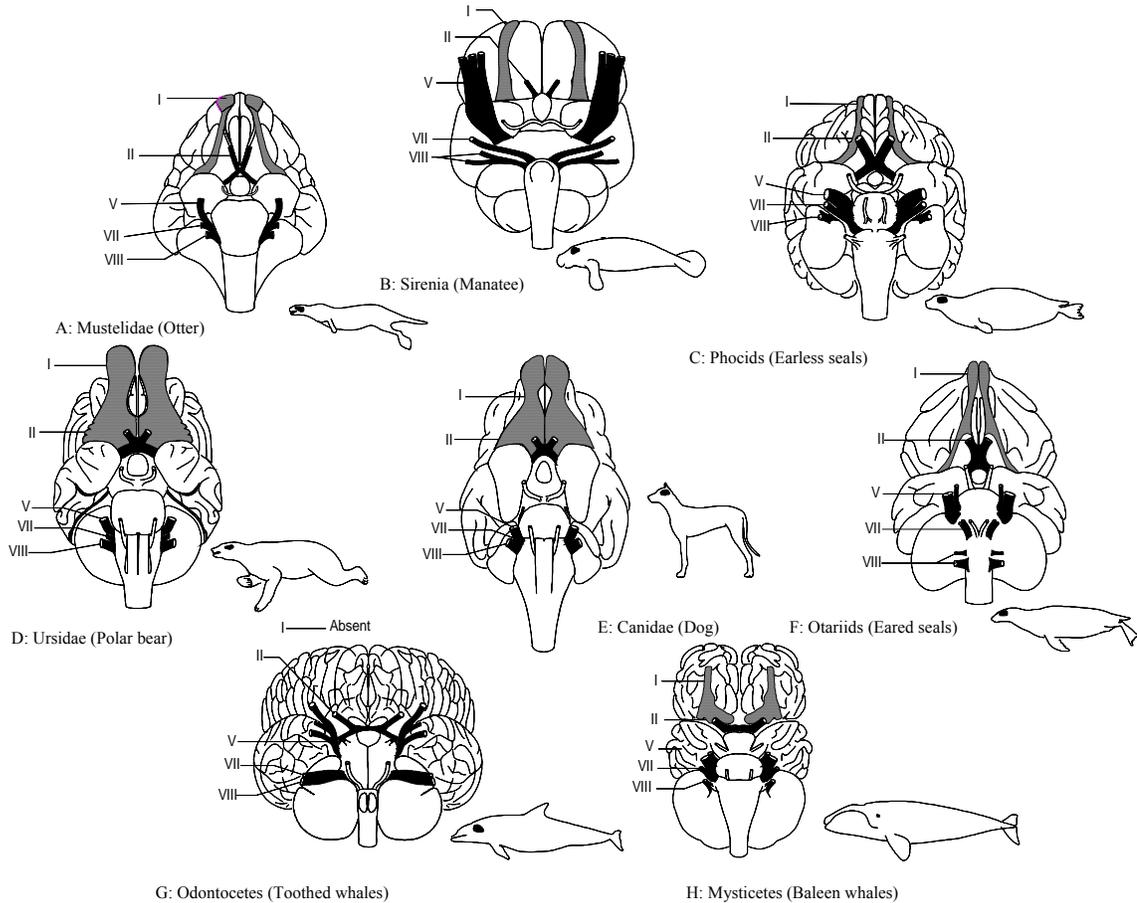


Figure 2-1. Size comparison of cranial nerves I, II, V, VII and VIII among the different mammal species and with the dog. Used and adapted with permission from Reynolds JE, Rommel SA, eds. "Biology of marine mammals". 1999; Washington, DC: Smithsonian Institute. Figure 2-25 on page 60. © S.A. Rommel.

Cranial nerve 0 is the terminal nerve. It was only discovered after the other nerves were named. It was labeled zero to follow the anatomical naming scheme. This nerve is present in several species of cetaceans.<sup>104,105</sup> These terminal nerves have been

found to contain gonadotropin-releasing hormone (GnRH).<sup>106,107</sup> This has led to the hypothesis that this nerve may be involved in chemoreception for sexual reproduction however the function of this nerve is still unknown. There is currently no recognized method to test it properly.

Cranial nerve I is the olfactory nerve. It is interesting to note that this cranial nerve is completely absent in the odontocete species although they still retain an olfactory lobe.<sup>108,109</sup> As indicated by Figure 2-1, cranial nerve I size varies among different species (with the polar bear much larger than phocids, for example). This is likely due to the fact that polar bears rely heavily on smell to hunt.<sup>110</sup> Traditionally, the olfactory nerve is tested in small animals by the use of a noxious odor such as alcohol. In marine mammals this may be difficult if not impossible. If a lesion is suspected in a captive animal, food can be hidden in the enclosure prior to allowing the animal allowed to enter. Testing of this nerve is very subjective. The reduced size of this nerve in many of the marine mammal species suggests that it is not as important a sense for them as it is for terrestrial animals.

Cranial nerve II (CN II), the optic nerve, is responsible for sight. The optic nerves of odontocetes and sirenians are much reduced in size compared to other marine mammals.<sup>110</sup> Their reliance on sight is less than the other species and therefore, their optic nerve function is more difficult to assess. Odontocetes possess echolocation and captive animals may be able to move around their tank with little difficulty even with a damaged optic nerve. Sirenians possess sensory tactile hairs on their body which have been hypothesized to function in a manner similar to the lateral line system of teleosts.<sup>111</sup> Pinnipeds possess well developed vibrissae that have been shown experimentally to allow

them to identify objects, even when their vision was completely inhibited experimentally with eye cups.<sup>112</sup> Therefore, observation alone should not be used to evaluate CN II function in these species.

In animals that can be handled, signs indicating loss of function include a dilated pupil and the loss of the pupillary light reflex. In most other mammals, the eyes project the majority of their nerve fibers to the contralateral cerebral hemisphere but there are still a good number of fibers that project to the ipsilateral side. This results in the observation of pupillary dilation of the contralateral eyes during examinations. This does not appear to be the case in dolphins examined both anatomically and physiologically.<sup>113-115</sup> Therefore, a direct pupillary light response should be observed normally, but not necessarily the indirect pupillary light response.

Observation of the animal in its environment can help detect visual deficits. For example, when new objects are introduced in their environment are they easily avoided by the animal? Even domestic animals accommodate for any loss of sight. Often, the animal will rely on its other senses when moving around and finding food and thus can be difficult to assess. Polar bears, otters, and pinnipeds are more affected than cetaceans with damage to CN II as they rely more on sight.

Cranial nerves III, IV, and VI innervate the muscles of the eyes. Cranial nerve III, the oculomotor nerve, functions to innervate all the extrinsic muscles of the eye except for the superior oblique and the external rectus muscles. Cranial nerve IV, the trochlear nerve, functions to innervate the superior oblique muscle of the eye. Cranial nerve VI, the abducens nerve, functions to innervate the external rectus and retractor oculi muscles of the eye. Comparative studies have not been performed on these nerves

for most marine mammals. In odontocetes the nerves appear slightly reduced but the cranial nerve function is assumed to be similar to that in other animals.<sup>116</sup> It should be noted that odontocetes and sirenians possess limited mobility of their eyes which can make detecting a strabismus difficult.<sup>117</sup> Despite that, it has been shown that dolphins are capable of moving their eyes independently.<sup>118,119</sup> This leads to difficulty in assessing dolphins because if a strabismus is pathologically present, it may not be noticed. If a strabismus is noticed, it may, in fact, be normal. Conversely, mysticetes appear to have great mobility of their eyes.<sup>120</sup> Dysfunction of CN III results in a ventrolateral strabismus. Dysfunction of CN IV results in a dorsomedial strabismus. Dysfunction of CN VI results in a medial strabismus and poor retraction of the globe on corneal reflex.<sup>103</sup>

Cranial nerve V, the trigeminal nerve, is primarily sensory to the face including the eyelids, nasal mucosa, and cornea. It is composed of three major branches, the ophthalmic, the maxillary, and the mandibular which also has a motor component that innervates the muscles of the lower jaw. In most marine mammals, this is a very large nerve (Figure 2-1).<sup>110</sup> In mysticetes, it is the largest cranial nerve.<sup>121</sup> This makes functional sense in these marine mammals as large mysticetes require enormous power for their lower jaws. In manatees and pinnipeds, this nerve is responsible for the innervation of their tactile vibrissae on the face.<sup>122,123</sup> Sensation of the face can be tested by touching areas of the face such as the palpebra, cornea, and nasal mucosa.<sup>103</sup> Dolphins have a well developed corneal reflex.<sup>114</sup> Lack of a facial response may be due to either CN V or CN VII dysfunction. Loss of corneal reflex may reflect dysfunction of either CN V or VI. With observation alone, dysfunction can be identified by the presence of atrophy of the temporalis and masseter muscles, and a perceptible loss of sensation of the

vibrissae of sirenians and pinnipeds. Animals may have a more difficult time manipulating their food and may present with a dropped jaw if the lesion is bilateral.<sup>103</sup>

Cranial nerve VII, the facial nerve, provides motor innervation to the muscles of the face. In odontocetes, it is quite large due to the musculature involved in echolocation.<sup>109</sup> Therefore, dysfunction in odontocetes would limit echolocation and may be associated with difficulty maneuvering and difficulty finding food. With good eyesight and a clear tank, these signs may not be present. The same test for CN V is used in testing CN VII, however, that requires a hands-on approach. Observable signs include a lip, eyelid, and ear droop. Cetaceans cannot present with those signs based on their anatomy. Pinnipeds may exhibit a lip or eyelid droop. Sirenians may exhibit a lip droop. Otters and polar bears can exhibit all signs.

Cranial nerve VIII, the vestibulocochlear nerve, provides innervation to the inner ear for hearing and vestibular function. In sirenians and cetaceans, ears have been modified evolutionarily for underwater hearing and may no longer be able to detect airborne sounds.<sup>112</sup> Odontocetes possess extremely large vestibulocochlear nerves. This has been hypothesized to allow the rapid and high speed conduction of acoustical information.<sup>124</sup> Therefore, as with cranial nerve VII, dysfunction may result in difficulty maneuvering since they rely heavily on echolocation. In the other species, dysfunction results in ataxia, head tilt, nystagmus, and/or deafness. Testing of this nerve in marine mammals is difficult as it requires assessing hearing which is often quite subjective. The absence of any vestibular signs should indicate that vestibular function is normal.

Cranial nerve IX, the glossopharyngeal nerve, controls the motor function to the swallowing muscles. No extensive studies have been done examining this nerve in

marine mammals. Traditionally, loss of the gag reflex in animals is a sign of dysfunction. Cetaceans do not have a gag reflex and there is no easy way to elicit this reflex safely in the other species. Dysphagia may be the only visible sign in most animals.

Cranial nerve X, the vagus nerve, provides both sensory and motor innervation to the swallowing muscles, as well as innervation of the heart and lungs. Here again, the only visible sign may be a dysphagia. In the vocal animals, there may be a laryngeal paralysis resulting in a voice change or an inspiratory stridor, if bilateral.<sup>103</sup>

Cranial nerve XI, the accessory nerve, innervates the muscles of the neck. Dysfunction of this nerve results in associated muscle atrophy. Cetaceans and sirenians do not possess a well defined neck area, therefore, atrophy of these muscles would not be clearly evident. In pinnipeds, polar bears, and otters, examination of the neck muscles should be performed through palpation in those animals that can be handled. Animals with suspected dysfunction should be sedated for further examination.

Cranial nerve XII, the hypoglossal nerve, controls motor function to the tongue. Dysfunction results in a loss of tongue strength or a deviation, if unilateral. Many captive odontocetes are trained to open their mouth for examination and the tongue can be palpated. In other animals, careful observation may be the only option.

## **West Nile Virus**

### **Taxonomy**

West Nile virus, an arbovirus, is an arthropod-borne virus. The term arbovirus includes many unrelated virus families grouped together based on their mode of vector transmission. The arboviruses most commonly refer to members of four virus families including *Bunyaviridae*, *Togaviridae*, *Arenaviridae* and *Flaviviridae*.

West Nile virus is a member of the family *Flaviviridae*. *Flaviviridae* is composed of three genera: *Flavivirus* (from the Latin *flavus* meaning yellow), *Pestivirus* (from the Latin *pestis*, meaning plague) and *Hepacivirus* (from the Latin *hepar* or *hepatos*, meaning liver).<sup>1,125,126</sup> At one time, the flaviviruses and pestiviruses were both considered members of the family *Togaviridae*, but as methods of virus identification have broadened to include antigenic and genomic similarity, members of these groups were reclassified.<sup>127</sup>

The flavivirus genus consists of over 70 types of related positive-stranded RNA viruses which can be further divided into antigenically related serocomplexes.<sup>128,129</sup> The three main serocomplexes include the Dengue serocomplex, the Japanese encephalitis serocomplex, and the tick-borne encephalitis serocomplex. West Nile virus is a member of the Japanese encephalitis serocomplex which also includes Japanese encephalitis (JE), Kunjin, Murray valley encephalitis, and St. Louis encephalitis viruses (SLEV).<sup>129</sup> Recent studies into the sequence homology of Kunjin has led to its proposal as a subtype of WNV.<sup>130</sup>

Two major strains of WNV have been described based on genetic relatedness.<sup>131,132</sup> Strain I has a wide distribution encompassing Africa, Europe, and North America while strain II has been mostly limited to Africa and Madagascar. Both strains are detected serologically in humans and horses with strain I resulting in more severe human and equine disease.<sup>131-133</sup>

### **Life Cycle**

West Nile virus is maintained in a life cycle involving birds and mosquitoes. Infected mosquitoes feed on birds, which amplify the virus in their system. Feeding on a viremic bird infects the mosquitoes. Once the infected mosquito has built up sufficient

viremia levels ( $\geq 10^5$  PFU/ml), it is then able to infect new hosts and the cycle repeats.

Many host species do not produce a high viremia and therefore do not have enough virus present to infect mosquitoes.<sup>129</sup> These species, such as humans and horses, with viremia levels averaging  $10^3$  PFU/ml, are considered dead-end hosts.<sup>134</sup>

A host can be infected with WNV when fed upon by an infected mosquito. The virus is released from the salivary epithelial cells of the mosquito and introduced subcutaneously into the host.<sup>135,136</sup> Contrary to popular belief, the virus is not deposited in the blood because any virus deposited in the blood is reingested. Instead, the virus replicates locally and studies have shown that the primary target for infection of the host may be Langerhans cells.<sup>137,138</sup> Virus replication extends into regional lymph nodes where the virus is then carried through the lymphatics to the bloodstream via the thoracic duct.<sup>129,139</sup> This primary viremia seeds the extraneural tissues which act as a site of further viral replication for release into the circulation.<sup>135,136</sup> In unaffected hosts, the viremia is controlled by clearance through macrophages and the appearance of circulating antibodies which usually occurs around one week after the infection.<sup>129</sup>

In order to become infective, an arthropod vector must ingest a sufficient concentration of virus to exceed the mesenteron infection threshold. A vertebrate host must have viremic titers in excess of  $10^5$  PFU/ml in order to infect a feeding mosquito. Once in the mosquito, the virus multiplies in the mesenteron epithelial cells. There the virus is released systemically to further infect many tissues of the mosquito, including the salivary glands. From the salivary glands, the virus is released and transmitted to other vertebrate hosts during feeding.<sup>129</sup>

The mosquitoes remain chronically infected for life and produce extremely high levels of infectious virus particles in their salivary glands.<sup>127</sup> Although most adult mosquitoes live for only a few weeks, some species can overwinter while infected.<sup>140</sup> In addition to transmission via feeding, recent studies have shown that flaviviruses can also be vertically and horizontally transmitted between mosquitoes. Virus can infect fully developed eggs at the time of fertilization and oviposition through the micropyle in female mosquitoes.<sup>129,141</sup> Venereal transmission from male to female mosquitoes has also been demonstrated.<sup>142</sup> In addition to mosquitoes, the virus has been found to infect hard and soft ticks under natural and experimental conditions. The capacity of ticks to infect new hosts, however, remains unclear.<sup>129</sup>

There is evidence to suggest that flavivirus infection can be persistent. The term persistence has been defined by Kuno as “the prolonged presence of infectious virus, virion components (protein, antigen and nucleic acid), and virus-specific immunoglobulins in vertebrate hosts after infection.”<sup>143</sup> Latent infections of WNV have been reported in several cell cultures lines.<sup>144,145</sup> The majority of the cells in these cultures expressed viral antigen but a minority actually produced infectious virus.<sup>133</sup> The viruses that occur in these persistent infections often undergo phenotypic alterations including a reduction in plaque size, temperature sensitivity, host-range restriction, and loss of neurovirulence. Some cultures often exhibit alterations in the composition of their viral proteins.<sup>133</sup> In vivo, latent infections of WNV have been reported in ducks, pigeons, mice, and monkeys.<sup>146-148</sup> Whether or not there is persistence of WNV in human or equine disease is largely undetermined.

## Morphology

WNV virions are small, enveloped, and spherical shaped, measuring around 50 nm in diameter.<sup>126</sup> Virions consist of a spherical ribonucleoprotein core surrounded by a lipoprotein envelope with small surface projections.<sup>129</sup> The envelope is a lipid bilayer with two or more envelope (E) proteins surrounding the nucleocapsid. The genome of WNV consists of a positive-sense single-stranded RNA, approximately 11 kb in length<sup>127</sup> consisting of 199,029 nucleotides.<sup>132</sup> The genome of WNV encodes 10 mature viral proteins.<sup>127</sup> All the viral proteins are initially made as part of a single long polyprotein of more than 3,000 amino acids. The polyprotein is cleaved using both host and viral proteases. The structural proteins are encoded in the N-terminal portion of the polyprotein with the non-structural proteins in the remainder.<sup>127</sup> The three structural proteins are the capsid (C), membrane (prM/M) and the E-proteins. The M-protein is a small proteolytic fragment of the prM-protein which is involved in the maturation of the virus into an infectious form.<sup>127</sup> The seven nonstructural proteins are named NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.<sup>126</sup>

Studies have shown that binding and uptake may involve receptor-mediated endocytosis via cellular receptors specific for the viral envelope proteins.<sup>127,149</sup> Several types of cell surface receptors have been hypothesized for flavivirus entry including expression of highly sulfated glycosaminoglycans<sup>150,151</sup> and antibody-dependent enhancement.<sup>152-160</sup> The pattern of receptor expression in animals is probably a determinant of their tropism.<sup>127</sup>

To gain entry into the cell, the virion envelope fuses with the cellular membranes and the nucleocapsid is delivered to the cytoplasm. Once in the cytoplasm, translation of the genome RNA occurs using the non-structural protein NS5, which is a viral

RNA-dependent RNA polymerase (RdRp).<sup>127,149</sup> NS5 works in conjunction with other viral non-structural proteins and possibly cell proteins to copy complementary minus strands from the genomic RNA template. These minus-strand RNAs, in turn, serve as templates for the synthesis of new genomic RNAs.<sup>126,127</sup> Viral RNA production is asymmetric in vivo with the plus-strands accumulating more than 10 times that of the minus strands.<sup>161,162</sup> Virion assembly occurs within the rough ER membranes where they bud through intracellular membranes into cytoplasmic vesicles.<sup>126,127</sup> These vesicles follow the host secretory pathway, fuse with the plasma membrane, and cause the release of mature virions into the extracellular compartment.<sup>127</sup>

The primary determinant of tropism and the primary target of virus-neutralizing activity is due to the E-protein.<sup>125</sup> The E-protein has two major functions: it is involved in virus attachment to susceptible cells and in the pH dependant virus-cell membrane fusion.<sup>127,129,149,163</sup> The E-protein is composed of three domains, of which domain III has been proposed as a putative receptor-binding domain.<sup>125,149</sup> Domain III contains a fold, like an immunoglobulin constant domain, and studies have shown that altering the Domain III structures of various flaviviruses can affect their virulence.<sup>149,151</sup>

Because the E-protein is required for entry into the cell, E-reactive antibodies are effective at interfering and therefore, neutralizing virus infectivity.<sup>163-165</sup> The E-protein is responsible for eliciting high titers of virus-neutralizing, and cell membrane fusion-blocking antibodies. However, not all E-protein-reactive antibodies neutralize virus infectivity, which suggests that this biological activity is localized to only certain areas or epitopes on the E-protein.<sup>163</sup> The authentic expression of E-protein epitopes appears to require co-expression of the prM-protein.<sup>163,166</sup>

## **History**

West Nile virus was first discovered in 1937 in the blood of a woman with a mild febrile illness in the West Nile province of Uganda.<sup>167</sup> The first reported epidemic of WNV was in Israel in 1951 where a total of 123 human cases were reported with no fatalities.<sup>168</sup> West Nile virus was subsequently isolated from humans, birds, and mosquitoes in Egypt in the early 1950s.<sup>169,170</sup> A serosurvey performed on humans in the 1950s in Egypt along the Nile showed that WNV antibodies were present in more than 60% of the human population.<sup>170</sup>

The first reported occurrence of equine encephalitis was in Egypt and France in the 1960s.<sup>171,172</sup> An outbreak in France occurred in the summer of 1962 with several horses exhibiting neurological signs including ataxia, weakness, and amaurosis.<sup>173</sup> Several human cases occurred in the area in subsequent years.<sup>174,175</sup> West Nile virus was eventually isolated from several sick horses in the area in 1965.<sup>176</sup> In South Africa, an outbreak in 1974 resulted in an exposure rate of nearly 55% of the human population with clinical cases being mostly mild.<sup>177</sup>

Recently, outbreaks in humans and horses have become more frequent with outbreaks occurring in Romania and Morocco in 1996 where nearly 500 clinical cases were seen with a fatality rate near 10%.<sup>178-180</sup> Other outbreaks of note have occurred in Tunisia in 1997, Italy in 1998, Russia in 1999, and the first appearance in the U.S. in 1999.<sup>1,181-184</sup>

## **Pathogenesis and Clinical Signs**

Three main patterns of pathogenesis occur with flavivirus infections: a fatal encephalitis with viremia, a subclinical encephalitis with a low viremia, or an inapparent infection characterized by trace viremia and no neuroinvasion.<sup>129</sup> The severity of the

infection on the host is dependent on several factors including the virus strain's virulence, the level of virus transmitted and route (intranasal vs. hematogenous), as well as the ability of the host's immune system to clear the virus.<sup>129,133</sup>

The mechanism by which the virus particles cross the blood-brain barrier remains unknown, however there are several hypotheses. One proposed pathway into the CNS includes the transport of the virus across the cerebrovascular endothelium.<sup>185,186</sup> The ability of the virus to replicate in vascular endothelial cells suggests that it may grow across capillaries.<sup>129</sup> In fact, studies have detected infectious virus in multiple sites in the brain and spinal cord which would support a hematogenous route.<sup>187</sup> Another proposed method involves gaining access to the CNS after a loss of the blood-brain barrier integrity.<sup>188</sup> Cytokines could play a role in the disruption of the blood-brain barrier.<sup>189</sup> The third hypothesis involves entry through the olfactory epithelium.<sup>139,190</sup> The olfactory system has been previously recognized as an alternative pathway to the CNS. Olfactory neurons are unprotected by the blood-brain barrier. In a study involving mice and hamsters inoculated with SLEV, virus was detected in the olfactory neurons by day 4 postinoculation.<sup>190</sup> It was further identified in the olfactory bulbs on day 5 and by day 6, it was identified in the brain. This study shows that the olfactory system may not only be an entry way for the virus, but may result in a pathway for aerosol transmission.

## **Humans**

Once infected, the incubation period is 1 to 6 days in humans.<sup>129</sup> The typical case in humans is mild, characterized by fever, weakness, headache, backache, nausea, generalized myalgia, and anorexia.<sup>129,191,192</sup> In European outbreaks of WNV, a roseolar or papular rash was commonly seen, however, this has occurred less often in the U.S.<sup>193</sup> Cases which lack a neurologic component are termed West Nile fever.<sup>192</sup> West Nile

meningitis is defined as a meningitis with no encephalitis. West Nile encephalitis is the most severe form and involves the brain. West Nile poliomyelitis is a flaccid paralysis syndrome that rarely occurs with WNV infections.<sup>194</sup>

In less than 15% of patients, the symptoms are more severe and involve the CNS.<sup>129,195</sup> Younger patients (<50) are more likely to be affected with a meningitis while an encephalitis occurs more often in older patients.<sup>129,192,195,196</sup> A distinctive feature of the North American strain of WNV is a poliomyelitis-like flaccid paralysis.<sup>129,192,195,196</sup> In encephalitic patients, the brainstem is most severely affected and pathologically, there are microglial nodules, perivascular cuffing, and mononuclear inflammation along the cranial nerve roots.<sup>195,197,198</sup> Those surviving encephalitis sometimes have residual weakness and memory loss.<sup>195</sup> Interstitial myocarditis and pancreatitis has also been associated with WNV infections.<sup>129</sup>

## **Horses**

As with humans, infections in horses can be inapparent, mild, or severe. Experimentally about 10% of challenged horses develop clinical signs.<sup>134</sup> Clinical signs commonly seen in horses include ataxia, limb weakness, recumbency, and muscle fasciculations.<sup>199</sup> Less common signs include low grade fever (38.4 to 39.4°C), paralyzed or drooping lip, hypermetria, face or muzzle twitching, teeth grinding, hypersensitivity, and blindness.<sup>199,200</sup> An interstitial myocarditis has been also been associated with WNV infection.<sup>129</sup>

Lesions in horses have been characterized as a multifocal lymphocytic polioencephalomyelitis.<sup>183</sup> This is located in the ventral and lateral horns of the thoracic and lumbar spinal cord and is often associated with moderate to severe hemorrhage. In addition, there is moderate lymphocytic and monocytic inflammation with scattered foci

of microgliosis in the medulla oblongata and pons, and to a lesser extent in the basal nuclei, thalamus, and mesencephalon. In the most affected areas there is neuronal degeneration with central chromatolysis. There are no pathognomonic lesions in horses.

### **Birds**

Although WNV rarely affected birds clinically in Europe, over 200 species of birds have been reported clinically affected with WNV in the United States.<sup>192</sup> As with mammals, birds can experience a range of clinical signs, from asymptomatic to death and include lethargy, recumbency, and hemorrhage in some cases.<sup>201</sup> Emaciation, brain hemorrhage, splenomegaly, hepatomegaly, meningoencephalitis, and myocarditis are the most common pathological findings on clinically affected birds, however, there are not always pathological lesions present.<sup>202,203</sup>

### **Other species**

West Nile virus has been reported in many other wildlife and domestic species. Clinically affected mammalian species include squirrels, bats, chipmunks, rabbits, raccoons, dogs, and cats.<sup>8,204-207</sup> There have been reports of several canids with clinical WNV infection.<sup>9</sup> A wolf pup and an adult 8 year old dog exhibited clinical signs including anorexia, weakness, ataxia, ptyalism, head tilt, and blindness.<sup>9</sup> The dog had a concurrent immune-mediated thrombocytopenia which might have made him more susceptible to disease. The encephalitis affected the gray matter with poorly demarcated aggregates of lymphocytes and microglial cells and mild necrosis. Marked myocarditis was also present.

Many more mammals have seroconverted to WNV without signs of clinical disease. While seroconversions in marine mammals have been reported, captive *P. vitulina* and *M. schauinslandi* have also been clinically affected.<sup>3,5,6</sup> These seals

exhibited clinical signs including weakness, head tremors, anorexia, and abnormal swimming. Lesions included a nonsuppurative encephalitis, meningitis and myocarditis.

Reptile and amphibians are also susceptible to WNV. Experimentally infected lake frogs (*Rana ridibunda*) not only developed a high titer, but were able to transmit the virus to *Culex pipiens*.<sup>208</sup> More recently, WNV infected captive alligators have exhibited clinical signs such as “star gazing”, ataxia and muscle spasms.<sup>7</sup>

### **Epidemiology**

As of 2004, WNV has been detected in all of the continental U.S. and has expanded into Canada, Mexico and several Caribbean islands.<sup>209-211</sup> West Nile virus will likely continue spreading through the remainder of North America, and into Central and South America.

### **Mosquitoes**

Mosquitoes are involved in an enzootic cycle, transmitting the virus to additional hosts. The main mosquito species involved in maintaining the bird/mosquito cycle are *Cx. restuans* and *Cx. pipiens*.<sup>212</sup> The high prevalence of virus detected in these ornithophilic vectors early in its introduction helped WNV spread rapidly through the U.S. via the bird populations. The mosquito species of greatest interest to human research are those who feed on both birds and mammals, as they provide the link between the viremic birds and mammalian hosts. As of 2004, WNV had been isolated from 43 different species of mosquitoes.<sup>213</sup> In the U.S., the majority of isolates have come from the *Culex* species, primarily *Cx. restuans*, *Cx. pipiens*, and *Cx. salinarius*, as well as some *Aedes* species.<sup>214,215</sup> As WNV spread southward, additional species such as *Cx. nigripalpus* and *Cx. quinquefasciatus* became involved in the transmission cycle. *Cx. salinarius* appears to be mammalophilic compared to *Cx. pipiens* and *Cx. restuans* and as

such, may be an effective bridge from birds to mammals.<sup>192</sup> In the western states the species *Cx. tarsalis* is an indiscriminate feeder of both mammals and birds, and may be the reason why western states such as Colorado had such high infection rates in 2003.<sup>192,216</sup> *Culex* is also an efficient vector because it can overwinter as an adult mosquito.<sup>140</sup>

## **Humans**

The first report of WNV in humans in the U.S. was in August of 1999.<sup>217</sup> The introduction of WNV became evident as several clinical cases of encephalitis in humans were reported at the same time that unexplained mortality of birds, primarily crows, was occurring.<sup>1</sup> Testing of the samples using RT-PCR showed >99% similarity with a strain from Israel.<sup>132,163,217</sup> The mechanism by which the virus was introduced into the U.S. is not known. Hypotheses include introduction of an infected bird or mosquito.

Since its introduction, human cases have occurred in nearly every state in the continental U.S., moving in a westerly direction.<sup>218</sup> Most infections have occurred via mosquito transmission and as such, increased exposure to mosquitoes directly increases the chances of becoming infected. Therefore, the amount of time spent outdoors, as well as the season, can affect one's chances of infection. In some areas there is seasonal blood-feeding of the mosquitoes and this results in higher infection rates during certain time periods.<sup>192</sup> However, as WNV moves into tropical areas, the mosquitoes feed year round and there will likely be no seasonality.

Once infected, the determination of the pathogenesis is a result of host factors including age, sex, genetic susceptibility, and pre-existing immunity to heterologous agents.<sup>129</sup> Immunosuppression and age (>60) have both been hypothesized to increase

the chances of developing clinical signs.<sup>129,219</sup> In addition, older patients were more likely to die from the disease as compared to younger patients.<sup>219</sup>

Several other modes of WNV transmission in humans have occurred in the U.S. There are reports of WNV transmission from the mother both transplacentally (1 reported case),<sup>220</sup> and via breast milk (1 reported case).<sup>221,222</sup> In addition, WNV clinical cases have occurred after blood transfusions from infected individuals (4 reported cases) and organ transplants (4 reported cases).<sup>222-225</sup> Transmission has also occurred through exposure in the work place at a turkey farm and after accidental inoculation in a laboratory.<sup>226,227</sup>

## **Birds**

Birds amplify WNV as part the virus' life cycle. In 1997, a new strain of WNV was identified clinically affecting birds in Israel.<sup>228</sup> Previously, WNV was rarely associated with clinical disease in birds. As previously mentioned, the strain of WNV that arrived in North America was nearly identical to the strain from Israel.<sup>132,163,217</sup> This strain has resulted in significant avian mortalities in North America.

Thus far, WNV has caused fatal infections in 198 species of birds.<sup>192</sup> Incidence in certain species, such as the American crow (*Corvus brachyrhynchos*), has been extremely high, with some areas approaching near 100% mortality.<sup>192,203</sup> A closely related species, the blue jay (*Cyanocitta cristata*), has also been severely affected.<sup>192</sup> Studies performed in New York during the outbreaks in 1999 and 2000 found that nearly one third of the avian species tested had virus-neutralizing antibodies to WNV.<sup>203,229,230</sup>

To be an effective reservoir for WNV, a bird species must be frequently exposed to infection, abundant in relation to other bird species, and capable of producing viremic levels high enough to infect mosquitoes.<sup>229</sup> Several bird species develop extremely high

levels of viremia and are very competent amplifying hosts.<sup>201</sup> The Passerine species such as the blue jay, common grackle, house finch, house sparrow, and American crow all are competent at transmitting virus back to mosquitoes as are the Charadriiformes such as gulls, terns and plovers. Because of their high abundance in many areas, house sparrows are considered an important reservoir host even though their seroprevalence is not generally as high as crows.<sup>229</sup> In contrast, the Piciformes (woodpeckers, toucans), Psittaciformes (parrots), and Galliformes (chickens, turkeys) have all been identified as incompetent vectors.<sup>201</sup> These birds are fairly resistant to disease and do not develop high viremic levels.

Early on in the outbreaks it was clear that dead birds provided early warning surveillance for WNV. In 2000, dead bird surveillance was initiated in New York to provide a method of detecting early virus activity before the onset of human cases.<sup>203</sup> The best and earliest warnings were provided by dead crows. Although studies have shown that the presence of WNV positive birds does translate to local virus transmission, this does not indicate the levels of viral transmission in the area.<sup>212</sup> In order to detect local virus transmission, several species were considered as possible sentinels. The ideal sentinel candidate would seroconvert to WNV upon exposure, but not show any clinical signs or develop a viremia high enough to infect mosquitoes. Experimental infections in goslings, poults, and chickens have been performed to evaluate their use as sentinel species.<sup>202,231-233</sup> Results showed that chickens shed low amounts of WNV per cloaca and per os and that they developed low level viremias.<sup>231,233</sup> In the case of the goslings and poults, both produced viremias higher than the chickens. The goslings were clinically affected.<sup>202,232</sup> In addition there was low level cloacal shedding in the poults and low

level oral shedding in the goslings. Because of this, chickens have been viewed as safe to use as sentinel species.

Oral and cloacal shedding of virus has been demonstrated in many bird species, however, virus levels are generally low.<sup>192</sup> Oral transmission via infected water, dead birds, mice, and mosquitoes has been shown experimentally.<sup>201</sup> Horizontal transmission among birds confined together has been demonstrated in several species.<sup>192,201,202</sup> This is important because despite effective control of mosquito vectors, the virus may continue its life cycle in this manner in some areas.

### **Horses**

Significant morbidity and mortality has been reported in equids in several outbreaks in Europe and the Middle East.<sup>171,234,235</sup> A WNV outbreak in France in 2000 resulted in 76 equine clinical cases with 21 deaths.<sup>171,236</sup> As a result, the local animal health authority performed a serosurvey on all equids located within a 10 km radius of the confirmed cases.<sup>236</sup> Results on 1,429 horses showed a prevalence of IgG antibodies of 19.2% (274 horses). The first confirmed case of WNV infection in a horse in the Western Hemisphere was in October of 1999.<sup>199</sup> In 2000, 60 horses were confirmed as having a case of WNV encephalitis with a fatality rate of 38%, either due to natural causes or euthanasia. With each year, the number of equine cases has grown exponentially.<sup>192</sup> This may be due in part to the movement of WNV into areas where the *Culex* vectors feed more frequently on horses.

Horses are considered dead-end hosts because they produce a maximum viremia of  $10^3$  PFU/ml, which is well below the  $10^5$  PFU/ml threshold to infect mosquitoes.<sup>134</sup> Studies in horses have failed to show any correlation between age or gender and infection

rates in horses.<sup>192</sup> The primary risk factors for horses involves their proximity to communal bird roosts or waterfowl congregations.

### **Other species**

Beginning in 1999, serum samples were taken from dogs and cats in New York to determine their exposure to WNV.<sup>237</sup> Antibodies were detected in 5% of the dogs and in 0% of the cats, and none demonstrated clinical signs. Similar studies have been performed in Europe to examine the prevalence of WNV antibodies in dogs using the hemagglutination test.<sup>238</sup> There was a reported seroprevalence of only 0.7%, however, these results could not be completely interpreted because tick-borne encephalitis is also present in the area and is cross-reactive.

Recent studies have evaluated experimental infection of dogs and cats with WNV to determine their response to infection as well as their potential to serve as amplifying hosts.<sup>205</sup> The dogs were exposed to WNV through the experimentally caused bites of infected mosquitoes. None of the dogs exposed developed any clinical signs, nor developed high levels of viremia. Therefore, dogs are considered dead-end hosts.

Cats were exposed to WNV through the experimentally caused bites of infected mosquitoes, while four additional cats were exposed via oral ingestion of mice previously infected with WNV through an intraperitoneal inoculation.<sup>205</sup> All of the cats tested developed higher viremia levels than the dogs. Of the four cats inoculated via mosquitoes, three of the four exhibited lethargy and a febrile response, but no neurological signs. The four cats who ingested WNV infected mice developed similar levels of viremia but clinical signs were absent. This was the first time transmission via ingestion was reported. Cats have high enough virus levels to infect mosquitoes, although it is probably much less efficacious when compared to many avian hosts.

Studies into the disease status in most exotic and wildlife species are lacking. Recent serosurveys have been performed on captive primates in Louisiana, including rhesus macaques, pigtail macaques, and baboons.<sup>239</sup> Results indicated that approximately 36% of the primates had been exposed to WNV, indicated by PRNT testing. WNV has also recently been found to affect alligators.<sup>7,240</sup> The manner in which the alligators were originally exposed is still unclear. The alligators were farmed and had been fed horsemeat that tested positive via PCR for WNV. This has been suggested as a means of transmission, but has not been proven. The alligators were also under stressful growth conditions including year-round warmed-water and being housed in complete darkness, which may have resulted in immunosuppression. Alligators may prove an important reservoir host as they develop high viremia levels and may harbor virus overwinter.<sup>240</sup> In addition, lateral transmission and transmission via ingestion has also been shown experimentally.<sup>240</sup>

### **Clinical Diagnosis**

Diagnosis can be difficult because WNV resembles other related viruses and there are no pathognomonic signs for WNV. Most hematological and biochemical parameters remain within normal limits. Cerebrospinal fluid findings include mononuclear lymphocytic pleocytosis and elevated protein.<sup>195</sup>

The viremia in most mammals is relatively low and of short duration, so virus isolation is rarely performed. Growth of virus in cell culture remains the gold standard for viral detection but requires time to grow and to be identified.<sup>241</sup> Flaviviruses can be cultured in whole animals such as chick embryos, suckling mouse brain, and mosquitoes, as well as in primary or established cell lines of mammalian, avian, or insect origin.<sup>127</sup>

Serological testing is based on detection of IgM and/or neutralizing antibodies after exposure to WNV. Strongly neutralizing antibody is the most virus-specific.<sup>163</sup> In addition, IgM antibodies are more specific than IgG antibodies.<sup>242,243</sup> The PRNT is the gold standard for the serological diagnosis of WNV infection. This test has the advantage of differentiating between WNV and closely related flaviviruses.<sup>244</sup> The disadvantage of this test is that it requires the use of live virus in biosafety level 3 containment and is time consuming.<sup>245</sup> The MAC ELISA is highly specific and measures serum IgM antibody levels. These levels rise and fall rapidly in the horse compared to neutralizing responses to WNV.<sup>199</sup> A positive MAC ELISA is interpreted as exposure to WNV within 30 days. IgM antibodies are detectable around day 4 post-infection which usually coincides with the first signs of clinical disease.<sup>133,244</sup> Neutralizing antibodies generally appear around 4 to 5 days after the onset of illness.<sup>133,243</sup> In the CSF, the switch to IgG appears to be earlier, occurring around day 3 to 4 of illness. In humans, IgM can occur in the CSF before detection in the serum. Because this occurs, it is assumed that IgM production occurs locally due to intrathecal viral infection and not from the systemic circulation. Neutralizing antibodies are detectable for several months to years after acute infection. Over 90% of convalescent sera are positive 2 and 3 months after the onset of illness.<sup>243</sup>

In addition to the MAC ELISA, blocking or competitive inhibition (CI) ELISA testing has been performed for several different flaviviruses using monoclonal antibodies.<sup>246-249</sup> Studies using monoclonal antibodies, namely 5H10, 3A3, 7H2 and 5C5, have shown that all monoclonals strongly neutralized WNV strain I but not strain II.<sup>125</sup>

These experiments have shown that each monoclonal recognizes spatially distinct epitope structures.

RT-PCR has the advantage of speed, specificity, and sensitivity for the detection of viral RNA, but its utility is also dependent on the viremia.<sup>241,250</sup> Although the sensitivity and specificity of the assay are excellent, the many steps involved and the specialized lab necessary for the work made it an inefficient method to detect WNV for large numbers of birds.<sup>251</sup> The VecTest WNV/Saint Louis encephalitis virus Antigen Panel Assay (Medical Analysis Systems, Inc., Camarillo, CA) was originally developed to detect WNV or SLEV in infected mosquitoes.<sup>252</sup> However, recent studies have used this assay to detect the presence of WNV in oropharyngeal and cloacal swabs taken from birds.<sup>251,252</sup> Results indicated that oropharyngeal swabs were more sensitive than cloacal swabs when used for antigen detection.<sup>251</sup> In American crows, the sensitivity of the test was 83.3% with the specificity being 95.8%, making the assay an acceptable screening test for field use. The main advantage of this test is its speed, with results available within 15 minutes. The main disadvantages are that it has a lower sensitivity when compared to the RT-PCR TaqMan assay, as well as a decreased efficacy when working with non-corvid species.

### **Treatment and Prevention**

There is currently no treatment for WNV infection and treatment is primarily supportive. *In vitro* studies have found antivirals such as IFN- $\alpha$ 2b to be effective against the virus while ribavirin was protective but not therapeutic.<sup>191,253,254</sup> *In vivo* studies using mouse and hamster models has shown promise with IFN- $\alpha$ 2b therapy, while ribavirin treatment has been unsuccessful.<sup>255,256</sup> Recent studies with Dengue virus infections have

shown that mycophenolic acid, a non-nucleoside inhibitor of IMP dehydrogenase, has inhibited Dengue virus infection by reducing the levels of RNA produced.<sup>257</sup>

Epidemiological studies support a point source exposure to WNV, thus vaccination is a primary focus of prophylaxis. A formalin-inactivated vaccine has been marketed since 2001 for horses. Both horses and birds have been vaccinated using this product. Inactivated vaccines have the advantage of being relatively safe for animals and are relatively simple to develop. The main disadvantage of these vaccines is that they generally require multiple inoculations to produce an effective response. These vaccines generally do not elicit a sustained cellular immune response.<sup>258</sup>

Live attenuated vaccines should elicit a much stronger humoral and cellular immune response and have the advantage of requiring only a single dose. A recombinant vaccine against WNV using yellow fever 17D as a live vector for the envelope genes of WNV and other flaviviruses has been developed and is presently in human safety trials. Similar vaccines for Japanese encephalitis and dengue are currently under development and testing.<sup>258,259</sup> Additional vaccine candidates include recombinant DNA vaccines expressing various proteins and recombinant E-protein subunit vaccines.<sup>260-262</sup> The final option to prevent WNV infections is through mosquito control.

CHAPTER 3  
DEVELOPMENT OF A COMPETITIVE INHIBITION ENZYME-LINKED  
IMMUNOSORBENT ASSAY

**Introduction**

West Nile virus (WNV) is an arthropod-borne virus introduced to the New World in 1999 that causes neurological disease primarily in birds, horses, and humans.<sup>1</sup> Since its initial appearance, this virus has spread rapidly across the North American continent.<sup>3</sup> During 2003, WNV was established in much of the U.S., affecting over 9000 people with WNV encephalitis and WNV fever. Thus far, harbor seals, monk seals, alligators, gray squirrels, alpacas, llamas, a wolf, and one dog have been clinically affected by natural infections.<sup>4,12,13,17,18,22,23,28</sup> A wide range of other animals have seroconverted to WNV, although actual clinical status in these instances has not been documented. The infection and disease status has not been completely assessed for many species because of limited federal and state resources. Since WNV has been detected in a variety of birds and mammals, assessment of multiple species of free-ranging and captive wildlife species is necessary to assess the risk of WNV infection to these populations. A survey of WNV status in non-domestic animals in the state of Florida should include St. Louis encephalitis virus (SLEV) since this closely related Flavivirus cross-reacts with WNV antigens and could confound testing.<sup>21</sup>

The tests of choice, allowing highly specific serologic testing for viruses, are neutralization tests. These tests are applicable to multiple species, but are expensive as screening tools, requiring viral expertise, special facilities, and often special permits for

zoonotic agents. The goal of this research was to develop and validate a WNV specific ELISA using monoclonal antibodies in a competitive inhibition format (CI ELISA).

Currently, there are several serological methods utilized to identify WNV virus exposure in animals. The hemagglutination-inhibition test (HIT) is a commonly used screening test for WNV. These assays are labor intensive and require the use of nonspecific inhibitors. In addition, since related flaviviruses share many of the same epitopes, there is a high degree of cross-reactivity.<sup>26</sup> While the reverse transcription-polymerase chain reaction (RT-PCR) is an extremely sensitive and specific method of detecting WNV antigen in tissues, it has limited value with serum or cerebrospinal fluid (CSF).<sup>14</sup> Since the viremic stage occurs before the onset of clinical signs in mammalian hosts, virus is no longer present in plasma once clinical signs occur. Immunoglobulin M antibody capture ELISAs (MAC ELISA) and IgG direct ELISAs are both utilized.<sup>24</sup> The MAC ELISA has a high degree of sensitivity and specificity for both CSF and serum, but requires the use of species-specific antibodies. The IgG ELISA also requires the use of species-specific antibodies and has a higher degree of cross-reactivity than the MAC ELISA. Various monoclonals have been tested in their ability to react in a CI ELISA format for agreement with the PRNT in domestic mammals and birds.<sup>6,7</sup> Utilizing a panel of monoclonals and several antigens, this research tested whether or not the reagents could be used in a CI ELISA as a rapid WNV screening assay with broad applicability to multiple wildlife species.

### **Materials and Methods**

Serum samples from marine mammals were obtained from various U.S. zoological institutions. All blood collection was taken for routine purposes by the institutions or researchers, and not for the purpose of this study. Sites included: Miami seaquarium,

Columbus zoo and aquarium, Lowry Park Zoo, Rio Grande zoo and Six Flags Ohio. In addition, samples from wild manatee captures were provided on a yearly basis by the Florida Fish and Wildlife Conservation Commission's Fish & Wildlife Research Institute (FWRI), and by the United States Geological Survey (USGS) via their Sirenia project. Samples were tested at the University of Florida, College of Veterinary Medicine in Gainesville, Florida. It was conducted under authorization of the IACUC, number C411, the United States Fish and Wildlife (USFW) permit numbers MA791721-3 and MA067116-0 and the National Oceanic and Atmospheric Administration (NOAA) permit number 1054-1731-00.

In addition to marine mammals, several other species of animals were tested. In conjunction with the Ringling Brother's circus, elephant serum samples obtained for routine blood work were provided from several populations of elephants in the state of Florida. Alligator serum samples were provided by Dr. Elliot Jacobson. Archived horse and domestic cat serum was also used. The remaining serum samples were from various exotic species collected for routine clinicopathological testing and were provided by the Clinical Pathology department at the University of Florida's Veterinary Teaching Hospital from the years 2002 and 2003.

### **Reagents**

Two sources of WNV antigen were utilized for the CI ELISA and included WNV mouse-brain antigen from Bioreliance (Rockville, MD) and inactivated WNV recombinant antigen developed by Acambis Inc. (Cambridge, MA gifted by Intervet Incorporated). Positive and negative controls consisted of equine serum obtained from NVSL (Ames, IA). The monoclonal antibodies, 7H2, 3A3, and 5H10, were purchased from Bioreliance (Rockville, MD). Horseradish peroxidase (HRP)-conjugated goat

anti-mouse IgG antibody was purchased from Amersham Biosciences (Piscataway, NJ). The substrate used was 3, 3', 5, 5'-tetramethylbenzidine solution (TMB) (KPL, Gaithersburg, MD).

### **Plaque Reduction Neutralization Test**

A total of 262 serum samples were tested representing 28 different species of animals. Samples were kept frozen and shipped overnight to the Florida Department of Health where they were tested using the plaque reduction neutralization test (PRNT). The samples consisted of 212 different individual animals. Several animals were sampled more than once.

Briefly, samples were titered out from 1:10 and 1:100 for testing in a sample buffer composed of M-199 salts, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 units/mL penicillin, and 100 mg/L streptomycin in 0.05 Tris, pH 7.6 in sterile microtiter plates. Virus (75 mls-100 PFU) and 75 mL of serum was mixed and incubated at 37°C for 75 minutes. After incubation, the mixture was added to flasks containing confluent monolayers of Vero cells. Following incubation at 37°C for 60 minutes, flasks were overlaid with agar and incubated for 72 hours. A second agar overlay containing neutral red was added and the flasks examined after 24 hours. Plaque reduction of >90% was considered positive. A PRNT titer of at least 1:10 in serum was considered positive and specific for WNV.

### **Competitive Inhibition Enzyme-Linked Immunosorbent Assay**

A 96-well Immulon 2HB plate (Fisher, Pittsburgh, PA) was coated with mouse-brain antigen at a dilution of 1:100 in coating buffer (carbonate/bicarbonate pH 9.6), and allowed to incubate overnight at 4°C. The plate was washed and the wells

blocked with 150  $\mu$ L 5% Blotto (5% instant powdered milk in 20 mM Tris pH 7.6, 0.15 NaCl, 0.05% Tween20), sealed and incubated for one hour at room temperature. The serum/plasma samples were diluted 1:2 in the serum/control diluent and 60  $\mu$ L placed on a transfer plate (Fisher, Pittsburgh, PA).

Monoclonal antibodies were diluted at 1:1000 (3A3 and 5H10) and 1:5000 (7H2) in the serum/control diluent. These dilutions were based on individual reactivity against mouse-brain WNV positive and negative antigen (data not shown). Sixty  $\mu$ L of the monoclonal (MAb) mixture was added to each well of the transfer plate, except for the blank and the non-specific binding (NSB) wells. The MAb wells contained the serum/control diluent and the monoclonal antibody mixture only. The NSB and blank wells contained the serum/control diluent only. All other wells contained the sample and the monoclonal antibody mixture. A serum/control diluent was prepared consisting of 48 mL of phosphate buffered saline with Tween-20 (PBST) and 2 mL of normal goat serum. A conjugate diluent was prepared consisting of 48 ml of 5% Blotto and 2 ml of normal goat serum. Following blocking, the Immulon 2HB plate was washed and 100  $\mu$ L of the sample/monoclonal combination was transferred to the corresponding wells of the Immulon 2HB plate. The plate was incubated for one hour at 37°C. Following incubation, the plate was washed and 100  $\mu$ L of HRP-conjugated goat anti-mouse IgG antibody diluted at 1:1000 in conjugate diluent was added to each well except the blank, which contained PBST only. The plate was incubated for 30 minutes at 37°C. The plate was washed and 100  $\mu$ L of TMB substrate was added to each well. The plate was allowed to develop for 15 minutes at room temperature in a dark drawer. The reaction was stopped after 15 minutes with 100  $\mu$ L of 1% HCl solution and the plate read

at 450 nm using a Bio-Tek plate reader (Bio-Tek Instruments, Winooski, VT). Positive samples were determined by measuring the percent inhibition of the negative control using the optical density (OD). The formula used for this was  $[100 - (\text{sample OD} / \text{negative control OD} * 100)]$ .

## **Optimization Procedures**

### **Monoclonal optimization**

In order to determine whether or not a combination of three monoclonal antibodies was superior to running a single monoclonal, plates were tested using varying dilutions of the monoclonal antibodies. Plates were coated using the WNV positive mouse-brain antigen at 1:100 and the CI ELISA was performed as previously described. Each monoclonal was run both separately and as a group to compare results. Positive, negative, blank, and NSB controls were run on all plates. All monoclonals were compared against each other and against the monoclonal combination. The best performing monoclonal preparation was then serially diluted to determine optimal dilution on a checkerboard plate.

### **Antigen and monoclonal optimization**

Separate checkerboard plates were tested to determine the optimal dilution of two separate antigens in combination with several dilutions of the monoclonal antibody 7H2. One plate was coated with two dilutions of the mouse-brain antigen, 1:50 and 1:100. Another plate was coated with inactivated West Nile ChimeriVax® antigen (Intervet, Millsboro, DE) in a checkerboard fashion with dilutions including 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128. For both antigens, different dilutions of the 7H2 monoclonal were tested including 1:2000, 1:4000, and 1:5000. Positive, negative, blank, and NSB controls were run on all plates.

### **Statistical Analysis**

Available samples that had been tested using the PRNT were aliquotted, blinded, and randomized for testing with the CI ELISA. After the completion of testing of all the samples, the sample numbers were matched up with previous PRNT test results to determine the proper cut-off value to be used for the CI ELISA based on statistical analysis. One plate was selected at random and run in triplicate on different days to determine inter-assay variability. Sensitivity and specificity values were performed by the following formulas: Sensitivity was calculated by using the formula  $[\text{True positives} / (\text{True positives} + \text{false negatives})]$ . Specificity was calculated by using the formula  $[\text{True negatives} / (\text{True negatives} + \text{false positives})]$ . Statistics were calculated using a commercial statistics software program (MedCalc, Mariakerke, Belgium). The coefficient of variance was calculated by the formula  $(\text{standard deviation} / \text{average}) \times 100$ .

### **Results**

There were 262 samples from 28 species collected and tested by PRNT for the presence of neutralizing antibody against WNV and SLE (Table 3-1). Seven species (12 individuals) were positive for WNV neutralizing antibody and these included alpacas, camels, cougars, elephants, lions, tigers, and a box turtle. Four species (11 individuals) were positive against SLEV and these included camels, elephants, a lion and a tiger. When positive, WNV titers ranged between 10 and 640 and SLEV titers ranged between 20 and 640 (Table 3-1). 3 species (8 individuals) had titer values for both WNV and SLEV.

Table 3-1. Plaque reduction neutralization results by species

Collection dates	Species	# WNV/ #Tested	# SLE/ #Tested	WNV titer
				SLE titer
Oct 2002	Alpaca	2/3	0/3	>320;>40
				Not applicable
May 2002	Bat	0/1	Not tested	Not applicable
May-Aug, Oct 2002	Bovine	0/10	0/10	Not applicable
Dec 2002	Camel	2/2	2/2	1:640; >320
				1:40; 1:40
Jun 2002	Capuchin	0/1	0/1	Not applicable
Nov 2002	Cougar	1/2	0/2	1:320
				Not applicable
Jun, Sep 2002	Deer	0/3	0/2	Not applicable
Mar, May, Jul, Aug, Oct, Dec 2002	Bottlenose dolphin	0/12	0/12	Not applicable
Jan, Apr 2003	Asian elephant	4/26	7/26	1:10; >40; 1:80; 1:80
				1:10; 1:10 1:20; 1:40; >40; 1:80; 1:160
Jun 2002	Ferret	0/1	0/1	Not applicable
May, Jun, Aug-Dec 2002	Goat	0/12	0/5	Not applicable
Nov 2002	Kangaroo	0/1	0/1	Not applicable
May, Aug, Oct 2002	Killer whale	0/3	0/3	Not applicable
May 2002	Leopard	0/1	Not tested	Not applicable
Jun, Aug 2002	Lion	1/2	1/1	>320
				1:40
Aug, Sep 2002	Llama	0/5	0/1	Not applicable
Jul 2002	Macaque	0/1	Not tested	Not applicable
Jan-Apr, Jun-Sep, Dec (1999-2003)	Manatee	0/136	0/69	Not applicable
Dec 2002	Monkey	0/1	0/1	Not applicable
Jan, Feb 2003	Monk parakeet	0/21	0/21	Not applicable
Jul, Aug 2002	Porcupine	0/2	Not tested	Not applicable
Sep, Nov 2002	Rabbit	0/2	0/1	Not applicable
Mar, Apr, Jul, Dec 2002	Sea lion	0/4	0/4	Not applicable
Aug 2002	Sheep	0/1	0/1	Not applicable
Jun 2002	Spider monkey	0/1	0/1	Not applicable
Jan, May, Jun, Dec (2002-2003)	Tiger	1/4	1/4	1:160
				1:640
Aug 2003	Box turtle	1/1	0/1	1:10
Aug 2003	Tortoise	0/1	0/1	Not applicable

## Monoclonal Optimization

The highest optical densities were achieved with monoclonal 7H2 alone or by all three monoclonals used simultaneously. The optical densities for 7H2 MAb alone was nearly identical to that obtained by using all three MAbs (Figure 3-1).

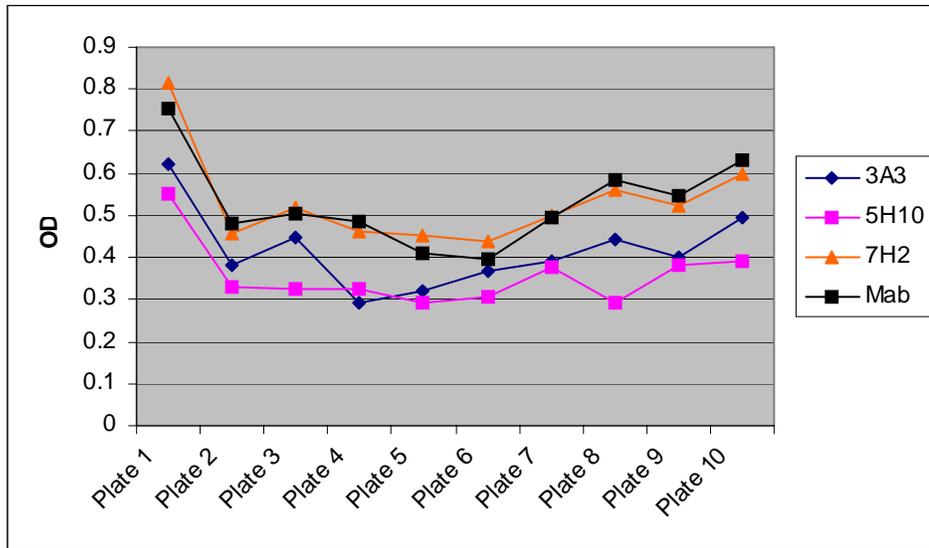


Figure 3-1. Comparison using MAbs 3A3, 5H10, and 7H2 separately and as a group (Mab). Plates were coated with mouse-brain derived antigen at a 1:100 dilution. Monoclonal dilutions were 1:1000 (3A3, 5H10) and 1:5000 (7H2).

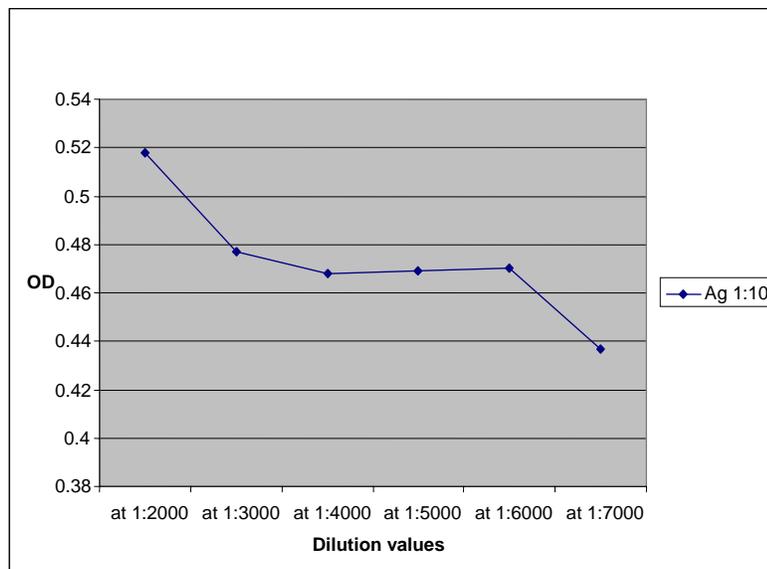


Figure 3-2. Determining optimal 7H2 dilution by testing several dilutions on the CI ELISA. Plates were coated with mouse-brain antigen at a 1:100 dilution.

Because there was no additional contribution of 3A3 and 5H10, serial dilutions of 7H2 were investigated. Results indicated that a 7H2 dilution of 1:4000 was appropriate (Figure 3-2).

### Antigen and Monoclonal Antibody Optimization

A total of nine plates were run using the mouse-brain antigen and compared to each other for inter-assay variation. Results using the mouse-brain antigen were inconsistent with an inter-assay variation based on a calculated coefficient of variance (CV) of 21.8% with a standard deviation of 7%. The NSB using the mouse-brain antigen was extremely high, ranging from 0.08 to 0.33. The two dilutions of mouse-brain antigen exhibited little difference. The dilution of 1:50 resulted in a slightly higher optical density when compared to the 1:100 dilution (Figure 3-3).

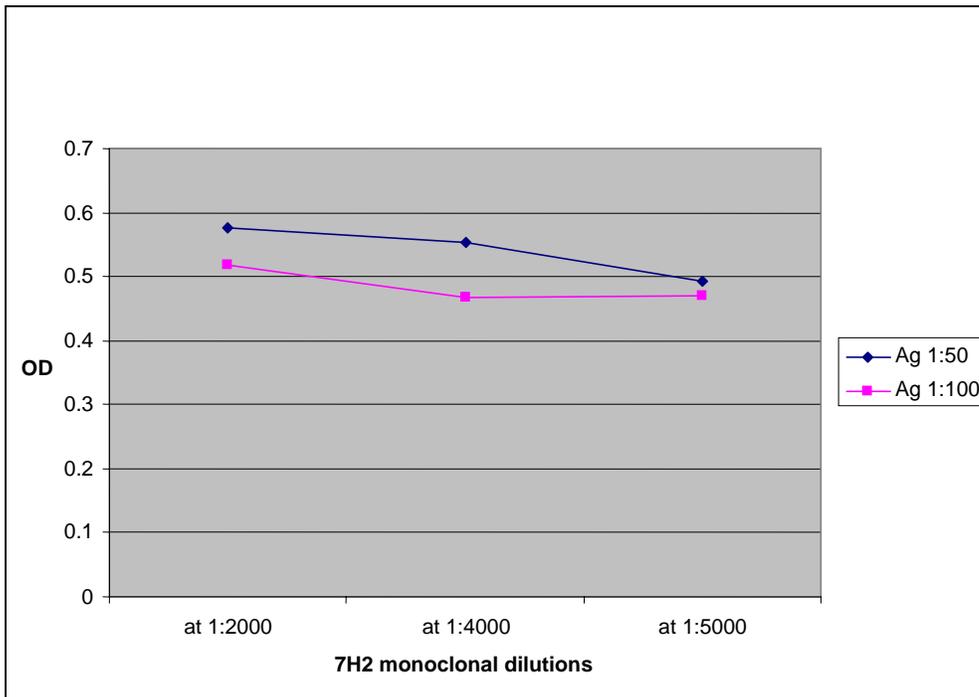


Figure 3-3. Plates were coated with mouse-brain antigen at two separate dilutions (1:50, 1:100). Several dilutions of the 7H2 monoclonal were tested against the antigen dilutions for reactivity.

The 7H2 performed similarly at the dilutions 1:2000 and 1:4000, but levels dropped off at 1:5000. Using the ChimeriVax® antigen at dilutions 1:2 through 1:32 resulted in optical densities that were nearly identical for all controls (Figure 3-4). The dilution of 1:64 resulted in the highest optical density with good separation between positive, negative, and monoclonal controls. The 1:128 dilution was slightly higher, but still not at the level of 1:64. Inter-assay variation based on a calculated coefficient of variance (CV) for three plates consisting of 35 samples was 7.9% with a standard deviation of 4.9%.

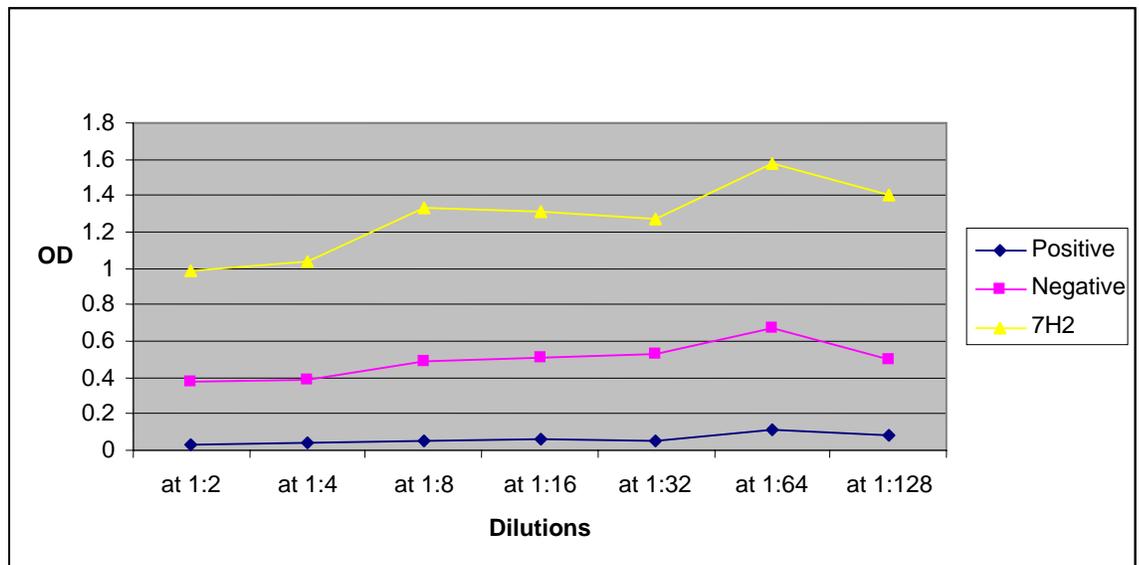


Figure 3-4. ChimeriVax® antigen was serially diluted and used to coat a plate. Positive, negative, and 7H2 controls were tested against each dilution for reactivity.

### Statistical Analysis

The cut-off for interpretation of the CI ELISA results was determined by two different values, raw optical density and percent inhibition using only the horse and alligator samples. The first method was based on the OD compared to the results of the PRNT. Utilizing a receiver operating characteristic (ROC) curve analysis, the OD cut-off value was determined to be at 0.550 with a sensitivity of 92.9% with a 95% confidence interval of (76.5 to 98.9) and a specificity of 97.2% and a 95% confidence interval of

(90.2 to 99.6). All animals above the 0.550 cut-off would be considered negative for WNV exposure (Figures 3-5 and 3-6).

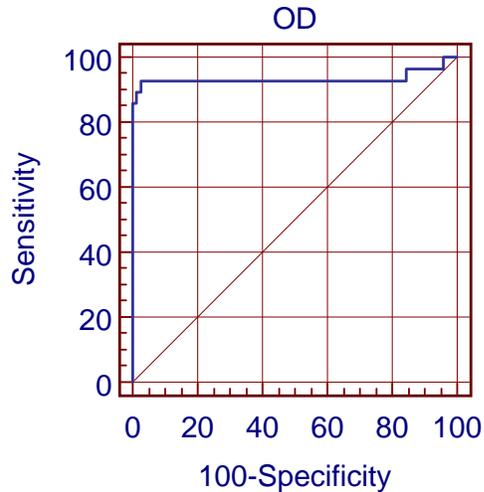


Figure 3-5. An ROC curve demonstrating the sensitivity and specificity of the CI-ELISA using an OD cut-off value of 0.550.

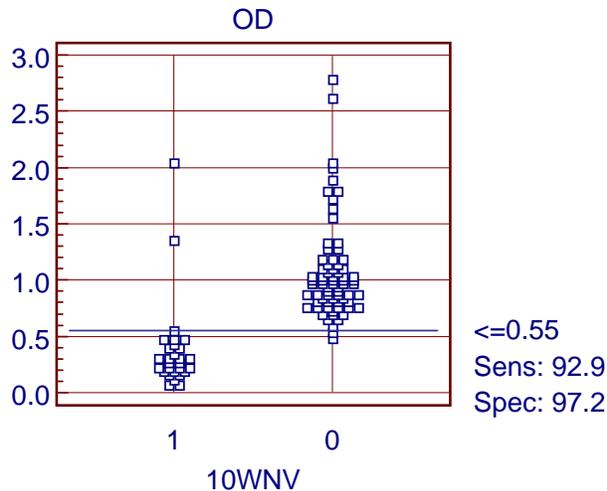


Figure 3-6. Scattergram demonstrating the separation of the positive WNV animals (1) and the negative WNV animals (0).

Using an ROC curve analysis, the percent inhibition cut-off was determined to be at 28% inhibition. Any sample testing greater than 28% inhibition would be considered WNV positive. Using this cut-off there was a sensitivity of 92.6% with a

95% confidence interval of (75.7 to 98.9) and a specificity of 100.0% with a confidence interval of (94.9 to 100.0) (Figures 3-7 and 3-8).

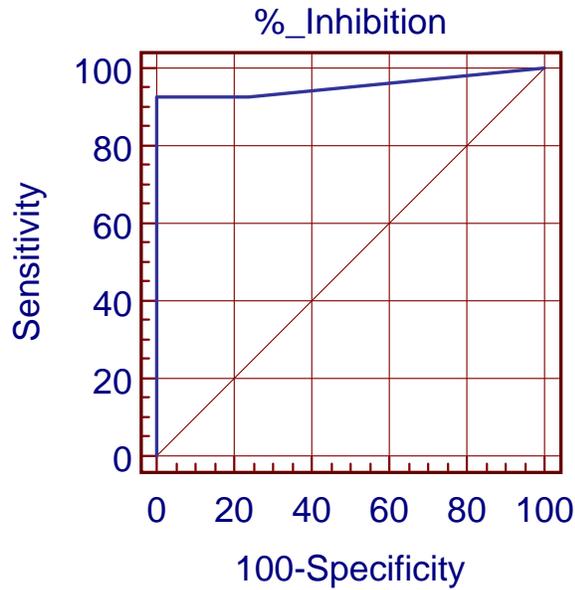


Figure 3-7. An ROC curve demonstrating the 100% specificity and 92.6% sensitivity with a large area under the curve using a percent inhibition cut-off value.

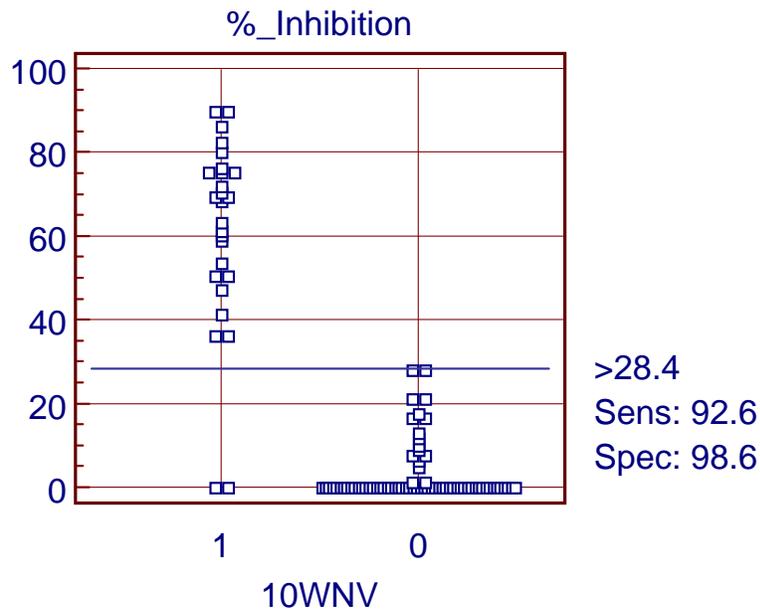


Figure 3-8. Scattergram demonstrating the separation of the positive animals (1) and the negative animals (0) using the 28% inhibition cut-off value.

The sensitivity and specificity were also analyzed by separate species. Horse samples had a sensitivity of 84.6% and a specificity of 96.2% using an OD cut-off of 0.549. Their distribution is demonstrated in Figure 3-9. When examining the alligator data alone, there is 100% specificity and sensitivity using a 28% inhibition cut-off value (Figure 3-10). Domestic cats were tested using the CI ELISA; however, none of the available samples were positive by either WNV or SLEV PRNT. A small number of exotic cats were also examined. There was 100% agreement between the CI ELISA interpretation and the PRNT results using the predetermined 28% cut-off for both the exotic and domestic cats (Table 3-2).

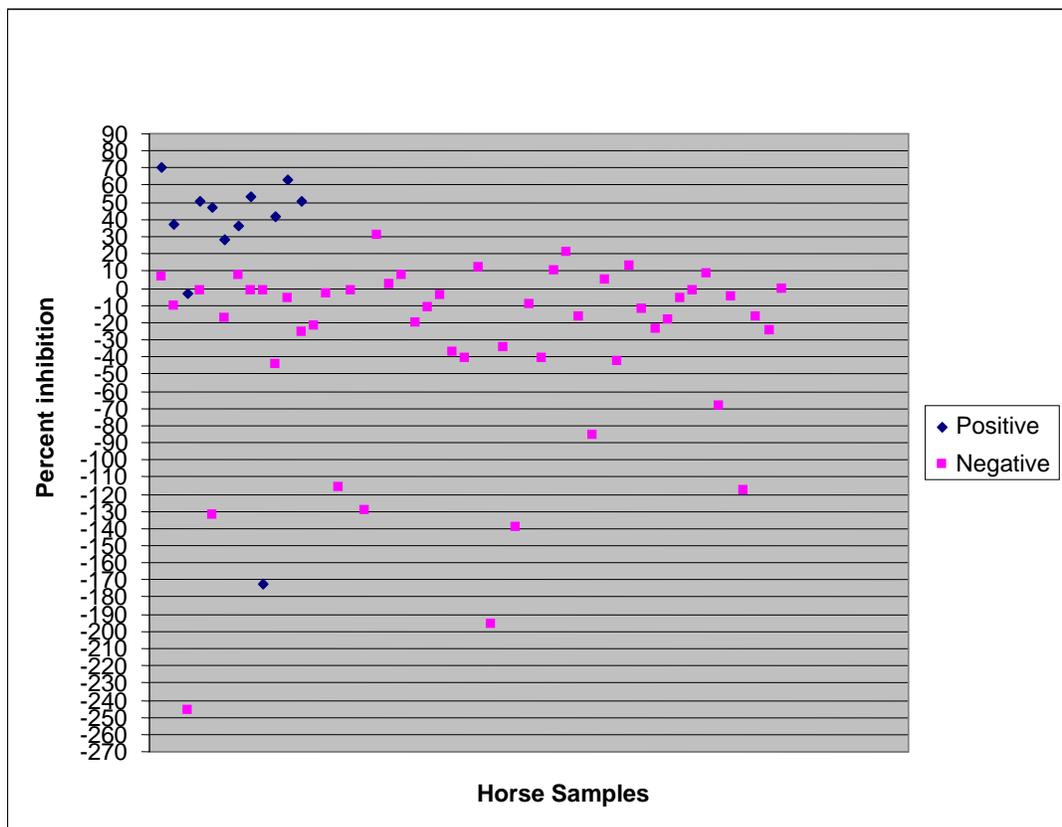


Figure 3-9. Distribution of PRNT-tested positive and negative horses tested on the CI ELISA and compared to a 28% inhibition cut-off value.

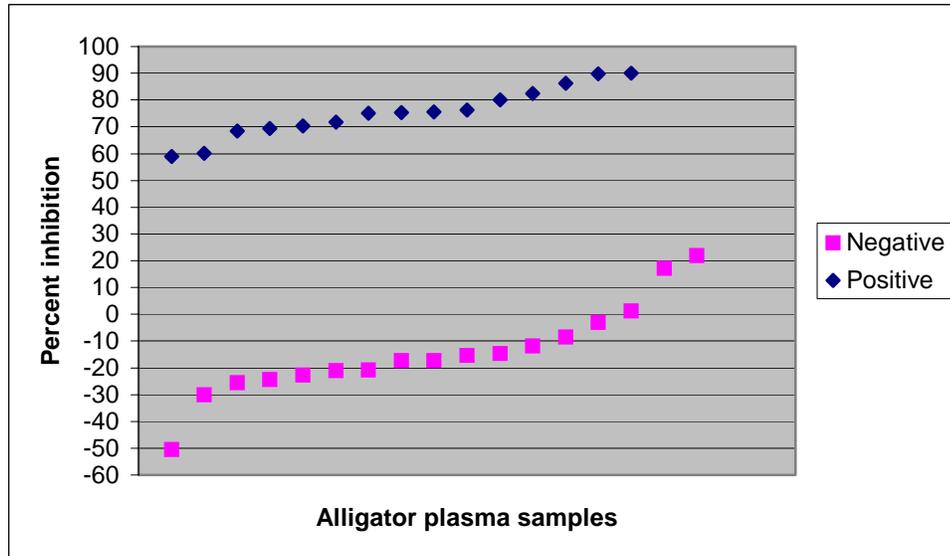


Figure 3-10. Scattergram representing results of known positive and negative alligator plasma samples as tested on the CI ELISA.

Table 3-2. Agreement between cat samples on the CI ELISA and the PRNT. All samples are domestic cat unless otherwise noted.

Animal #	Date sampled	WNV PRNT titer	Percent inhibition of MAb/Optical density
4697	2/27/04	<10	0/0.9255
4715	2/27/04	<10	0/0.756
4717	2/27/04	<10	0/1.0835
4735	2/27/04	<10	13.8/0.647
4795	2/27/04	<10	0.03/0.7485
93192	8/7/02	<10	0/1.067
96191	7/23/02	<10	0/0.9805
103160	9/4/02	<10	0/1.26
130393	8/21/02	<10	0/0.9505
152563	8/7/02	<10	0/0.9575
152563	8/14/02	<10	0/0.9215
158542	7/19/02	<10	0/0.718
158721	8/27/02	<10	0/1.1495
160137	8/15/02	<10	0/0.869
160660	7/16/02	<10	0/0.9625
160739	8/27/02	<10	0/1.2825
161429	8/14/02	<10	0/1.159
164597	7/29/02	<10	0/1.1565
164714	8/12/02	<10	0/1.019
165326	8/15/02	<10	0/1.975
165356	7/29/02	<10	0/1.304
165399	7/24/02	<10	0/0.85
165408	7/29/02	<10	0/1.132

Table 3-2. Continued.

Animal #	Date sampled	WNV PRNT titer	Percent inhibition of MAb/Optical density
165476	8/5/02	<10	0/1.1125
165750	7/30/02	<10	0/0.93
165804	8/12/02	<10	0/1.105
165908	8/14/02	<10	0/1.0435
165927	8/14/02	<10	0/1.019
166186	9/4/02	<10	0/1.043
166566	8/12/02	<10	0/0.9905
A1B5	2/27/04	<10	8.4/0.6875
A1C7	2/27/04	<10	3.2/0.7265
A1F7	2/27/04	<10	20.9/0.594
AGD6	2/27/04	<10	11.1/0.7345
AGK5	2/27/04	<10	14.1/0.71
AHD2	2/27/04	<10	19.6/0.6475
AHS5	2/27/04	<10	14.4/0.6425
AHT4	2/27/04	<10	10.7/0.67
AIC6	2/27/04	<10	24.5/0.591
AICY	2/27/04	<10	13.9/0.712
IFH3	2/27/04	<10	0/1.0635
IGK4	2/27/04	<10	0/0.8585
IGN2	2/27/04	<10	18.9/0.6085
IGW2	2/27/04	<10	3.1/0.801
Q1G2	2/27/04	<10	16.2/0.693
QDCI	2/27/04	<10	0/1.4825
QGS6	2/27/04	<10	0/0.9665
QHV4	2/27/04	<10	22.0/0.5855
QJA5	2/27/04	<10	13.7/0.648
QJG1	2/27/04	<10	19.8/0.602
163563 (leopard)	5/3/02	<10	0/1.047
163099 (tiger)	5/8/02	<10	0/0.9195
167804 (cougar)	11/6/02	<10	0/0.978
144566 (tiger)	1/13/03	>40	38.7/0.507
144566 (tiger)	1/10/03	1:160	54.9/0.353

Of the 20 non-manatee marine mammal samples tested, only one was PRNT positive. This particular animal was a clinically ill harbor seal (gift from Dr. Ned Gentz at the Rio Grande Zoo). Using the 28% cut-off, the distribution of the samples is shown in Figure 3-11. Of the 126 manatees tested, all tested negative on the PRNT assay. Figure 3-12 illustrates the manatees that tested above the 28% cut-off using the CI ELISA. There are four false positives.

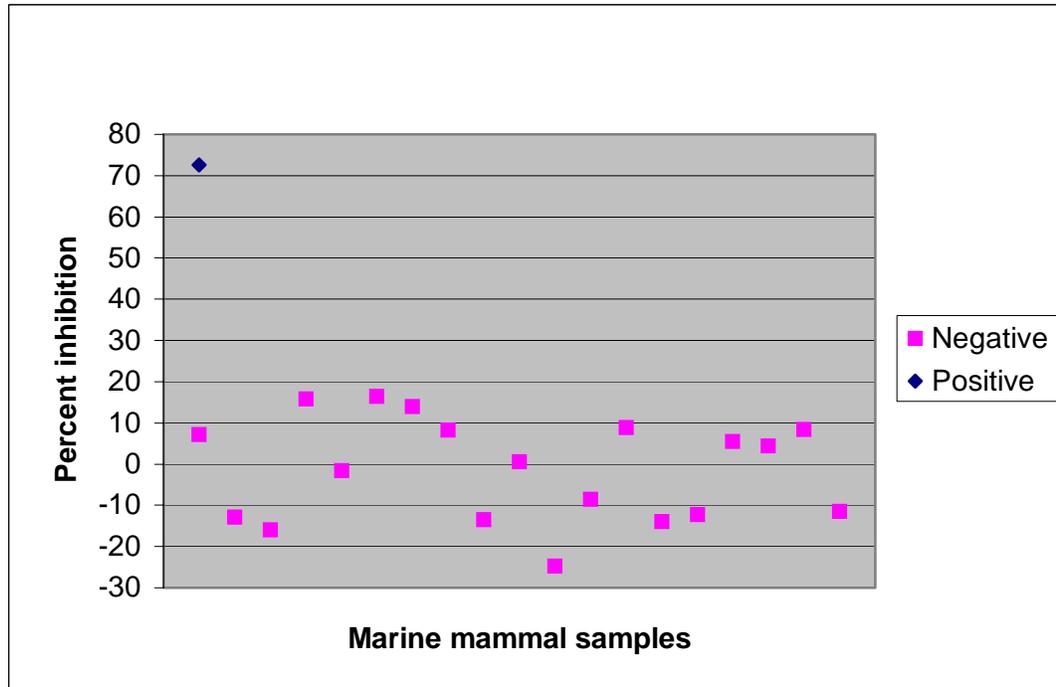


Figure 3-11. Scattergram of the non-manatee marine mammal samples as tested on the CI ELISA using the percent inhibition cut-off.

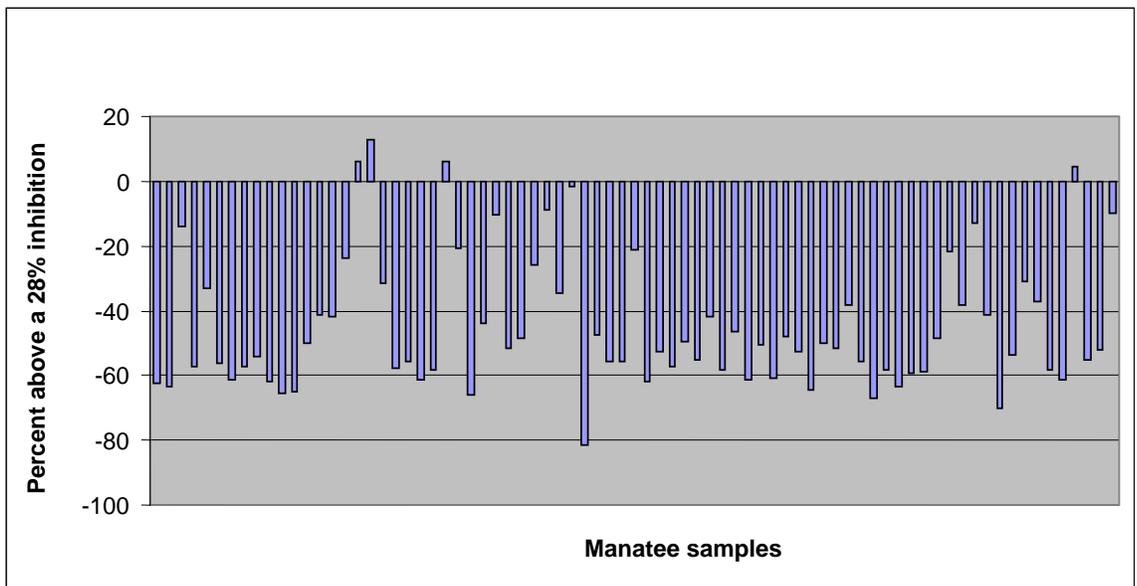


Figure 3-12. Level above a 28% cut-off for each manatee tested. Note that any bars going above the zero line would be suspect positives requiring further PRNT testing.

There is disagreement of the positive and negative elephants (Table 3-3) based on their performance on the CI ELISA. Using a 28% cut-off, there are three false negatives and there is no correct identification of positive animals using this assay. Since the elephant serum may react differently with the monoclonal, an ROC curve analysis of the elephant samples was performed. This resulted in a sensitivity of 100% with a 95% confidence interval of (30.5 to 100), but a specificity of only 20% with a 95% confidence interval of (5.9 to 43.7). The best cut-off that could be determined was at 0% inhibition which essentially renders the CI ELISA inappropriate for this species under these assay conditions.

Table 3-3. Disagreement between PRNT results and CI ELISA results of the elephant samples.

Elephant sample #	Date sample taken	WNV PRNT titer	% Inhibition of MAb	Agreement
1	1/30/2003	<10	0	Yes
2	1/30/2003	<10	0	Yes
3	1/30/2003	<10	0	Yes
4	1/30/2003	<10	0	Yes
5	1/30/2003	<10	0	Yes
6	1/30/2003	1:10	0	No
7	1/30/2003	<10	22.7	Yes
8	4/8/2003	<10	0	Yes
9	4/8/2003	<10	0	Yes
10	4/8/2003	<10	0	Yes
11	4/8/2003	>40	0	No
12	4/8/2003	<10	0	Yes
13	4/8/2003	<10	0	Yes
14	4/8/2003	<10	0	Yes
1	4/11/2003	<10	0	Yes
2	4/11/2003	<10	6.8	Yes
4	4/11/2003	<10	0	Yes
6	4/11/2003	<10	0	Yes
15	4/11/2003	>40	9.1	No
7	4/11/2003	<10	11.5	Yes
4	10/16/2003	<10	0	Yes
4	10/30/2003	<10	0	Yes
4	11/3/2003	<10	0	Yes

## Discussion

Historically, there had been no arboviruses described in any aquatic species. There have been recent reports of alphaviruses in rainbow trout (*Oncorhynchus mykiss*),<sup>25</sup> Atlantic salmon (*Salmo salar*),<sup>27</sup> and southern elephant seals (*Mirounga leonina*).<sup>19</sup> This latter finding is of particular interest since the virus was isolated from the elephant seal louse (*Lepidophthirus macrorhini*) which routinely infests the population of southern elephant seals. It has been suggested that this new virus is transmitted by the louse, primarily since the island has no mosquitoes and the seals are only rarely affected with ticks. There was a report of a captive killer whale clinically affected with SLEV which later died of the disease.<sup>8</sup> Confirmation was made using virus isolation. More recently, several captive harbor seals (*Phoca vitulina*) and a Hawaiian monk seal (*Monachus schauinslandi*) were clinically affected with WNV.<sup>11,12,23</sup> These seals exhibited clinical signs including weakness, head tremor, anorexia, and abnormal swimming. Lesions included a nonsuppurative encephalitis, meningitis, and myocarditis.

West Nile virus has been reported in many other wildlife and domestic species. Clinically affected terrestrial mammalian species include squirrels, bats, chipmunks, rabbits, raccoons, dogs, and cats.<sup>2,4,13,15,20</sup> There have been reports of several canids with clinical WNV infection.<sup>18</sup> A wolf pup and an adult 8-year old dog exhibited clinical signs including anorexia, weakness, ataxia, ptyalism, head tilt, and blindness.<sup>18</sup> The dog had a concurrent immune-mediated thrombocytopenia which may have contributed to disease susceptibility. Many more mammalian species have seroconverted to WNV without development of clinical disease.

Reptiles and amphibians are also susceptible to WNV. Experimentally infected lake frogs (*Rana ridibunda*) not only developed a high titer, but were able to transmit the

virus to *Culex pipiens*.<sup>16</sup> More recently alligators have exhibited clinical signs with WNV infection.<sup>22</sup> Clinical signs seen in these animals included “star gazing”, ataxia, and muscle spasms.

Our CI ELISA screening of blinded samples did not identify any positive marine mammals. Early experience with WNV suggests that pinnipeds may be the marine mammal group most affected. Our sampling primarily consisted of cetacean and sirenian sampling with few pinniped samples. Although it would appear that cetaceans are most likely not affected by WNV, the SLEV infection reported previously in a captive killer whale illustrates that such infections cannot be entirely ruled out.<sup>8</sup>

Several WNV and SLEV positive elephants were identified during the screenings performed in this current study. All of these elephants have been in the U.S. for several years, removing the likelihood of exposure prior to arrival into the U.S. There are no published reports identifying seropositive elephants. There were no clinical signs reported in any of the elephants with elevated titers. It would appear, based on this preliminary data, that WNV infections remain unapparent in Asian elephants.

Previous CI ELISA formats described in the literature relied on the use of only one monoclonal with sensitivities and specificities of 87% and 86%, respectively.<sup>6,7</sup> The competitive inhibition format with three monoclonals was investigated to enhance the performance of this test. The surface located WNV E-protein has multiple functions associated with virulence which include cell attachment and invasion. Complete neutralization is difficult to achieve with only one monoclonal. Hence it was hypothesized that multiple monoclonals that neutralized or reacted with this large protein would be more correlated with an ELISA format. A competitive format using Japanese

encephalitis virus demonstrated 82.1% and 100% sensitivity and specificity when compared to a neutralization format.<sup>10</sup> In studies done by Beasley et al., three mouse monoclonal antibodies were tested for their reactivity with the WNV E-protein domain III.<sup>5</sup> Results indicated that all three monoclonal antibodies strongly neutralized the virus. However, no enhancement was observed regarding inhibition in the ELISA format. The monoclonal 7H2 demonstrated reaction at or near the level of the monoclonal combination and the benefit of using all three was not evident. The protocol was changed to use monoclonal 7H2 only for all the future CI assays that were performed.

The original CI assay used mouse-brain antigen which resulted in problematic results. There was a high degree of non-specific binding that was interfering with the results. In addition, the inter-assay variation was quite high. Other antigens were tested (data not shown) but were cost prohibitive. The WNV chimera antigen was far superior to the mouse-brain antigen for several reasons. There was lower non-specific binding, and had better reactivity antigen specific sera and monoclonals than the mouse-brain antigen.

Two species were used to determine a cut-off for the CI ELISA. A domestic species, the horse, was selected because they are clinically affected with disease. Alligators were chosen because they represented a wild species which also develops clinical disease. In addition, there were many representative samples from these two species.

There was a good degree of consistency using this assay based on the CV results. The cut-off value selected using the ROC curve analysis allowed identification of the area on the curve with the highest sensitivity and specificity. By this analysis, this assay

has higher specificity than sensitivity. Using the ROC, this value can be increased by changing the cut-off value. The specificity of the test would be decreased. However, since the PRNT is the gold-standard, confirmatory testing would then be used to validate the CI ELISA results in a diagnostic laboratory setting.

When a cut-off value using the percent inhibition is calculated, the sensitivity remains nearly identical to that obtained using the OD cut-off. The specificity, however, improves to 100%. Using the percent inhibition cut-off is more accurate, not only based on these results, but also allows for the inter-assay variation that occurs between plates. Therefore, it is recommended that calculation of percent inhibition be based on the negative control of each plate.

Different species performed very differently on the assay and the assay should be validated for each species. Although the original intention was for the CI ELISA to be used as a screening assay, it is a test that has a high degree of specificity rather than a high degree of sensitivity. It is clear, based on these findings, that the assay would need to be validated for each individual species or group of animals prior to use as a screening test. The assay performed moderately well on horse samples. Most horses today are vaccinated against WNV and it remains unclear how this may be affecting the results. The assay performed extremely well for alligator samples, giving 100% specificity and sensitivity. All the positive alligators were clinically ill and likely had high viremic levels. Based on this preliminary data, this may be an excellent assay for alligators and demonstrates that both serum and plasma can be used. Further sampling should be performed using wild-exposed alligators to determine whether the test will be sensitive enough to identify those animals.

Cats are another group of animals where WNV may be of clinical importance and serosurvey value. It has been demonstrated experimentally that domestic cats develop higher viremic levels than most species, ranging from  $10^{3.2}$  to  $10^{4.0}$  PFU/mL.<sup>4</sup> In addition, they have been shown to be clinically affected by WNV and have the ability to become infected through the ingestion of infected mice. The viremia in the cats is not consistently above the  $10^5$  PFU/ml threshold necessary for the host to transmit the virus to mosquitoes.<sup>9</sup> However, this level is only an average and can vary between mosquito species, therefore, cats could play a minor role as an amplifying host. The exotic cat sampling consisted of only 5 samples. This is not enough to determine the validity of the test. The preliminary results show 100% agreement with the PRNT and more samples are necessary to further validate this format as a potential screening tool for this family. The domestic cat samples obtained were all PRNT negative, therefore, no conclusions could be made concerning the assay. A separate study concerning cats and WNV is currently in progress.

There were not enough non-manatee marine mammal sample numbers to determine whether or not this test will be of value in those species. And of those, there was only one PRNT positive animal. In general, marine mammals do not seem to have significant exposure to WNV. The only group of marine mammals where WNV has been a reported problem, and could continue to be in the future, has been the captive pinnipeds.<sup>11,12,23</sup> If more of these infections become apparent, these samples could be used to validate the test for pinnipeds.

As was the case with the cats, all of the manatees tested on the PRNT assay were negative. Therefore, this assay is not currently validated for this species given that no

animals confirmed with WNV exposure were found in our PRNT confirmed dataset. Most manatees did stay under the ~28% cut-off that was developed using the horse and alligator samples. If used as a screening assay, those few animals that tested above the cut-off level would have needed to be confirmed using the PRNT assay, where they would have been found negative.

There was no discernable method to develop a cut-off value with the elephant samples. There were numerous false positives and false negatives. It is unclear why the elephants reacted so poorly with this assay compared to other species. In order to verify whether the PRNT assay results are accurate, rescreening of the elephants is necessary. Further validation with a Western blot format may be necessary. If they are confirmed, then this assay should not be considered for screening elephants and only serves to illustrate the importance of validating the ELISA for each individual group of animals.

CHAPTER 4  
USE OF A COMPETITIVE INHIBITION ENZYME LINKED IMMUNOSORBENT  
ASSAY FOR DETECTING WEST NILE VIRUS EXPOSURE IN A POPULATION OF  
CAPTIVE BATS

**Introduction**

West Nile virus (WNV) is an arthropod-borne virus introduced to the New World in 1999 that causes neurological disease primarily in birds, horses, and humans.<sup>1</sup> Since its initial appearance, this virus has spread rapidly across the North American continent.<sup>2</sup> Thus far, harbor seals, monk seals, alligators, gray squirrels, alpacas, llamas, a wolf, and one dog have all been clinically affected by natural infections.<sup>3-5,7-10,205</sup> A wide range of other animals has seroconverted to WNV with unknown clinical status. During 2003, WNV was established in much of the U.S., affecting over 9000 people with WNV encephalitis and WNV fever. The actual infection and disease status has not been completely assessed for many species. West Nile virus has been identified several species of bats including big brown bats (*Eptesicus fuscus*) and little brown bats (*Myotis lucifugus*).<sup>207</sup> Serosurveys have been performed on some species of bats (*Eptesicus fuscus*, *M. lucifugus*, and *M. septentrionalis*) indicating a low level of seropositivity (2.4%) to WNV.<sup>270</sup> No studies have been performed with the species in this study or in bats in Florida. A competitive-inhibition enzyme-linked immunosorbent assay (CI ELISA) has been developed (see chapter 3) to detect seroconversion to WNV for various wildlife and domestic species. Our specific aim is to perform a serosurvey of several species of captive bats and determine their exposure levels to WNV.

## Materials and Methods

Bat plasma samples were obtained from routine blood samples taken from bats at the Lube bat conservancy (Gainesville, FL). Although these animals are captive, they are kept outside in pens, and consequently are exposed to mosquitoes. A total of 39 animals representing five species were sampled (Table 4-1).

Table 4-1. Sample breakdown of the bat species tested

Species	Number sampled
<i>Pteropus vampyrus</i> (Large flying fox)	18
<i>Pteropus pumilus</i> (Little golden-mantled flying fox)	6
<i>Pteropus hypomelanus</i> (Island flying fox)	5
<i>Pteropus giganteus</i> (Indian flying fox)	5
<i>Pteropus poliocephalus</i> (Gray-headed flying fox)	5

## Testing

Samples were tested using the CI-ELISA as previously described. Briefly, a 96-well Immulon 2HB plate (Fisher, Pittsburgh, PA) was coated with WNV recombinant antigen and allowed to incubate overnight at 4°C. The wells were blocked with 150 µL of 5% Blotto for one hour at room temperature. The plasma samples were diluted 1:2 and added to the plate with monoclonal antibody 7H2 (Bioreliance, Rockville, MD). The plate was incubated for one hour at 37°C. Following incubation, a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Amersham Biosciences, Piscataway, NJ) was added. The plate was incubated for 30 minutes at 37°C after which 3, 3', 5, 5'-tetramethylbenzidine solution (TMB) (KPL, Gaithersburg, MD) was added to each well and the plate allowed to develop for 15 minutes at room temperature in a dark drawer. The reaction was stopped after 15 minutes with 1% HCl solution and the plate read at 450 nm using a Bio-Tek plate reader (Bio-Tek Instruments, Winooski, VT). Positive samples were determined by measuring the percent inhibition of the negative

control. The formula used for this was  $[100 - (\text{sample OD} / \text{negative control OD} * 100)]$ . Greater than 28% inhibition was considered positive.

### Results

Results showed that a majority of animals tested above the cut-off OD (Table 4-2). The average OD of all samples was 0.396 with an average percent inhibition of 55%. Even assuming a cut-off value of 50% or higher, this would still result in 32/39 with a positive interpretation. Although *P. vampyrus* has a lower prevalence, this may be due to the higher number of samples tested for that species.

Table 4-2. Competitive inhibition ELISA results on bats tested showing a large percentage of positive samples

Species	Number testing positive	Percent positive
<i>P. vampyrus</i>	13/18	72.2
<i>P. pumilus</i>	6/6	100
<i>P. hypomelanus</i>	5/5	100
<i>P. giganteus</i>	5/5	100
<i>P. poliocephalus</i>	5/5	100
TOTAL	34/39	87.17

### Conclusions

Although many of these bats tested well above the cut-off, this is the highest degree of seropositivity in any of the species tested thus far. Either these are true positive samples and there is a high degree of subclinical exposure in this species in Florida or there is a very high degree of background reactivity in bat testing. None of the bats are reported to have shown any clinical signs. As no overt clinical signs were reported in any of the animals, it is possible that most infections are unapparent or this test is inappropriate for use in this species.

Based on testing of this assay in multiple species, identification of true positive and negative based on the gold standard, PRNT will be required to determine the true prevalence of WNV in bats in Florida. Validation of this CI ELISA using bat plasma should be performed to optimize the test for bat samples. Further testing of these samples will be performed using virus neutralization to confirm infection of bats in Florida with WNV.

## CHAPTER 5 SUMMARY

The purpose of this study was to develop a rapid screening assay to test numerous wildlife and domestic species for WNV exposure. This assay could then be used to screen a broad range of species for WNV exposure. Rapid, broad-based screening assays require a test to be highly sensitive and easy to use. The PRNT assay is time consuming (a minimum of 4 days) and requires the use of live virus and a BSL-3 laboratory in which to perform it.<sup>a</sup> This CI ELISA has the benefit of taking only two days to perform and does not require the use of a BSL-3 laboratory.

The mouse brain WNV antigen, a commercially available inactivated antigen, while having acceptable antigen specific reactivity, proved unreliable. The antigen resulted in high interassay variation and high non-specific binding (NSB). Because of the requirement for live virus and live animal technology, this antigen is expensive and difficult to obtain. The WNV chimera antigen was selected for use in this assay because it was far superior to the mouse brain antigen. There was lower NSB and it had improved reactivity with antigen-specific sera and monoclonals than the mouse brain antigen.

The two species used to determine a cut-off between negative and positive for the CI ELISA were the horse and the alligator. Both of these species are clinically affected by the disease and represent domestic and wild species. Two methods were tested to establish a cut-off value; an OD cut-off, and a percent inhibition cut-off. The percent

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<sup>a</sup> Centers for Disease Control and Prevention and National Institutes of Health. Biosafety in microbiological and biomedical laboratory (BMBL). 4th edition. Washington: U.S. Government Printing Office; 1999.

inhibition cut-off value provided a better degree of sensitivity and controlled for interassay variation.

Using the ROC analysis, the cut-off value can be modified to allow for improved sensitivity. If an assay has increased sensitivity, specificity will decrease and more false positives will occur which will need to be confirmed using the PRNT assay. However, since the PRNT is the gold-standard, confirmatory testing would then be used to validate the CI ELISA results in a diagnostic laboratory setting, irrespective of CI ELISA findings.

Of the various species tested, only the horses and the alligators provided enough positive and negative samples for the assay to be validated. The assay resulted in excellent sensitivity and specificity when tested with the alligator samples. All the positive alligators were clinically ill and likely had high viremic levels. Based on this preliminary data, this is an excellent assay for the alligator population and demonstrates that both serum and plasma can be used. Further sampling should be performed using wild-exposed alligators to determine whether the test will be sensitive enough to identify those animals. The horses samples, when tested on the assay, had better specificity than sensitivity. Most horses today are vaccinated against WNV and it remains unclear how this may affect overall test performance. Irrespective this assay needs validation for each individual species for use.

The marine mammal CI ELISA screening did not result in any positive marine mammals. The only positive sample tested on this ELISA was a clinically ill harbor seal sample. Because of the lack of positive samples, validation was not possible for this group of animals. The only group of marine mammals where WNV has been a reported

problem are the pinnipeds.<sup>3,5,6</sup> If more of these infections become apparent, these samples could be used to validate the test for the pinnipeds. All of the manatees tested on the PRNT assay were negative. Most manatees did stay under the ~28% cut-off that was developed using the horse and alligator samples. If used as a screening assay, any animals testing above this cut-off require confirmation with the PRNT assay.

The elephant screening resulted in several positive elephants for both WNV and SLEV exposure. One elephant was positive for both. None of these elephants were clinically ill; therefore, it appears that WNV infection may not be a concern for this species. Using an ROC curve, it was apparent that this version of the CI ELISA does not agree well with the PRNT testing in this species.

The cat screening included only a few positive exotic cat samples, but preliminary results demonstrate 100% agreement with the PRNT. More samples are necessary to validate this format as a screening tool for this exotics. A separate study concerning cats and WNV is currently in progress.

The bats screened had a large number of potentially positive animals in their population. These animals are very commonly exposed to mosquitoes and their exposure may be similar to that of birds. However, this assay requires validation for each species, so further testing of these samples is required before we can confirm whether these bats have been exposed to WNV.

Few animals were SLEV positive. Therefore the extent, if any, of cross-reactivity could not be determined. The degree of specificity may change when testing multiple species that have a high degree of positivity for SLEV.

Although the original intention was for the CI ELISA to be used as a screening assay, this project indicates that the test has a high degree of specificity rather than a high degree of sensitivity. The ROC curve can be used to adjust the cut-off value and increase sensitivity if desired. This assay reacts differently across species and requires validation on each individual species before any broad-based testing applications can be done.

APPENDIX A  
TESTING OF VERO CELL ANTIGEN ON THE CI ELISA

Inactivated West Nile virus (WNV) Vero cell antigen (gift from Dr. Elliot Jacobsen) was tested for reactivity on the CI ELISA. As with the mouse brain WNV antigen, a checkerboard plate was designed using Vero cell antigen dilutions of 1:250, 1:500, 1:1000, and 1:2000. Based on previous results, only the 7H2 monoclonal was used at a dilution of 1:4000. The remainder of the CI ELISA plate was run as previously described.

Results indicated that the Vero cell antigen outperformed any results previously obtained from the mouse brain antigen. In addition, the plate development occurred much faster than expected and the reaction needed to be stopped at 10 minutes as compared to 15 minutes with the mouse brain antigen. The Vero cell antigen was cost prohibitive and as a result, we did not continue to pursue this antigen.

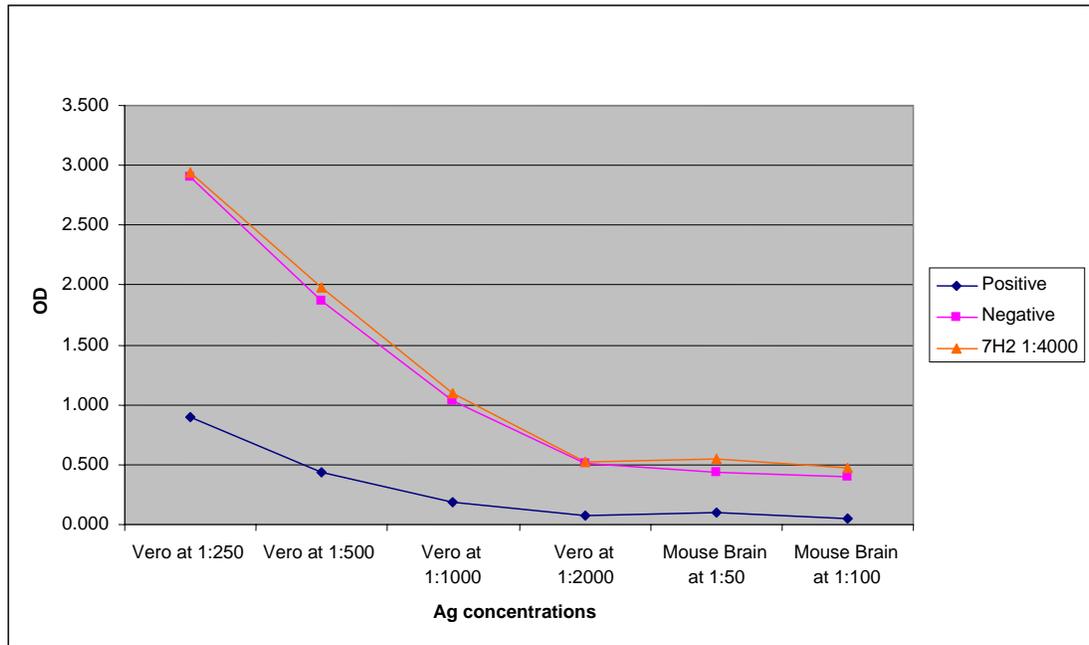


Figure A-1. Composite graph comparing the results obtained using the Vero cell antigen and the mouse brain antigen. Positive, negative, and NSB controls were tested on all plates.

APPENDIX B  
OPTIMIZATION OF THE CI ELISA USING THE CHIMERIVAX® ANTIGEN

**Antigen Dilutions**

A checkerboard plate was designed to determine the optimal dilution for the new ChimeriVax® antigen. Dilutions were tested at 1:1, 1:2, 1:8, 1:16, 1:32, 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600. Positive, negative, 7H2, blank, and NSB controls were tested for all dilutions.

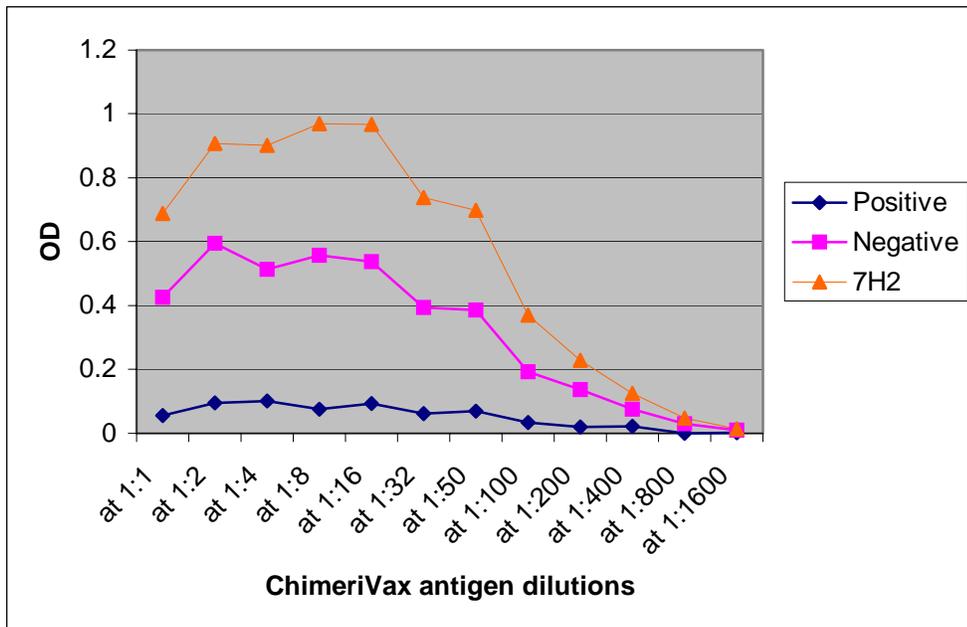


Figure B-1. Competitive inhibition ELISA plate coated with the ChimeriVax® antigen. Positive, negative, 7H2, and NSB controls were tested.

The assay performed well (Figure B-1) using the ChimeriVax® antigen until the 1:32 dilution, where levels began to drop off. Based on these results, a dilution of 1:16 was selected for all future plates using this antigen.

### Monoclonal Antibodies

As with the mouse brain WNV antigen, all three monoclonal antibodies were tested using the antibodies alone at a 1:1000 dilution (3A3 and 5H10) and a 1:4000 dilution (7H2). A combination of all three antibodies together (MAb) was also tested. Positive, negative, blank, and NSB controls were run on all plates.

As with the mouse brain WNV antigen, the optical densities of the 7H2 and the MAb were closely associated (Figure B-2). In contrast, the 3A3 and the 5H10 resulted in lower optical densities. As a result, the 1:4000 dilution of the 7H2 was continued for use in all future assays using the ChimeriVax® antigen.

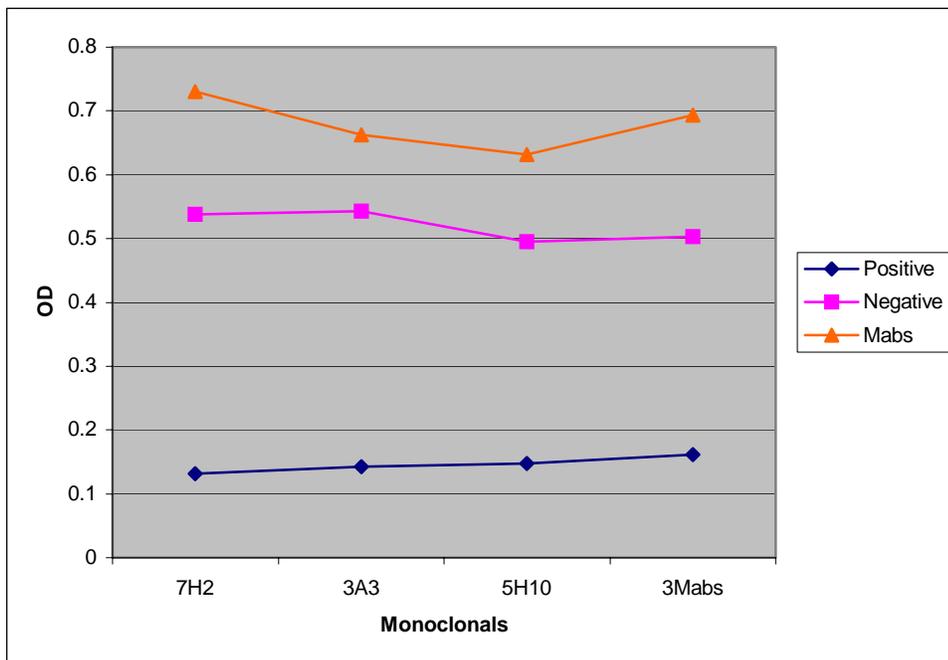


Figure B-2. Competitive inhibition ELISA plate coated with the ChimeriVax® antigen at a dilution of 1:16. Monoclonals 7H2 (1:4000), 3A3 (1:1000), and 5H10 (1:1000) were tested against this antigen for reactivity.

### Comparison of Conjugate Diluents

The conjugate diluent originally used for the mouse brain antigen was 5% Blotto with normal goat serum. As noted in Chapter 3, the original mouse brain antigen resulted

in very high non-specific binding (NSB). Because of this, several blocking agents were used throughout the assay to control the NSB. Using the Blotto was an attempt to reduce the NSB. Once the antigen was changed, testing was performed to determine whether this extra blocking step should be continued or should be eliminated.

### **Procedure**

A plate was coated with the ChimeriVax® antigen at a 1:16 dilution. Standard controls were tested including positive, negative, NSB, and blanks. The left half of the plate was tested using PBST only as the diluent for the conjugated antibody. The right half of the plate used the conjugate diluent as previously described. In addition, the samples and controls were run at dilutions ranging from 1:2 to 1:16 to determine optimal performance.

### **Results**

Using conjugated antibody diluted in PBST alone resulted in improved results with no increase in NSB. The NSB average for the Blotto plate was .0485 while the NSB for the plate using the PBST only was .0455. For the positive control, there was more reactivity when using the non-blocked conjugate (Figure B-3). In addition, the dilution of 1:2 resulted in the best reactivity. Results were similar for the negative control (Figure B-4).

Using these results, the procedure for the CI ELISA was modified to dilute the conjugated antibody in PBST only instead of the blocking solution. All CI ELISA procedures in the body of the thesis were performed using PBST diluent alone.

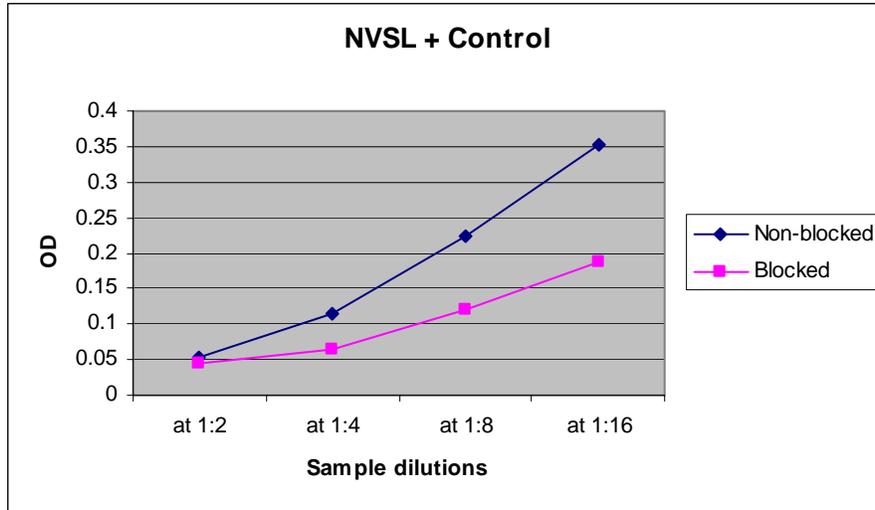


Figure B-3. Testing the NVSL positive control at varying dilutions to compare diluting the conjugated antibody in a blocking solution versus using PBST alone.

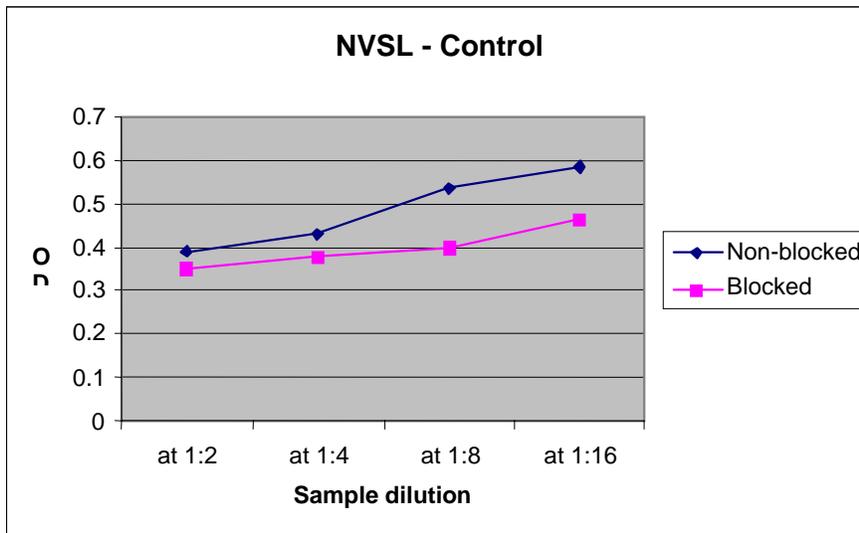


Figure B-4. Testing the NVSL negative control at varying dilutions to compare diluting the conjugated antibody in a blocking solution versus using PBST alone.

### Negative Control

Although the ChimeriVax® antigen was performing well, the negative control was not optimal and several additional negative controls were tested. Serum from several screened negative horses was collected and pooled to serve as a negative control. This control was tested against the positive, negative, NSB, and MAb controls and against two

known positive animals; India, a borderline PRNT positive elephant at 1:10 and Frankie a PRNT positive horse.

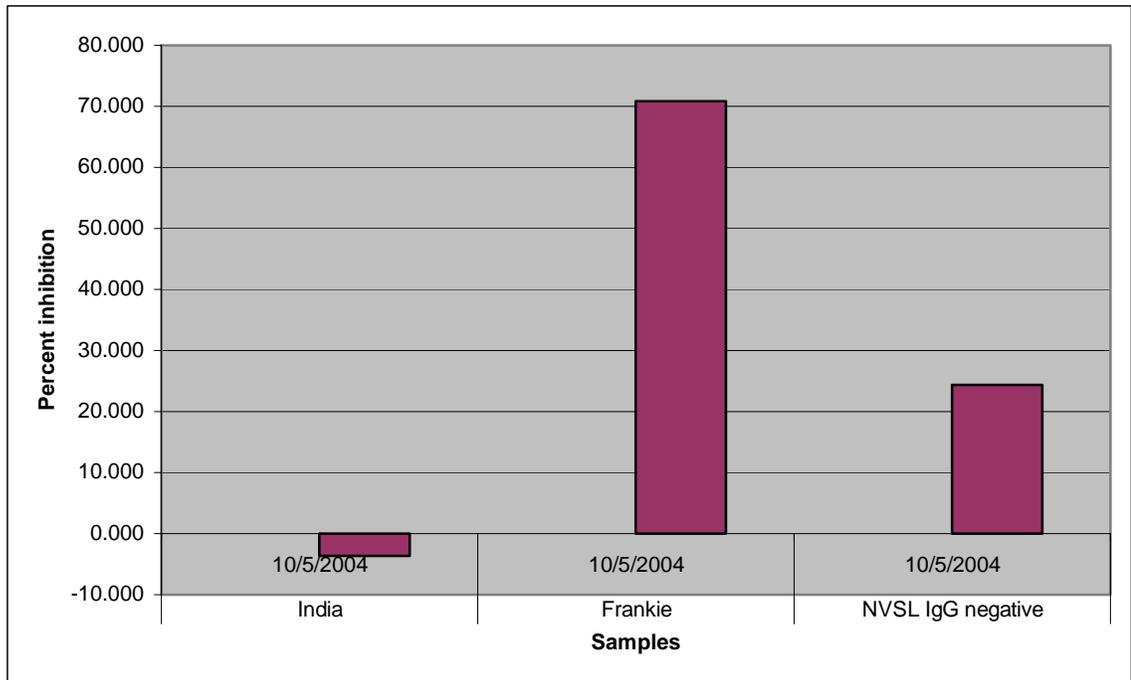


Figure B-5. Comparison of known positive serum samples and their percent inhibition using the new negative control and the new recombinant antigen.

The pooled negative control (Figure B-5) clearly identified Frankie as a positive horse with over 70% inhibition and placed India at the borderline. As a comparison, the NVSL IgG negative control that had been used has quite a high inhibition compared to the new control. This negative control was adopted for use in all future CI ELISA validation procedures.

### **Second ChimeriVax® Antigen Batch**

Once the CI ELISA protocol had been finalized, we requested a new shipment of antigen. This new shipment proved to be a different dilution than the previous one. Therefore, a new checkerboard plate was run to determine the level of optimal performance. This procedure is described in Chapter 3 and was determined to be 1:64.

Therefore, all validation procedures performed in the thesis were done with the new batch of antigen at a 1:64 dilution.

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## BIOGRAPHICAL SKETCH

Martha Keller began her studies as a music education major at the University of Tulsa. She pursued her music education degree for 3 years before deciding that that career was not for her. She eventually enrolled as an Animal Science/Pre-Vet major at Oklahoma State University. She was accepted into veterinary school before completing her bachelor's degree and was awarded her Doctor of Veterinary Medicine from Oklahoma State University in 2000. She spent 1 year in private practice at a small animal/exotics practice before accepting an internship position at the University of Florida. She completed her internship in small animal medicine and surgery in July of 2002 and began her graduate studies at the University of Florida.