# PROTECTIVE IMMUNE RESPONSES TO WEST NILE VIRUS (WNV) INFECTION IN HORSES

By

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To my parents for their love and support.

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West Nile Virus (WNV) is a mosquito-borne encephalitic flavivirus affecting humans and horses since its emergence in the United States in 1999. From epidemiological studies it is known that natural exposure results in life-long immunity with other flaviviruses and is expected with WNV. The immunologic components which determine this protective immunity have only been partially defined and primarily in murine models. The objective of this study was to determine what mediates long-term immunity to WNV utilizing an equine model of WNV induced encephalomyelitis. The hypothesis was that a protective immune response against WNV infection was induced by vaccination with a modified live WNV vaccine and was composed of an antigen specific $T_H1$ $CD4^+$T cell immune response.

Immunological characterization of the intrathecal model of WNV was studied with injection of virulent WNV in a serologically naïve weanling colt (ID1) and a non-naïve filly (ID2). ID1 developed WNV encephalomyelitis at seven days post-infection with detection of viremia, and histopathological changes. ID2 was completely protected from disease with no viremia.

The efficacy of three commercially available equine WNV vaccines was tested in horses using the model of WNV encephalitis. Twenty-four healthy, WNV-seronegative horses of

varying ages and gender were placed into three randomized, blinded trial groups consisting of eight horses with two horses in each group receiving 1) an inactivated (K-WN) WNV vaccine, 2) a modified-live vaccine (CP-WN), containing the WNV prM and E proteins expressed by a canarypox vector 3) a live-chimera vaccine (WN-FV) containing WNV prM and E proteins expressed in a YF17D vector, or 4) diluent control. Challenge by this model caused grave neurological signs, viremia, moderate to severe histopathologic lesions in the brain and spinal cord, and an outcome of 0% survivorship in all six control horses. In contrast, challenge in horses at between 28 days post-vaccination of the chimera vaccine or 56 days post vaccination of the commercial inactivated or modified-live vaccines resulted in 100% survivorship, protection from the onset of WN encephalitis and viremia. Horses vaccinated with the live-chimera vaccine showed significantly fewer clinical signs than horses vaccinated with inactivated vaccine ($p=0.035$) and the control horses ($p \leq 0.01$).

Protective recall responses were seen in vaccinated horses with WNV challenge and data was supportive of the hypothesis that vaccinated horses have WNV antigen specific $T_H1$ $CD4^+$ T cell response. WNV proliferative responses were most significantly increased in the K-WN and WN-FV vaccinates at 21 days after challenge, with an increased IFN-γ/IL-4 ratio and both groups had increased $CD4^+$ T cell populations. A protective response was also characterized by the rapid induction of an antibody response mediated at least in two of the vaccines by IL-5. WNV antibodies for IgG total, IgGa, IgGb were detected after vaccination in the K-WN and CP-WN horses and there were marked increases in antibodies in the CP-WN and WN-FV horses by D14PC. Successful response also appeared to be correlated with low expression levels of TNF-α. In contrast, naïve control horses had significant expression of TNF-α after virulent challenge

and developed a primary immune response to WNV infection characterized by detectable IgG total, IgGa, IgGb at D7, and no WNV antigen specific lymphocyte proliferation.

# CHAPTER 1
# INTRODUCTION

Since its emergence into the United States in 1999, West Nile Virus (WNV) has killed a total number of 928 humans, approximately 6,000 horses, and an estimated hundreds of thousands of birds. [1,2] This mosquito-borne encephalitic flavivirus has caused clinical disease in 23,962 humans (both West Nile Fever and West Nile Encephalitis (WNE)) and 24,824 horses (WNE).[1,2] With the heightened attention surrounding this serious pathogen, scientific focus has centered with the development of diagnostic tests, therapeutics, and prophylaxis, including the three WNV (killed, modified live, DNA) vaccines that have been licensed for use in the horse. Several other vaccines are currently in various levels of experimental evaluation and licensure. These newer vaccines stimulate both humoral and cell-mediated immune responses. [27,76,91,127,160] Cell-mediated immunity has been shown in murine models to be an important player in the long-term resistance to infection with flaviviruses.[31] The relevance of the immune response in mice to actual development of protective responses in natural disease is yet unknown.

The overall goal of this dissertation was to determine what mediates protective immunity to West Nile Virus in horses. It was hypothesized that a protective immune response against WNV infection, induced by vaccination with a modified live WNV vaccine, was composed of an antigen specific $T_H1$ $CD4^+$ T cell immune response. The following specific aims were identified:

- **Specific aim 1:** To determine whether or not a protective immune response against intrathecal WNV infection is induced by vaccination in horses.

  The objective was to compare the clinical efficacy in the CNS and periphery of three equine WNV vaccines using an intrathecal WNV infection model.

  It was hypothesized that, in modified live vaccinated horses there was protection against WNV challenge in terms of onset of clinical disease and outcome.

- **Specific aim 2:** To determine whether or not vaccinated horses have a WNV antigen specific $T_H1$ $CD4^+$ T cell immune response.

  The objective was to characterize the lymphocyte subsets of equine PBMC, lymphoproliferative responses, *ex vivo* and *in vitro* cytokine mRNA profiles, and immunoglobulin isotypes of vaccinated horses.

  It was hypothesized that, modified live vaccinated horses would have a WNV antigen specific $T_H1$ $CD4^+$ T cell immune response.

# CHAPTER 2
# LITERATURE REVIEW

## Central Nervous System Infections in Horses

Infections of the central nervous system (CNS) of horses are uncommon but when they do occur, they are some of the most devastating and frequently fatal diseases in horses. Diseases such as equine protozoal myeloencephalitis (EPM) and West Nile virus encephalomyelitis (WNE) have had a significant economic impact on the equine industry in recent years and stimulated investigations into preventative, diagnostic, and therapeutic alternatives for CNS infections in horses.

Viral, bacterial, rickettsial, protozoal, parasitic and fungal pathogens may cause CNS infections in horses (Table 2-1). In small animals and in people causes of meningoencephalitis in order of decreasing frequency are viral, bacterial, protozoal, rickettsial, parasitic, and fungal while, in the horse, the most commonly diagnosed CNS infections are probably of viral and protozoal origin.[37,51] In a 1999 Australian study, 30 of 450 horses with neurological disease had an infectious or inflammatory disease and 11 of these 30 had meningitis.[150] This study does not reflect the emergence of WNV in the United States in 1999 nor does it account for CNS pathogens that are present in North America such as Eastern equine encephalomyelitis (EEE) or EPM.

Regardless of the type of etiologic agent involved, CNS infections require an accurate and rapid diagnosis and implementation of an appropriate course of treatment by the attending clinician. Central nervous system infection should be suspected in horses with abnormal mentation, seizures, blindness, multiple cranial nerve abnormalities, and general proprioceptive deficits. Infections involving primarily the spinal cord may manifest as limb weakness,

incoordination, and stiffness with or without associated cerebral dysfunction. The following is an overview of CNS infection, pathogenesis, diagnosis and treatment.

The appropriate term for infection and resultant inflammation of the CNS is determined by the specific area of the nervous system affected. Inflammation of the brain, meninges, spinal cord, and peripheral nerves is termed encephalitis, meningitis, myelitis, and neuritis, respectively. Rhomboencephalitis and cerebellitis refers to localized inflammation of the brain stem and cerebellum, respectively.[28,52] Frequently more than one tissue or anatomical site may be affected. Meningoencephalitis is inflammation of the meninges and brain and meningoencephalomyelitis is inflammation of the meninges, brain, and spinal cord. Inflammation of the brain and spinal cord, without meningeal involvement, is termed myeloencephalitis.

Infection of the CNS can also result in focal suppuration of the brain parenchyma or spinal cord and formation of abscesses. Localized areas of infection between the outermost meningeal layer (dura mater) and the skull and vertebral column are termed epidural abscesses. Inflammation between the outer two layers of the meninges (the dura mater and arachnoid) is termed subdural empyema.[51]

**Neuroanatomy and Disease**

**Brain and meninges**

Inside the protective barrier of the skull, the brain is surrounded by three layers of meninges: the outermost dura mater or pachymeninges and the leptomeninges consisting of the inner arachnoid and pia mater. The pia mater is continuous with the external surface of the brain and spinal cord, forming cuffs around penetrating vessels and merging with the ependymal lining of the fourth ventricle. Cerebrospinal fluid (CSF) occupies the subarachnoid space between the pia mater and arachnoid. Acute bacterial infections within the subarachnoid space typically

begin with the leptomeninges of the brain and spinal cord and spread inward through the foramina of the fourth ventricle. Infections between the dura mater and arachnoid (subdural empyema) can spread over the entire cerebral hemisphere. The dura mater adheres to the periosteum of the skull and limits the spread of epidural abscesses (between the dura and skull) except where it invaginates into the cranial cavity to form four rigid septa: the falx cerebri, falx cerebelli, tentorium cerebelli, and diaphragma selli.[51]

The brain rests within the anterior, middle, and posterior cranial fossae which are associated with the paranasal sinuses. The anterior fossa forms the roof of the frontal and ethmoidal sinuses. The sella turcica is located between the left and right middle fossa and forms the roof of the sphenoid sinuses. Infection in these paranasal sinuses can spread through the respective fossa centrally to the brain resulting in epidural abscesses and subdural empyema. In the horse, these infections can either be bacterial or fungal.[3,7,19,22,68] Middle ear infections (otits media) within the petrous temporal bone may extend into the middle fossa to involve the temporal lobe of the brain or into the posterior fossa to involve the cerebellum or brain stem.[51,48]

Neuroanatomical localization of brain disease in the horse has been described by others elsewhere.12,85,107 Briefly, infectious neurological disease in the horse is either diffuse (e.g., viral or protozoal) or can have a single neuroanantomical signal (e.g., brain abscess). Neuroanatomical localization should be define in terms of the five major regions of the CNS and/or the cranial nerves[12]: disease of the cerebrum, basal nuclei, rostral brainstem, caudal brainstem, and cerebellum.[12]

Seizure activity and moderate to severe obtundation are the most common signs of cerebral disease in the horse. Although the cortex controls conscious proprioception, this is difficult to assess localize to the cerebrum in the horse and in the absence of other clinical signs.

Blindness secondary to lesions in the visual cortex occur. Cortical blindness presents as decreased normal reactions to visual cues with normal pupillary light reflexes.[12,85,107] A full ophthalmic examination is paramount to assessing cortical blindness. The most common clinical sign of focal disease of the basal nuclei is the inability to chew and form coordinated eating movements with the tongue, teeth, and oropharynx.[12,85,107] However, diffuse disease in this area involves caudate nucleus, globus pallidus, putamen, and substantia nigra and should result in losses of coordination of movement. Extension to the reticular formation may result in abnormalities of wake/sleep.[105]

Clinical signs referable to lesions of the rostral brainstem can be differentiated on the basis of signs of abnormalities of cranial nerves (CN) II through IV. Vision, pupillary responses and eyeball placement can be affected by disease in these areas.[12,85,107] Post-ganglionic Horner's syndrome (ipsilateral ptosis, miosis, enophthalmis and localized ipsilateral sweating) can occur if there are lesions rostral to the foramen lacerum involving the sympathetic fibers as they course to the sphincter pupillae muscle.[12,85,107]

The hypothalamus, reticular formation and pituitary gland are included within the diencephalon and mesencephalon of the brainstem. Hypothalamic and pituitary disease generally result in endocrine dyscrasia. The reticular formation is very important for arousal and coordination of motor function. Lesions associated with the caudal brainstem can be identified on the basis of clinical signs indicating abnormalities of CN V through XII.

**Cranial nerves**

All of the cranial nerves exit through the meninges at the base of the brain and are susceptible to injury in horses with meningitis due to direct spread of infection or increased intracranial pressure. The clinical signs of multiple versus single CN abnormalities are important for ruling out specific etiologies.[12,85,107] For example, a weak horse with primary dysphagia and

slow papillary responses has evidence of a diffuse disease process such as botulism. A horse with unilateral masseter atrophy consistent with CN V paralysis is a common finding in horses with clinical disease due to *S. neurona* infection.

**Spinal cord**

The spinal cord has a central core of gray matter surrounded by the ascending and descending nerve tracts of the white matter. Intramedullary lesions (within the spinal cord) produce neuronal injury at one or more spinal cord segments and then expand laterally to involve motor and sensory nerve tracts. Clinical signs are observed caudal to the site of the spinal cord lesion because of damage to descending motor tracts. [12,85,107] While clinical signs of spinal cord disease are most commonly bilateral in horses, severity is frequently asymmetrical; a close examination will reveal that most intramedullary lesions, unless extremely focal, will have some degree of abnormality associated with the contralateral limb. On the other hand extramedullary or lesions of the peripheral nerves will involve a singls limb. Peripheral or extramedullary lesions can produce signs of nerve root irritation. When a lesion is compressive on the spinal cord, from outward to inward, there is a stepwise loss of proprioception then weakness. After onset of weakness, further compression results in loss of sensation followed by loss of deep pain. A typical presentation for a horse with cervical vertebral myelopathy is a young, strong two year old racehorse with spontaneous loss of balance.[85,86,109] Diffuse spinal cord disease is commonly observed in viral infections like arboviruses, neurologic EHV syndrome, rabies, and *Halicephalobus* infection while multifocal, asymmetrical disease is observed in horses with EPM.[15,80] West Nile virus can be highly variable with either diffuse spinal signs or highly asymmetrical disease.[8,21,105,155]

Spread of infectious agents can be limited by neuro-anatomical boundaries. The anatomical arrangements of the meningeal layers (pia mater, arachnoid, and dura mater) are the

same as described for the brain and a plane of infection is possible between the arachnoid and dura. The spinal dura and periosteum diverge at the foramen magnum. At the level of the seventh cervical vertebra they are separated by a fat-filled epidural space that cannot prevent longitudinal spread of infection, hence infection may extend over many segments.[51] In the horse the spinal cord ends as the cauda equina as the spinal cord tapers into the conus medullaris with distally coursing spinal nerve roots. Unlike other species, the meninges end caudally between S2 and S3.[85,86] The cauda equina is a site associated with CNS inflammatory diseases and occasional peripheral neuritis.[162]

**Vascularization**

The blood supply to the CNS includes an extensive network of intercranial arterial and venous vessels fed by two sources, the basilar and internal carotid arteries, with multiple communications to the external circulatory vessels via the circle of Willis to ensure collateral circulation of the brain. The horse is distinct from other species because the internal carotid artery does not receive any blood from the maxillary artery. The details are beyond the scope of this review, however some salient features are worth mentioning. The ophthalmic artery is a branch of the internal carotid artery which is a branch of the main intercranial artery, the basilar artery. Hence, CNS infection could result in septic emboli to the ophthalmic artery and consequent retinal lesions and loss of some visual fields. The middle cerebral artery has the greatest blood flow volume and is considered the area of greatest risk for septic embolization and mycotic aneurysms in the brain.[150] Brain infection most likely arises from infections with *Aspergillosis* and mucoracious fungi in lungs, uterus and intestine.[145] In descending order of frequency, the internal carotid artery, external carotid artery and maxillary arteries are the most common equine vessels to be affected with mycotic aneurysm and extracranial (guttural pouch) infection.[51]

Despite an extensive network of collateral circulation, there are three areas of the brain supplied only by one or two vessels. These areas are highly vulnerable to ischemic injury and abscess formation. They include the middle and posterior cerebral arteries at the junction of the parietal, occipital, and temporal lobes, the medial surface of the hemispheres of the cerebellum and cerebral white matter. No valves are present in the venous supply to the CNS and the direction of flow may change with hemodynamic changes due to pressure changes in the CSF, cerebral edema or other pathological events. The anterior spinal cord is supplied by the cervical and intercostal arteries from the descending aorta and generally has a higher likelihood of infection in than other parts of the spinal cord.[51,150] Data that supports this has not been evaluated in the horse, although osteomyelitis of the cervical and thoracic spinal column is not uncommon in foals.[84,99]

**Blood brain barrier and cerebrospinal fluid**

The capillary system of the CNS is unique in that it consists of endothelial cells with tight junctions and no fenestrations creating an effective blood brain barrier (BBB).[51] The BBB is the primary protective barrier of the CNS and acts as filter preventing access of large proteins, immunoglobulins, antigens, pathogens, and some antimicrobial agents (e.g,. gentamicin and amphotericin B) to the brain.[51] Injury to the BBB either by ischemic insult (e.g., septic emboli), vasculitis induced by inflammation, or increased levels of TNFα can disrupt this protective barrier and predispose to CNS infection. Disruption of the BBB permits the entry of radiodense agents into the CNS for early visualization of abscesses on contrast magnetic resonance (MR) and computed tomography (CT) studies.[7,140] The blood supply to the pituitary gland, choroid plexus and brain stem does not have tight junctions and these areas are considered to exist outside of the BBB.

Cerebral spinal fluid is an ultrafiltrate of plasma produced by active secretion from the choroid plexus in the lateral, third, and fourth ventricles and by diffusion across the meninges.[51,83,130,139] Cerebrospinal fluid protects and sustains the CNS.[61,78] It circulates outward through the ventricular foramina into the subarachnoid space and is reabsorbed over 3 to 4 hours through cells of the arachnoid villi along the superior sagittal sinuses. Blockage of the villi due to inflammation, blood in the subarachnoid space or occlusion of the superior sagittal or lateral sinuses prevents the reabsorption of CSF and communicating hydrocephalus develops. Obstructive hydrocephalus results from blockage of CSF circulation at the ventricles due to inflammation or compression of the ventricles such as might occur with an abscess or hemorrhage. Unlike communicating hydrocephalus, redistribution of increased quantities of CSF and cerebral edema into the subarachnoid space is not possible and there is increased likelihood of brain herniation and death.[51,61]

**Pathogenesis**

**Entry of pathogens**

Most neurotropic viruses gain initial entry to the body through the bite of an infected mosquito or insect (e.g., arboviruses), the respiratory tract (e.g., herpesvirus), or the gastrointestinal tract. Dendritic cells or phagocytes at the site of initial infection transport virus to local lymph nodes where it undergoes primary replication with subsequent viremia. Initial infection with bacterial or fungal organisms most commonly occurs via the respiratory, gastrointestinal, reproductive, or urinary tracts.[37] Septic emboli from vegetative endocarditis are another potential source of bacteria or fungus for hematogenous spread to the CNS. Regardless of the initial route of infection, the majority of CNS pathogens probably enter the nervous system of the host via a hematogenous route.[37,51,52]

The exact mechanism by which pathogens cross the BBB and enter the CNS is uncertain for most viruses and bacteria but several mechanisms have been proposed. Bacterial infections of the CNS frequently involve the meninges. A breakdown in the BBB due to ischemia of meningeal vessels secondary to emboli and inflammation may provide a route of access to the brain parenchyma with subsequent abscessation.[146,147,148] The initial systemic immune response to viral infection in the periphery results in release of cytokines that stimulate increased expression of adhesion molecules on CNS endothelial cells and increased surveillance of the CNS by activated T cells.[52] Some viruses enter the CNS using these cells as "Trojan horses". Other viruses utilize endothelial adhesion molecules to gain entry or induce release of TNF-α with subsequent increased BBB permeability.[52] Intracellular pathogens, such as *Listeria monocytogenes* and rickettsial species gain entry into the CNS by penetrating endothelial cells of the BBB or by travel within phagocytes.[34]

Other than hematogenous spread, pathogens may access the CNS by direct invasion (trauma or iatrogenic introduction), spread from contiguous structures (paranasal sinuses, otitis media), or retrograde entry along nerve roots.[37,52,146,131] Despite the frequency of infections involving the equine head (sinusitis, tooth root abscesses, guttural pouch empyema, etc.) the number of CNS infections resulting from direct spread to the CNS appears to be low in horses.[131] Rabies virus gains entry to the CNS by retrograde axonal transport along peripheral nerves. Herpes virus is thought to infect the peripheral trigeminal nerve during latent phases.[37] The potential for entry to the brain through the free nerve endings of the olfactory nerve in the nasal cavity has been proposed for rabies virus and arboviruses, especially Venezuelan equine encephalitis virus (VEE), but has not been proven clinically.[141]

**Immune response of the CNS**

The response of the CNS to infection plays an important role in the pathogenesis of disease. In bacterial CNS infections, CSF concentrations of complement and immunoglobulin G (IgG) are low compared to concentrations in the peripheral circulation.[146] Complement and specific antibody are important for opsonization of bacteria and a diminution of this function may be a critical factor in the pathogenesis of bacterial infections in the CNS. The presence of bacterial cell wall components in the CSF elicits the release of cytokines (e.g., IL-6, TNFα, MIP 1a, 1b, and 2) which stimulate the entry of neutrophils, increased BBB permeability, and vasculitis, CNS edema, and inflammation of tissues surrounding the meninges.

The peak inflammatory response is observed 72 hours after the start of infection.[146] Degenerating leukocytes release toxins that stimulate vasospasm, local ischemia, and further tissue edema. Inflammation of the arachnoid villi where CSF is absorbed could result in communicating hydrocephalus; inflammation of the ependymal lining and ventricles where CSF is circulated may result in obstructive hydrocephalus. Initially there is redistribution of increased spinal fluid and cerebral edema into the subarachnoid space with communicating hydrocephalus, but with severe edema and obstructive hydrocephalus this redistribution is not possible. Within the confining structures of the skull the increase in intracranial pressure may result in pressure necrosis of the brain parenchyma or death due to herniation of the cerebellum through the foramen magnum.[147] Vasogenic edema of the CNS is now viewed as a potentially fatal consequence of bacterial infection and treatment of human patients with both antimicrobial and anti-inflammatory medications has dramatically decreased the mortality rate associated with CNS infection.[141]

In the face of infection, the CNS must mount a controlled adaptive immune response that minimizes damage to brain cells.[52] Initially there is an innate immune response with production of IFN-β, chemokines, and pro-inflammatory cytokines. These mediators activate microglia to express IL-1, TNF-α, and chemokines that stimulate increased expression of endothelial adhesion molecules (e.g. vascular cell adhesion molecule [VCAM-1] and intracellular adhesion molecule [ICAM]). By three to four days post-infection, peripheral inflammatory cells that were activated in secondary lymphoid tissues enter the CNS. Unlike in the periphery, non-lytic clearance of viruses and infected cells occurs in the CNS to prevent secondary damage to surrounding neurons and tissues. Viruses which remain latent in neurons are controlled by continued secretion of antibody, IFN-β, and IFN-γ by long-term T and B cells.

As with bacterial CNS infections, control or elimination of viral infection in the CNS without inducing unacceptable damage to neural tissue, requires a delicate balance of the CNS immune response. Induction of apoptosis of neurons by microglia and stimulation of migration of T cells into the CNS are possible contributing factors to the neurodegeneration observed in degenerative diseases like Parkinson's and Alzheimer's diseases.[108] Overexpression of C protein, important in the complement system, causes bystander neurodegeneration and oligodendrocyte damage.[137] The immune response to EHV-1 may be important in the pathogenesis of the neurologic form of this pathogen. Localization of EHV-1 in the CNS endothelium induces vasculitis. Subsequent CNS damage results from ischemia rather than direct neuronal insult, hence the disease is termed a "myeloencephalopathy" rather than "myeloencephalitis".[151] The exact pathogenesis of herpesvirus neurologic disease in horses remains unclear. The disease is sporadic and seems to be more common in horses with a previous history of exposure to this ubiquitous pathogen and in pregnant or lactating mares.

Evidence of antigen-antibody complexes between EHV-1 antigen and EHV-4 antibody and decreased levels of complement activation have been observed in experimentally infected ponies.[32]

Other factors also play a role in the pathogenesis of viral infections. Non-survivors of Japanese encephalitis virus (JEV), a flavivirus infection, have increased levels of IL-6, IFN-α, and IL-8. Host genetic factors influencing production of these cytokines may be a factor.[157] Irrespective of the immune response, viruses have developed strategies to facilitate evasion of the CNS immune response. For example, West Nile virus may block the signaling pathway for interferon-alpha (IFN-α).[56]

Understanding of the host response to viral CNS infections is increasing and may explain the seemingly improper or inadequate immune response that allows the establishment of persistent infection. In human patients certain types of vaccination or viral infection result in a multifocal inflammatory demyelinating process or acute disseminated encephalomyelitis (ADEM).[28] An immune-mediated attack against antigen in brain myelin appears to be the cause of ADEM. The poliomyelitis-like acute flaccid paralysis observed in some people with West Nile encephalitis may be an example of ADEM.[40,119]

The old paradigm of the CNS as incapable of mounting an immune response to infection and being "immunologically privileged" has given way to our current recognition of the CNS as a specialized immune organ.[108] The innate and adaptive immune responses of the host play an important role in CNS infections. A better understanding of this role is important for ultimately developing novel therapeutic and preventative strategies.

## Cerebospinal Fluid Characteristics

### Collection techniques

Cerebrospinal fluid may be obtained antemortem from two sites in horses: the atlanto-occipital (cerebellomedullary) space or the lumbosacral (LS) space. The optimal site for sample collection is determined on the basis of the neuroanatomical localization of the suspected lesion and practical considerations regarding patient systemic health status and restraint options. In general, better diagnostic results are achieved if CSF is obtained from the site closest to the suspected lesion. The atlanto-occipital (AO) space is sampled under general anesthesia and may be preferable in nervous horses, horses undergoing anesthesia for another reason, or in horses with conformation preventing successful LS taps (LS subluxation). Conversely, a LS tap performed standing under sedation may be advantageous in an animal where recovery from general anesthesia is considered a risk due to the severity of neurologic disease.

Collection techniques for both AO and LS tap have been described in detail by several authors.[48,139,83] Briefly, atlanto-occipital CSF collection is performed with the horse under general anesthesia and lying in lateral recumbency. An area of the poll and neck (15 to 20 cm caudal to the ears and 8 to 10 cm on either side of the mane) is clipped and surgically prepped. The head is flexed so that the median axis of the head is at right angles to the median axis of the cervical vertebrae. A sterile 8.9 cm 20 gauge spinal needle with stilette is inserted at the intersection of the cranial borders of the atlas and the external occipital protuberance long the dorsal midline. The needle should be parallel to the ground, perpendicular to the skin and aimed towards the nose of the horse. The needle is gradually advanced until a “popping” sensation is felt with penetration of the atlanto-occipital membrane and cervical dura. The stilette is withdrawn and the appearance of clear CSF at the hub indicates a successful procedure. If no CSF appears when the stilette is removed, the needle is rotated 90 degrees. If fluid is still not

obtained, the stilette is replaced, and the needle is advanced carefully. The approximate depth of needle insertion for entry into the subarachnoid space is 5 to 8 cm. If the needle contacts bone at a depth of 2 to 5 cm, it should be withdrawn and repositioned appropriately. If blood appears at the hub of the needle when the stilette is removed and does not clear with CSF in 15 to 20 seconds, the stilette is replaced and the needle removed. A fresh needle is used for the following attempt. When CSF flows freely from the hub of the needle the sample is collected by free flow or gentle aspiration into an appropriate tube. After the sample has been collected and the needle is withdrawn, the head of the horse is extended to a normal or slightly extended position to prevent leakage of CSF from the puncture site.[83]

Lumbosacral CSF collection in the horse is typically performed with the sedated horse standing as squarely as possible.[83,85] Landmarks for the LS site are the intersection of imaginary lines joining the caudal borders of the tuber coxae along the dorsal midline or at the highest point of the gluteal region of the horse. In addition to sedation, adequate restraint with a twitch and use of stocks is advisable. In response to penetration of the dura mater, sedated horses may show no reaction, tail movement and slight flexion of the pelvic limbs, or violent kicking responses that can endanger the patient and the veterinarian. A 10 x 10 cm site is clipped and sterilely prepped. A 20 gauge 15.2 cm spinal needle with stilette is inserted in a sterile manner and advanced carefully a few millimeters at a time. Care should be taken to keep the needle perpendicular to the dorsum and on midline. A "popping" sensation may be felt with penetration of the LS interarcuate ligament, dorsal dura mater, and arachnoid membrane. The stilette is removed to check for CSF at the hub. Gentle aspiration with a syringe may be necessary to initiate flow of spinal fluid. If no fluid is obtained, the needle (with the stilette replaced) is advanced to the floor of the vertebral canal and then withdrawn with slow rotation of the needle

a millimeter at a time. A needle depth of 12-14 cm is commonly required for successful CSF collection. Large breed horses or obese horses may require longer needles. The Queckenstedt's maneuver (bilateral occlusion of the jugular veins) may be performed by an assistant to increase intracranial and intraspinal pressure and facilitate CSF flow up the spinal needle. Rotation of the needle 90$^{o}$ to remove occluding meningeal tissue and nerve roots from the needle point may also be helpful. Indirect aspiration with a syringe through an extension set connected to the spinal needle hub is recommended to minimize hemorrhage from excessive suction pressure and resultant occlusion of the needle with meninges. After adequate CSF is obtained, the stilette is replaced in the spinal needle and the needle is removed. Collection of CSF from the LS space while the horse is in lateral recumbency (under general anesthesia or in a tetrapelgic horses) is possible, but is considered more difficult than in the standing horse. Attempts may be facilitated by elevating the upper pelvic limb so that the tuber coxae is perpendicular to the floor or by advancing the pelvic limbs cranially to flex the pelvis and LS joint.

Both AO and LS collection techniques are regarded as safe procedures in the horse. A common complication is blood contamination of the sample with puncture of meningeal or spinal cord vessels. Initial blood contamination of CSF frequently clears after a few mL during collection; however even microscopic amounts of blood in the CSF sample may result in false positive results in testing for EPM in horses.[42] In humans, cerebellar herniation through the foramen magnum and herniation of the temporal cortex under the tentorium cerebelli are considered potential complications of CSF collection, especially in patients with increased intracerebral pressure, severe meningitis or brain abscesses with deteriorating condition.[51] This complication has not been reported as a frequent sequelae to CSF collection in horses. Evidence of extradural hemorrhage or formation of fibrous adhesions between the LS ligament and dorsal

LS dura mater have been observed in experimental subjects post-mortem. Penetration of the AO joint is another potential complication. Cellulitis and septic abscesses secondary to CSF collection in horses are rare.

**Analysis**

Analysis of the CSF may include measurement of CSF pressure and examination of sample cytology, total protein concentration, glucose concentration, biochemical alteration, turbidity, and color.[83,139] Cerebrospinal fluid pressure is measured by attachment of a manometer to the hub of the spinal needle prior to collection. Normal pressure in the horse is approximately 300 mm $H_20$ (150 – 500 mm $H_20$).[83,86,87] Increased opening pressure, when CSF is first obtained, may result from obstructive hydrocephalus. In addition to non-infectious congenital abnormalities, potential causes of obstruction include tumor, abscess, hemorrhage or edema. An increased opening pressure that decreases by 20-50% after removal of 1-2 mL of CSF is indicative of an intracranial mass or spinal cord lesion cranial to the site of collection. Because CSF flows caudad from the ventricles of the brain and jugular compression causes increased blood volume in the cranial cavity with subsequent increases in CSF pressure, failure of the CSF pressure to increase in the LS site with bilateral jugular vein compression may indicate a compressive thoracic or cervical lesion.

**Appearance**

Normal CSF is clear, colorless, and does not clot. Xanthochromia (yellow discoloration) of the CSF after centrifugation is caused by preexisting trauma, vasculitis, increased protein concentration (150 mg/dL), direct bilirubin leakage from high serum concentration or breakdown of the BBB. [83,86,87] Xanthochromia with increased protein concentration is typical of equine encephalomyelopathy due to vascular inflammation and increased BBB permeability.[32]

Clots may result from increased fibrinogen due to inflammation. A CSF sample may appear turbid if there is an increase in quantity of white blood cells (>200/ulL), red blood cells (>400 /uL), or epidural fat cells, or if there are significant numbers of bacterial, fungal, or amoebic organisms present.

**Cellular evaluation**

Cell counts and cytologic evaluation performed within thirty minutes of CSF collection are diagnostic. In normal horses and foals <10 white blood cells/uL are expected in the CSF. Cells are predominantly small (70-90%) and large (10-30%) mononuclear cells.[83,87] An initial neutrophilic pleocytosis followed by mononuclear pleocytosis is characteristic of EEE infections. However, CSF from horses with WEE and WNV encephalomyelitis is characterized by predominantly lymphocytic cells.[153] The increase in CSF nucleated cell count with viral infections is typically less (100-1000/ uL) than with bacterial meningitis. Eosinophilic pleocytosis with xanthochromia and increased protein concentration may be observed in CSF from horses with parasitic meningitis.[25,107] Infrequently, horses with parasitic meningitis can have a neutrophilic pleocytosis. Fungal organisms may be observed in the CSF of horses with fungal meningitis.[107] Although CSF analysis is useful to confirm the presence of an inflammatory process, to determine antibody titers to specific pathogens and to monitor for therapeutic response, culture of viral or bacterial pathogens from CSF of horses with infectious neurologic disease is often difficult. Identification of viral etiologic agents in CSF is rare.[28]

**Protein**

Normal protein concentration in equine CSF ranges from 20 to 124 mg/dL and is typically higher in CSF obtained from the LS site (93.0 +16.0 [58.0+11.0]mg/dL) than in samples obtained from the AO site (87.0+17.0, [53-11.6]mg/dL).[6,87,107] Differences in CSF protein between AO and LS samples that are greater than 25 mg/dL may indicate a lesion closer

to the site of origin of the sample with greater spinal fluid protein. CSF IgG and albumin concentrations may be determined by electrophoresis and radial immunodiffusion. These values are compared with serum IgG and albumin concentrations. An increase in the albumin quotient ([Alb Csf])/[Alb serum] x 100) is considered indicative of an increase in BBB permeability as may be seen with equine herpes virus myeloencephalopathy. An increase in the IgG index ([IgG Csf]/[IgGserum] x [Albserum]/[AlbCsf]) may reflect intrathecal IgG production due to inflammatory disease (EPM, meningitis, tumors, equine motor neuron disease).[6]

**Biochemical parameters**

Increases in CSF creatine kinase (CK) are an unreliable indicator of neurological disease in the horse and may be falsely elevated by contamination of the sample with epidural fat or dura during collection.[41,43,44,60] Lactic acid concentrations in the CSF may increase with some CNS diseases (EEE), head trauma, and brain abscesses.[6,107]

**Immunologic testing and molecular diagnostics**

Detection of specific antibodies or antigens within the CSF may be helpful for the diagnosis of some viral, fungal, or rickettsial diseases. Use of PCR for the diagnosis of viral encephalitis has become an important and sensitive tool.[28]

**General Therapeutic Considerations**

**Antimicrobials**

Antimicrobial selection for treatment of horses with bacterial infections of the CNS is based on initial Gram stain, culture, and susceptibility results whenever possible.[33] Desirable antimicrobial traits include the ability to penetrate the CNS and predicted activity in the low pH and high protein environment of infected CSF.[148] Low molecular weight antimicrobials that are lipid soluble and have a degree of protein binding and ionization at physiologic pH are favored.[33,148] With inflammation, BBB permeability increases to allow penetration and

accumulation of drugs that are normally actively transported out of the CNS (e.g. penicillin and cephalosporins).

To allow for maximum peak plasma concentrations, intravenous administration of antimicrobials is recommended initially. The rapid bactericidal killing needed for CNS infections in human patients requires drug concentrations that exceed the minimal bactericidal concentration by 10 to 20 fold.[146] Expected duration of therapy varies depending on the nature of the infection, but generally is 10 to 14 days.[33,146]

Antimicrobials with poor CNS penetration across the intact BBB include penicillins, cephalothin, cefazolin, ceftiofur, tetracycline, and aminoglycosides.[33,48,146] Good penetration is observed with fluoroquinolones, third generation cephalosporins (e.g., cefotaxime, ceftazidine, ceftizoxine, and ceftriaxone), sulfonamides, trimethoprim, pyrimethamine, doxycycline, chloramphenicol, rifampin, metronidazole, and macrolides. Enrofloxicin obtains therapeutic concentrations in the CSF for many Gram negative pathogens (e.g., *E. coli*, *Salmonella*, *Actinobacillus*, and *Klebsiella*) but is ineffective for treatment of most streptococcal and anaerobic pathogens. Its association with arthropathies in foals limits its use for treatment of neonatal bacterial meningitis. Potentiated sulphonamides (e.g., trimethoprim sulfa combinations) are attractive therapeutic agents for CNS infections as they have a broad spectrum of activity, are inexpensive, and are administered orally, but unfortunately antimicrobial resistance is common. Third generation cephalosporins are considered the antimicrobial of choice in human patients with bacterial CNS infection due to their activity against Gram negative bacteria but may be cost-prohibitive for use in horses. Although ceftiofur sodium is similar to true third generation cephalosporins, it does not effectively cross the intact BBB in horses. Chloramphenicol is a bacteriostatic broad-spectrum antibiotic with activity against Gram

positive, Gram negative, and anaerobic bacteria and is administered orally, but the associated human health risk (i.e. aplastic anemia) must be considered. Rifampin has activity against Gram positive and anaerobic bacteria and is distributed into the CSF but must be used in combination with other antimicrobials such as erythromycin due to the high frequency of development of bacterial resistance when used alone. Fluoroquinolones should not be used with rifampin as it is an RNA synthesis inhibitor. Metronidazole is effective against anaerobic bacteria and is used in combination with third generation cephalosporins for treatment of human patients with bacterial CNS infections.

**Glucocorticoids, osmotic agents, diuretics**

Increased intracranial pressure due to vasogenic edema or obstructive hydrocephalus is common in patients with bacterial CNS infections and its control is critical for successful treatment of these patients.[33,148] The use of corticosteroids in patients with CNS infection is controversial due to their immunosuppressive effects, however mortality rate was unaffected with corticosteroid administration to humans with brain abscesses.[158] Moreover, corticosteroids reverse the increased permeability of the BBB induced by inflammatory mediators (e.g., IL-6, TNF-$\alpha$, prostaglandins, leukotrienes, IL-1). Administration of dexamethasone (0.25-0.75 mg/kg) to two horses successfully treated for intracranial abscesses was thought to be beneficial.[22]

Mannitol causes an osmotic shift of water into the vascular space, decreases blood viscosity and increases cerebral blood flow and oxygen delivery.[22,33] The net result is vasoconstriction of the cerebral arterioles and a decrease in cerebral blood volume and intracranial pressure. A single dose of mannitol (0.15-2.5 g/kg IV) decreases intracranial

pressure experimentally within 5 minutes with peak effects at 10-40 minutes and lasting 90-120 minutes. Adequate hydration of the patient must be maintained.

The benefits of dimethyl sulfoxide (DMSO) for the reduction of intracranial pressure are unclear and most of the work has been performed in rodent models.[115] In one clinical trial, DMSO reduced intracranial pressure and improved clinical course of neurological recovery. In another trial, continued therapy was necessary for maintenance of decreased intracranial pressure.[62] Objective studies regarding the efficacy of DMSO in horses for the lowering of intracranial pressure are lacking.[3,22]

Furosemide prolongs the effects of mannitol but its effect as a sole agent for reduction of intracranial pressure is inconsistent and delayed. Controlled ventilation to prevent hypercapnia and subsequent cerebral arteriolar vasodilatation is advocated in human patients for the control of intracranial pressure.[51] Barbiturates reduce cerebral oxygen demand and are neuroprotective against brain injury.

**Supportive Therapy**

Properly trained nursing personnel and facilities equipped to handle horses with CNS dysfunction are essential as the size, strength, and severity of neurologic disease in some horses can render appropriate care extremely demanding and dangerous.[38] Rapid progression of disease is common in horses with CNS infections, necessitating the use of padded stalls and protective head gear, removal of shoes, and placement of leg wraps. Adequate bedding, periodic turning of the patient from side to side or the use of slings to prevent formation of decubital ulcers is essential in the care of recumbent horses. Control of hyperthermia with ice water, alcohol baths and fans may be indicated. Supportive care with intravenous fluids, parenteral nutrition and electrolytes is necessary in an inappetant animal.

## West Nile Virus

West Nile virus is a member of the family *Flaviviridae* composed of three genera (*Flavivirus*, *Pestivirus*, and *Hepacivirus*) of which many members are world-wide, pathogenic and arthropod-borne or arboviruses. The *Flavivirus* genus consists of over 70 types of related viruses which can be further divided into antigenically related serocomplexes. The members of the Japanese encephalitis serological group contain many that cause disease in horses and include Japanese Encephalitis Virus (JEV), Kunjin virus (KV), Murray Fever Virus, and West Nile Virus.[75]

### Morphology and Molecular Biology

WNV is a positive-sense, single stranded RNA virus measuring approximately 50nm. The virions are icosahedral, spherical, and enveloped with the capsid (C) protein, making up a nucleocapsid of 25nm. The genome consists of a single-stranded positive sense RNA with a type 1 cap (m 7 GpppAmp) present at the 5' end and 3' end without a poly A tract but with a CUOH terminus. The genome is 11,029 nucleotides (nt) in length consisting of a 5' noncoding region (NCR) of 96 nt, a single open reading frame (ORF) of 10,301 nt, and a 3' NCR of 631 nt. Ten mature viral proteins are produced from a single polyprotein precursor. There are three structural proteins: the capsid protein (C), the premembrane protein (prM) that is proteolytically cleaved by a cellular protease to form the membrane protein (M) in the mature virion and the envelope (E) protein. The seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, and NS4B, and NS5) are cleaved after translation and are required for vial replication and assembly.[18]

Intracellular replication of West Nile involves the binding of an unidentified but likely highly conserved cell protein (receptor) on the host cell. Virions enter cells via receptor mediated endocytosis followed by low-pH fusion of the viral membrane with the endosomal

vesicles membrane releasing the nucleocapsid into the cytoplasm. The genomic RNA is released and translated into a single polyprotein. Viral serine protease, NS2B-NS3, and several cell proteases cleave the polyprotein at multiple sites to generate mature viral proteins. Viral RNA-dependant RNA polymerase, NS5, with other viral nonstructural proteins copies the genomic (+) RNA into (-) complementary RNA. Synthesis of genomic RNA utilizes these templates and occurs ten times more efficiently than (-) cRNA synthesis. Nascent genomic RNA are thought to function as templates for translation and transcription and as substrates for encapsidation. Structural proteins are transported to cytoplasmic vesicle membranes and virions assembly occurs at the rough ER membranes. Immature virions with E and prM heterodimers accumulate in vesicles and are transported through the host secretory pathway. The glycosylated and hydrophilic N-terminal portion of prM is cleaved by cellular furin and the C-terminal portion M remains inserted into the envelope of the mature virion and may be necessary to maintain the E protein in a stable inactive conformation. Virions are transported to the plasma membrane in vesicles and are released by exocytosis.[17,18]

Based on what we know of the intracellular replication of the virus, sequences that would be expected to be conserved for efficient replication by viral encoded enzymes would include: (1) sequences for NS2 and NS3. The N-terminal third of NS3 contains a serine protease, but requires NS2B to form a stable complex and for activation. As stated above serine protease cleaves the polyprotein to generate mature proteins and deletions/mutations of these sequences would be expected have detrimental effects. The C-terminal portion of NS3 contains regions with homology to a family of helicases. Infectious clones containing deletion/ point mutations of the NS3 region for other viruses inactivated helicase activity and were essential for virus viability.[18] (2) sequences for NS5. NS5 is largest and mostly highly conserved of flavivirus

proteins and contains motifs characteristic for RNA-dependent RNA polymerases. The N-terminal region of NS5 contains a required methyltransferase domain, thought to function in the methylation of type I cap. Association with cellular proteins (elongation factor EF-Tu, EF-Ts, and S-1) for polymerase activity was shown to be necessary in other arboviruses.[18] (3) NS1 glycoprotein has also been shown to be essential for virus viability. It contains two conserved N-linked glycosylation sites and 12 conserved cysteines that are essential for virus viability. Temperature sensitive NS1 mutants for Yellow Fever virus were observed to have defective RNA accumulation in vesicle packets.[18]

It is not unexpected that molecular sequences of West Nile virus would be conserved to ensure successful viral replication in each of the multiple hosts (mosquitoes, mammals, and birds) that the virus infects during its transmission cycle. More definitive evidence for conserved molecular sequences have been found based on the prediction that critical cell proteins used by West Nile (and other flaviviruses) would be highly conserved among divergent host species.[17,18] Brinton *et. al.* were able to determine that the 3'terminal regions of both the genomic (+) RNA and complementary (-) RNA of WN were highly conserved. Specifically, there is a large, stable stem loop followed by a second short stem loop at the 3' terminus of the genome RNA and these interact with three cell proteins (p105, p84, and p52). Four cell proteins (p108, p60, p50, and p42) interact with the cRNA and p108 and p105 were found to interact with the 3'RNAs of both genomic and complementary strands. Purification and sequence studies of p52 identified it as elongation factor-1 alpha (EF-1$\alpha$) used to carry charged tRNA into ribosomes. WNV 3'(+) RNA has been found to bind to EF-1$\alpha$ in mammalian, chicken, and mosquito cell extracts. RNase footprinting assays identified a specific four nucleotide region on the 5' side of the 3'terminal stem loop for EF-1$\alpha$ and infectious WNV clones with mutations of this site resulted

in mutant progeny with defective growth.[17,18] Recent studies found conserved stretches of nucleotides near the 3' of the NCR and another 5'ACAGUGC 3' in the top loop. WN clone mutations of the top loop sequence revealed that 3 of the 7 nucleotides (5'ACAGUGC3') were critical for viral replication and were conserved in mosquito-borne viruses.[35] Further studies using capped mRNA chimeric constructs of 3'WNV, 3'rubella virus with poly AAA tract (to simulate cellular 3'mRNA), and no 3'sequence resulted in significant decrease in translational efficiency of the 3'WNV construct compared with the 3' rubella construct. This was suggestive that 3'-5'closed looped complex via 3' binding protein and 5'cap binding complex (seen with cellular mRNA translation) is not used in WNV translation. Additionally it also suggested that the 3'WNV (+) cRNA sequence could suppress host cellular mRNA translation.[71] Other studies have found conserved sequences (CS) upstream of the 5' region of the capsid gene that appear to be essential for virus replication. Lo et al. using WNV replicons mutations of CS1 abolished RNA replication and replicons with deletions in CS2, repeats of CS2, CS3, and repeats of CS3 resulted in compromised RNA replication. Mutations in the CS of the 3' NCR did not affect viral replication.[74]

Other molecular sequences predicted to be highly conserved would be those that code for the structural E and prM protein. Virus entry in cell is thought to be mediated by a highly conserved receptor protein but also equally dependent on a viral surface protein.[18] The E protein is the main neutralizing epitope for WNV and is expressed on the virion surface as a heterodimer with the M protein. The E protein is folded into three structural domains: the antigenic domain I with the N-glycosylation site, domain II involved with pH-dependent fusion to endocytic vesicles, and domain III involved with binding to host cells. Decreased neuroinvasiveness has been shown in viruses with N-glycosylation point mutations in the E protein. Phylogenetic

studies of WNV NY 99 strain (New World lineage 2 strain) and an attenuated Old World lineage 1 strain ETH76a revealed nucleotide (5.1%) and amino acid (0.6%) changes. A single A→U mutation at nucleotide 2273 that encoded a Thr→Ser change in E436 was found. Interestingly mutations in the NS2B, NS3, and NS4A genes were present in this attenuated strain.[9] As stated before, the glycosylated and hydrophilic N-terminal portion of prM is cleaved by cellular furin and the C-terminal portion M remains inserted into the envelope of the mature virion and may be necessary to maintain the E protein in a stable inactive conformation.

**History**

West Nile Virus is an arthropod-borne flavivirus maintained in endemic regions between mosquitoes and birds. It was first isolated from a febrile woman in Uganda in 1937 and has been associated with sporadic outbreaks in the 1950's of fever in humans and in horses in Africa, the Middle East, Europe, and Asia. A fatal encephalitic form of was seen in humans and in horses during epidemics in Egypt, France, Morocco, Israel, Italy, and in Russia.[50] Since its emergence into the United States in 1999, West Nile Virus (WNV) has become a major public health, veterinary, and wildlife concern. The virus has killed a total number of 928 humans, over 6,000 horses, and an estimated, hundreds of thousands of avian deaths.[1,2] Humans and horses are considered dead-end hosts whereas birds are considered the primary reservoir as they develop a high viremia. With the exception of bird deaths in Israel in 1998, birds were not clinically affected in previous Old World epidemics, but at least 198 bird species in North America, particularly corvids, appear to be quite susceptible to West Nile.[50] The American Crow has been used as a surveillance tool for detecting the spread of the virus across the United States.

## Clinical Disease

### West Nile infection in humans and horses

WNV infection in humans and horses is not clinically apparent in the majority of those exposed and has been reported as 80%.[103] Of the remaining 20% (1 in 5) of exposed humans, the majority may manifest clinical signs of West Nile Fever characterized by sudden fever (38-40°C) and including headache, abdominal pain, nausea, vomiting, diarrhea, malaise, anorexia, photophobia, mylagia, lymphadenopathy, and arthralgia. A transient, 1mm-4mm scattered erythematous blanchable macule and papule skin rash (punctuate exanthem)[5] mostly on the extremities is also a commonly reported symptom. Resolution of uncomplicated WN fever occurs in <1 week but fatigue and malaise may linger for weeks. In contrast, neurologic abnormalities are the most common initial reason for presentation for the equine patient. West Nile Fever is not a formally recognized manifestation of WN infection in the horse, but horses are suspected to have initial systemic lesions and fever. In one retrospective study for 2000 outbreak, in >60% of horses in the study fever was detected, and was used as a critical differentiating factor from other non-viral neurologic encephalitides. Recrudescence of increased rectal temperature was also predictive for resumption of neurologic signs.[105]

Less than 1% (1 out 150) of infected humans and <10% of infected horses develop severe neurological disease. WN virus is neurotropic and affects the gray matter of the brain and spinal cord to cause a polioencephalomyelitis. Affected horses typically have signs consistent with diffuse CNS disease (change in mentation, somnolence, agitation, aggressive behavior), spinal cord abnormalities, and cranial nerve deficits (facial nerve paralysis, tongue paralysis, dysphagia).[105] Greater than>90% of affected horses manifest spinal deficits of profound muscle weakness and ataxia Muscle and skin fasiculations, tremors, and hyperesthesia are common in horses with WNE. The overall mortality rate in affected horses was reported to be 38% in 2001

and disease and mortality were also reported to be greater in older horses.[105] In humans, three major categories of neurologic disease associated with WNV have been defined in and include: (1) WN Meningitis characterized by signs of meningeal inflammation including nuchal rigidity, Kernig or Brudzinski signs, and photophobia or phonophobia, (2) WN Encephalitis defined by depressed or altered level of conciousness, lethargy, personality change lasting > 24 hours and potentially progressing to coma, respiratory depression, and death, and (3) Acute Flaccid Paralysis or poliomyelitis characterized by acute onset of limb weakness areflexia/hyporeflexia of affected limb(s); absence of pain, paresthesia, or numbness in affected limb(s)with marked progression over 48 hours.[103] Similar to that seen horses, cranial nerve deficits and Parkinsonian-like movement disorders—axial rigidity, tremors, stereotypic orofacial dyskinesias, myoclonus, and ocular bobbing movements reminiscent of opsoclonus myoclonus—have been described in human patients.[134] Other clinical findings reported with human West Nile Virus infection, and not yet reported in horses are optic neuritis, chorioretinitis, labile vital signs, hypotension, and arrhythmia. Novel modes of transmission (in utero, transfusion/transplant related, and breast milk) have also been reported with humans.[50] In 2002 WNV infection of a pregnant woman during the second trimester resulted in transmission of the virus *in utero*. The infant born at term had bilateral chorioretinitis, severe bilateral loss of white matter in the temporal and occipital lobes of the brain, and cystic cerebral destruction in one temporal lobe. Sixteen cases of transplant and blood transfusion transmission of WNV have been reported since 2002. Among these cases, myocarditis, pancreatitis, and fulminant hepatitis due to WNV infection were noted and were likely due to the immunosuppressive drugs that the transplant recipients were taking. Transmission of WNV from infected breast milk from the mother to child was also seen in 2002. In the 1999 and 2000 outbreaks, 69% of cases of encephalomyelitis

were in patients older than 60 years of age and meningitis was predominantly in middle-aged and younger individuals. The case fatality rate of 15% was lower in humans than in horses and may be due to economic constraints and for humane reasons due to severe recumbency in equine patients.[50]

**West Nile infection in birds**

Unlike horses or humans, birds are considered the primary reservoir for West Nile. Passerine birds, including crows, house sparrows, and blue jays were found in an experimental study to be the primary amplifying hosts of the virus and develop a high-level of viremia for several days.[66] In this same study, cloacal shedding of virus in 17 of 24 species and oral shedding of virus in 12 of 14 species of birds was also found. Evidence of contact transmission (cage contact) and oral transmission was also found. WNV in the New World has been associated with significant avian mortality. Corvids (American crows, Blue Jays, Magpies) are particularly susceptible to West Nile.[66] In susceptible species, infection results usually in mortality and reported clinical signs seem to be a systemic disease with multiple organ involvement. In multiple experimental studies with mosquito or needle WNV infection, birds of a variety of species remained clinically normal until 4-5 days post inoculation (dpi), then became lethargic, had ruffled feathers, unusual postures, were unable to perch or hold their head up, and/or had sudden death.[156] These clinical signs correspond to low degree of lesions and virus recovered in the CNS of birds infected with WNV. To be complete, however, neurologic signs of sudden onset of recumbency, mild ataxia, tetraparesis, tremors, nystagmus, seizures, and disorientation were reported recently in ten captive wildlife birds positive for WNV infection and corresponded to CNS lesions found at post-mortem.[24] The picture of WN infection is far complete as a rock pigeon, a species not considered to be susceptible, was reported to be

susceptible to infection[4] and louse infestation was suspected as the transmission vector for WN in North American Owls.[45]

**Neurological manifestations of CNS lesions**

The neurologic clinical signs seen in affected horses and humans correlate with the histologic lesions seen in the brain and spinal cord. The signs of diffuse CNS are consistent with the histologic lesions of inflammatory foci (characterized by perivascular cuffing and gliosis) and detectable virus in the thalamus, medulla, and pons.[105,135] The thalamus integrates sensory input to the higher centers and the midbrain and rostral pons affects the reticular formation. The reticular formation projects to the thalamus which sends projections to the cortex and also to the base of the forebrain. The forebrain is the source of cholinergic stimulation to the cerebral cortex and hence lesions may induce the changes in behavior seen with WNE. Extrapyramidal movement disorders of tremors, myoclonus, etc. are associated with abnormalities of the basal ganglia (thalamus and substantia nigra).[134] In human patients, magnetic resonance imaging reveals increased uptake in the thalamus, basal ganglia, mid and hindbrain.[103] WNV is highly neurotropic and targets the neurons of the anterior horn of the spinal cord, where the motor neurons reside. In combination with lesions in the motor tracts of the hindbrain damage to these lower motor neurons in the spinal cord would account for the high degree of muscle weakness seen with WN with horses and in people.[39,104] Injury of the neurons has been shown in a rat model to be due to direct injury (cell death) induced by WN virus infection and not by impairment of the glutamate transporter-1 and subsequent glutamate excitotoxicity shown with related Sindbis virus.[26,124] In people WNV also has been found to target the sympathetic and dorsal root ganglia.[39] DRG loss would result in sensory deficits, electrophysiologic tests have shown reduced sensory nerve action potentials in WN virus affected patients. Disappearance of sympathetic neurons could also lead to autonomic instability and could explain the patients who

developed labile vital signs, hypotension, and arrhythmias. The cranial nerve deficits (particularly VII, XII, IX) are thought to be associated with lesions in the pons and medulla.[104]

**Virus load and localization**

Horses and humans are considered to be dead-end hosts for West Nile virus. Infection is characterized by low and transient levels of viremia. In humans, virus is detected in the blood from immunocompetent patients 2 days before onset of illness to 4 days after onset of signs.[152] The maximum viremia titer in horses infected with WNV via mosquito challenge on 5 dpi was 460 Vero cell PFU/mL and virus was detectable in the blood from 1 dpi to 6dpi.[20] In contrast, a maximum titer of $10^9$ PFU/mL of viremia was reported in experimentally challenged crows at 3 dpi and was detected up to 7 dpi. Birds with titers equal or greater than $10^5$ PFU/mL are considered competent reservoirs for mosquito vector transmission.[66] Furthermore virus was detected 1 dpi in the blood and spleen, lung and liver at 2 dpi and in the CNS by 5 dpi.[156] In an experimental infection study of 25 species, susceptible birds died acutely within 5 to 7 dpi, but evidence of viral persistence in organs was found in the surviving birds. WNV could be detected in any of 11 organs, including brain, eye, kidney, heart, spleen, liver, lung, intestines, gonads, esophagus, and skin. Eighteen of 41 birds sampled at 14 dpi had persistent infection in one or more organs and in two cases, birds with no detectable viremia.[66]

In horses the majority of virus recovered is in the CNS. Quantitative detection of WNV RNA using formalin fixed paraffin embedded tissue specimens revealed that the maximum viral transcripts were in the medulla and thalamus, followed by cerebellum, cerebrum, and spinal cord.[144] Interestingly only scant staining of these tissues for WNV antigen was seen with immunohistochemistry. In the one horse that developed clinical signs with experimental infection with a mosquito challenge model, ($10^4$-$10^{6.8}$ PFU/tissue) was recovered from several areas of the brain and spinal cord.[20] WNV staining studies of other systemic tissues in horses has

been reported to be negative, but viral RNA transcripts at low levels were recovered in the small intestine and heart. Poor detection of WNV antigen in non-neural tissue could be due to low level of viremia systemically, low levels of virus replication, or low sensitivity of IHC staining. In contrast, the maximum number of viral RNA transcripts in crows were detected in small intestine, followed by the liver, spleen, kidney, and heart and these corresponded to the degree of WNV antigen detected with IHC staining.[144] The least amount of virus was detected in the crow brain. Furthermore, in another study, antigen staining of CNS tissues revealed positive detection of WNV mostly with inflammatory lymphocytes and not with brain parenchymal cells in crows.[156]

Virus localization in the CNS is similar in humans as to horses. In humans it has been found that immunosuppression (with concomitant systemic illness or with immunosuppressive drugs) results in higher virus load in the CNS.[152] Extensive viral load in most of the neurons of the pons, medulla, midbrain, cerebellum, and cerebral cortex was seen in patients with AIDS or malignant cancer. Study of non-CNS tissues from nine patients were unremarkable except for one with AIDS had interstitial lymphocytic infiltrates in the heart. No WNV antigen staining was seen except for a focal area in the cytoplasm of epithelial cells in the renal tubules of an AIDS patient.

**Pathogenesis**

The compete story of WNV pathogenesis is still incomplete but has been extensively studied in murine models. Infection occurs in the muscle or other cells near the site of the mosquito bite or peripheral inoculation and is subsequently transmitted to regional lymph nodes. Viral replication in these tissues leads to a primary viremia, resulting in infection of tissues associated with the vascular system. Infection of secondary target organs such as the CNS or liver occurs. Recent studies have shown that with the mosquito penetration into the skin,

salivary antihemostatic molecules which prevent vertebrate hemostasis may enhance the initial replication of the virus in the skin by dampening the innate immune response.[11] Murine ear skin injection with West Nile virus results in the migration and activation of Langerhans cells (MHC I and MHCII+, CD80 and CD86 expressing) to the site and subsequent migration to draining lymph nodes.[64] Other studies provide evidence of up-regulation of MHC class I molecule expression with WNV infection. This may be a potential modulation of the virus to the innate immune response. With up-regulation of MHC class I molecules, natural killer cells would not be activated to destroy virus infected cells.

Experimental murine models demonstrate that WNV has primary predilection for neural tissues. Intraperitoneal injection of WNV ($10^2$ PFU) into 8 to 12 week-old mice results in dissemination into the CNS by 4 to 6 days after inoculation.[30] The time course of infection in the hamster is similar.[14] Experimental infection of horses results in viremia at days 3 to 5 and clinical signs in 7 to 10 days.[20,76,77] WNV inoculation into the CNS results in direct infection of nerve cell bodies. In rodent models, initial replication occurs in the basal ganglia, with subsequent dissemination to the cortex, cerebellum, and hippocampus. The large neurons of the ventral or anterior horns are infected later in the course of the disease.

Two routes of neuroinvasion are proposed for WNV infection.[112] In the first, WNV gains entry into the CNS across the blood brain barrier (BBB). It is hypothesized that systemic viral infection results in local cytokine responses that increased the permeability of the BBB to viral invasion. In particular, tumor necrosis factor alpha (TNF-α) in murine models increased vascular permeability and allowed infection of peripheral nerves. Evidence indicates that toll-like receptors are crucial for entry of WNV into the CNS, whether by neuronal or vascular route.[29,154] The second proposes direct axonal retrograde transport from infected peripheral neurons or

infection of the olfactory neurons and spread to the olfactory bulb. Several studies have been published data that provide evidence for and against both routes.[81,96,112,114154]

**Immune Response to WNV infection**

Most of the knowledge of what constitutes the successful immune response to WNV has been derived from in-bred murine infection models and appears to rely on several aspects of the innate and adaptive immune system. WNV replication in the periphery has been shown to be limited by the antiviral actions of interferon-alpha/beta (IFN-$\alpha/\beta$).[111] Mice strains susceptible to flavivirus have been shown to have a mutation in the interferon inducible Oas1b gene. This genetic alteration results in the truncation of the 2'5' oligoadenylate synthetase protein that normally mediates the activation of cellular RNase L which cleaves viral ds RNA.[82] Complement activation of all three pathways (classical, lectin, and alternative pathways) have been shown in mice to be important in the control of WNV infection and in the priming of humoral and cellular immune responses.[31,88] Antibody plays a clear role in protection against WNV. Passive transfer of WNV specific IgG was shown to protect mice if administered before or shortly after lethal challenge and, in another study, B cell deficient were protected from lethality by passive transfer of immune sera.[30,36] However, recent work has also demonstrated the importance of CMI in the control and recovery from WNV infection. $CD8^+$ T cells were shown to eliminate WNV infected cells via cytolytic mechanisms.[123] $CD4^+$ T cells also have a critical role in sustaining these WNV-specific effector $CD8^+$ T cell responses in the CNS for viral clearance and for the production of antiviral IgG.[129]

Little is still known yet of what determines secondary immunity to WNV, but is speculated to be a $CD4^+$ T helper type 1 ($T_H1$) biased response based on related flavivirus studies.[57] In one study, a $CD4^+$ $T_H1$ response was shown in immunized mice challenged with Yellow Fever virus

to be critical for survival.[57,95] A $CD4^+$ $T_H1$ response was present in the peripheral blood mononuclear cells (PBMC) in people subclinically infected with Dengue virus while those that developed clinical disease had a $T_H2$ response.[125]

A number of detailed histopathologic studies in human patients have been done where there may be evidence to support the theory that immunopathology may also be a factor in the development of WN encephalitis and poliomyelitis. In one study of four patients that developed acute flaccid paralysis the gray matter of the cervical and lumbar regions were severely affected and were characterized by chromolytic neurons, neuronophagia, and a feltwork of proliferating astrocytes indicative neuron loss. More importantly, immunohistochemical staining of the tissues revealed microglia CD68 and CD45 inflammatory cells around the vessels and within the gray matter with a predominance of T cells in immunologic reaction with the virus.[39] In another study of 2 cases, $CD8^+$T cell lymphocytes were the predominant lymphocyte population seen.[63] In horses, it has been noted that there is a paucity of virus recovered in the CNS despite the lesions that are seen and immunopathology has also been suspected in the pathogenesis of WN encephalitis.[21] Although cell lysis occurs with viral replication, WNV also induces apoptosis in neurons, as demonstrated in cell culture and in vivo. This apoptosis can be induced by the capsid protein through the caspase-9 pathway in mitochondria.[113,161] Another mediator of neuronal injury is the host immune response. Although $CD8^+$ T cells may be important in long-term protective immune responses, lesions in brains of mice with fatal WNV are predominantly composed of $CD8^+$ T cells.[123]

**Diagnosis**

**Testing modalities**

Testing methods available for the diagnosis of WNV in humans, horses, and birds include: (1) isolation of virus from various tissues, body fluids, and CSF, (2) serological assays, and (3)

molecular detection of viral RNA transcripts. Virus is isolated from application of homogenized tissues or fluid samples into Vero cell cultures followed by virus identification with immunofluorescence assay with WN virus-specific monoclonal antibodies. Serological assays include the plaque reduction neutralizing antibody testing (PRNT) for the presence of serum neutralizing antibody to WNV, hemagglutin inhibition (HI) assays, and IgM capture- enzyme linked immunoassays (ELISA). The presence of IgM antibody and serum or CSF is considered the most efficient and reliable indicator of infection in humans and in horses.[103] In humans infected with WN virus, neutralizing IgM antibody is detected in 38% of serum samples and 35% of CSF samples collected during the first seven days of illness, with IgM antibody appearing in CSF before serum. Switch to IgG antibody production occurs after 4-5 days of illness, again with those in the CSF preceding serum. The MAC-ELISA test, however, cannot distinguish between other related flaviviruses such as SLEV, JE, YF, and Dengue and must be interpreted in caution if patients have a history of exposure (due to vaccination, travel, previous infection, or in virus endemic areas) to these other related viruses. Furthermore, the presence of IgM antibodies has been detected in people for up to 6 months.[119] Serologic confirmation in these instances requires the detection of serum neutralizing antibodies in serum or CSF with PRNT. Virus isolation and serological assays are still considered the mainstay for diagnosis of WN but are labor intensive and time consuming with the assays taking usually one week for completion. Additionally, virus isolation is often difficult in humans and in horses, which have a low and transient viremia and low levels of virus in the CNS compared with birds and mosquitoes. Development of molecular diagnostics such reverse transcriptase-polymerase chain reaction (RT-PCR) for the detection of viral RNA transcripts as a complement to virus isolation and serologic testing allowed for more comprehensive detection of WN infection. Additional

molecular techniques since RT-PCR have made it possible for the rapid detection of WNV and high-throughput diagnosis of virus. Compared to the traditional RT-PCR assay, the TaqMan based real-time quantitative PCR assay developed could be performed in 3 hours and the detection limits was 10 fold more sensitive in detection of virus and set the stage for the a multitude of highly sensitive and specific nucleic acid amplification tests (NAT).[69] NATs have become quite important with cases of virus transmission in humans from infected blood and organ transplants.[152]

**Clinical diagnosis in horses and humans**

The human case definition for WNE is defined as febrile illness associated with one or more neurological manifestations (headache, aseptic meningitis, or encephalitis) plus at least one of the following: (1) Isolation of WN virus from tissue, blood, CSF, or other body fluid, (2) Demonstration of West Nile virus antigen or genomic sequences in tissue, blood, CSF, or other body fluid, (3)Demonstration of IgM antibody to WNV in acute CSF by IgM-capture enzyme immunoassay,(4) demonstration of both WNV IgM and IgG (screened by EIA or HI and confirmed by PRNT in single serum specimen, and (5) a four-fold or greater serial change in PRNT antibody of WNV in paired samples two weeks apart (IgG titers increase in people between days 7 and 21 post onset of illness).[103] The case definition for equine WN infection is based on the human case definition. Clinical signs of appropriate neurological abnormalities along with an IgM titer $\geq$ 1:400 on the MAC-ELISA on CSF or serum is considered highly suggestive of infection. History of vaccination with the killed equine WNV vaccine has been found not to interfere with interpretation of IgM antibody levels. The effect of the modified live equine vaccine, however, is not yet known. Horses, in contrast with humans, IgM antibody levels appear to last for three months. Additionally, the appearance of CSF IgM does not appear to be before antibody appearance in the serum.[104] Recently detection of WNV RNA transcripts

with TaqMan based Real Time RT-PCR for NS5 proved to be a more sensitive method of detection when compared to immunohistochemical staining of paraffin embedded formalin fixed tissues.[144]

Additional diagnostics in addition to serology and molecular detection of viral RNA include: (1) complete blood count and serum chemistry. In humans with WN infection normal or elevated total leukocyte counts, with lymphopenia, and anemia is seen. Hyponatremia has also been reported suspected to be due to inappropriate release of antidiuretic hormone.[103] Similar changes have been reported in horses.[105] (2) CSF analysis Cytologic examination of CSF fluid may also be helpful as horses have a mononuclear pleocytosis.[104] In humans pleocytosis with leukocyte counts ranging from 0 to 1782 cells/mm and a predominant lymphocyte population is seen in the CSF. Elevated protein levels (51 to 899 mg/dL) and normal glucose levels were seen., (3) diagnostic imaging in humans is feasible and may be helpful. No evidence of acute disease is seen on computed tomography and enhancement of the leptomeninges and/or periventricular areas are seen on magnetic resonance imaging.[103]

**Diagnosis of West Nile in birds**

The extensive mortality seen in American Crows have made dead bird testing an effective means of tracking the spread and activity of West Nile and have heavily relied on molecular amplification technology. The latest assay, the VecTest antigen-capture wicking assay could be used under field conditions and was found to be a rapid and reliable test in the detection of WN in mosquitoes and from orpharyngeal swabs of crows. The test, however, had a poor sensitivity than Real Time TaqMan PCR when antigen levels were low (early in infection) and was recently shown to have low sensitivity of detection in raptors (birds that shed lower quantities of virus). Usefulness of the VecTest as a screening test, however is recognized.[45]

**Treatment**

As with many other encephalitic viruses, there are still no known antiviral medications marketed that demonstrate reliable activity against infection. Clinical studies evaluating the efficacies of INF-α and WNV specific IV immunoglobulin as potential therapies for WNV are still in progress. In one study, interferon-alpha-n3 did not show any benefits in the treatment of Japanese encephalitis patients.[133] Hyperimmune WNV plasma administration in a blinded placebo controlled trial decreased the risk of development of recumbency in horses that received plasma but treatment did not change outcome and severity in WNV disease.[75] Hence treatment for WN infection is primarily supportive. In horses, the survival rate for WNV encephalitis is high compared with other infectious encephalitides. Most horses appear to begin recovery 3 to 5 days after onset of signs. Flunixin meglumine every 12 hours intravenously at 1.1mg/kg appears to decrease the severity of muscle tremors and fasciculations within a few hours of administration. To date, much of the mortality in WNV horses results from euthanasia of recumbent horses for humane reasons.[105,110]

**WNV Vaccines**

Early epidemiological studies in the horse indicated that vaccines were the best preventative strategy.[100] Although no human WNV vaccine is currently available, four WNV vaccines have been licensed for use in the horse by the United States Department of Agriculture (USDA) The first licensed vaccine (West Nile-Innovator®, Fort Dodge, Fort Dodge, IA) for horses has been available since 2001 and is composed of formalin-inactivated (K-WN) whole virus with adjuvant. A DNA plasmid vaccine was licensed in 2003, but has not been available commercially.[149] In 2004, a recombinant vaccine was licensed and consists of a canarypox virus vector (CP-WN) with insertion and expression of the membrane (prM) and envelope (E) proteins of WNV genes (Recombitek® Equine WNV Vaccine, Merial Limited, Athens, GA).[91] This

preparation also contains an adjuvant. Several epidemiological studies investigating the outbreaks throughout the United States have justified the use of these commercially available vaccines as a preventative strategy.[27,110,117,155]

The latest equine vaccine granted licensure in September 2006 is a single-dose, attenuated West Nile Virus, live flavivirus chimera (WN-FV) vaccine (PreveNile™, Intervet, DeSoto, KS) for horses and is marketed without an adjuvant. This vaccine was based on the technology used to create an attenuated human vaccine for Japanese Encephalitis virus (Chimerivax-JE).55 The recombinant chimera expresses the envelope (E) and membrane (prM) of WNV in a Yellow Fever vector (YF17D). The vaccine has been labeled for use in the horse for prevention of West Nile Virus viremia and as an aid in the prevention of WNV disease and encephalitis.

Table 2-1. Equine CNS pathogens

| Bacterial | Viral | Parasitic |
|---|---|---|
| Escherichia coli | West Nile Virus | Setaria |
| Actinobacillus equuli | Western Equine Encephalomyelitis Virus | Habronema |
| Streptococcus (equi, zooepidemicus, suis) | Eastern Equine Encephalomyelitis Virus | Hypoderma bovis |
| Salmonella | Venezuelan Equine Encephalomyelitis Virus | Parastronglyus sp |
| Pasteurella caballi | St. Louis Virus | Strongylus vulgaris |
| Pseudomonas aeruginosa | Murray Valley Virus | Halicephalobus gingivalus |
| Enterococcus | Louping ill Virus | Draschia megastoma |
| Actinomyces | Snowshoe hare Virus | Trypanosoma evansi |
| Rhodococcus equi* | Main Drain Virus | |
| Brucella abortus* | Semliki Forest Virus | |
| Mycobacterium bovis* | Japanese Encephalitis Virus | |
| Staphylococcus sp * | Hendra Virus | |
| Actinobacillus lingieresi* | Nipah Virus | |
| Klebsiella pneumoniae, type 1 | Powassan Virus | |
| Listeria monocytogenes. | Aujesky's disease Virus | |
| | Equine Herpesvirus type 1 | |
| | Equine Infectious Anemia Virus | |
| | Rhabdovirus (rabies) | |
| | Kunjin Virus | |
| | Borna Virus | |

| Protozoal | Fungal | Rickettsial |
|---|---|---|
| Balamuthia mandrillaris | Cryptococcus neoformans, | Anaplasma phagocytophilum |
| Sarcocystis neurona | Aspergillus niger, fumigatis* | |

* denotes vertebral body osteomyelitis

CHAPTER 3
IMMUNOLOGY OF THE INTRATHECAL MODEL OF WEST NILE VIRUS

## Introduction

Since its emergence into the United States in 1999, West Nile Virus (WNV) has killed a total number of 928 humans, over 6,000 horses, and an estimated hundreds of thousands of birds. This mosquito-borne encephalitic flavivirus has caused clinical disease in 23,962 humans and 24,824 horses.[1,2] Despite the heightened attention surrounding this serious pathogen, limited information is available that characterizes protective immunity in the outbred host. Basic questions regarding the protective immune response and effective prophylaxis still remain unanswered.

Like humans, the horse is a host that is susceptible to the development of central nervous system infection with WNV. Horses develop a clinical course of disease characterized clinically by changes in mentation, spinal cord ataxia and weakness, movement disorders, and cranial nerve paralyses with pathological changes in the brain and spinal cord consistent with encephalomyelitis.[21,132] Humans develop a meningitis and/or encephalitis characterized by movement disorders, change in sensorium and behavior, mentation deficits, and coma.[103] Direct infection of neurons occurs in both humans and horses.[122] West Nile virus pathology and immunity has been primarily characterized in the mouse and in particular, WNV mediated events have been associated with a combination of virus induced neuronal cell death, host immune responses, and brain parenchymal cell induced inflammation. Mice that develop signs of CNS disease have high levels of antigen in neurons and infiltrating leukocytes whereas mice that do not develop CNS disease have low levels of antigen, leukocyte infiltration, and neuronal injury.[124] *In vitro* studies demonstrate neuronal injury is due to apoptosis.[102,154,161] Elevated levels of TNF-α are secreted from activated microglia in wild type mice challenged with WNV

and may play a role in neuropathology.[154] An important unsubstantiated theory is that the TNF-α response causes loss of integrity of the blood-brain barrier and enhances viral localization in the CNS.[29] Neutralizing antibody has been classically considered an important protective mechanism against flaviviruses, but recent work has also demonstrated the importance of cell mediated immunity in control of WNV infection.[31,123]

Three WNV challenge models have been utilized to substantiate the efficacy of vaccines for horses. Two of these models, mosquito feeding and intravenous injection of live, virulent WNV, mirror the field infection ratio of asymptomatic:symptomatic of 11:1 and both induce viremia, but neither are able to induce significant clinical signs of WNV disease in horses.[20,98,127] The intrathecal model of infection induces grave, reproducible encephalomyelitis in all naïve subjects and was utilized to substantiate the efficacy of the two commercially available modified-live equine vaccines.[13,76,126] This data describes further development of the intrathecal model in a non-naïve horse to investigate mechanisms of immune protection in an outbred host.

## Materials and Methods

### Animals

All work with animals was performed with approval and under the guidance of the University of Florida (Gainesville, FL) Institutional Animal Care and Use Committee (IACUC). Horses used were of mixed breed, one horse was defined as no previous WNV exposure, as indicated by repeated WNV neutralization titers of ≤ 2 while the other horse was affected with clinical WNV which is described. Standard practices for determination of health status and preventative medicine (diet, parasite and infectious disease prevention, and podiatry) were performed under the guidance Animal Care Services, University of Florida. Rectal temperature, heart and respiratory rates, appetite, and attitude were recorded on a daily basis prior to commencement of study protocols.

**Clinical Disease**

Physical evaluations were performed daily upon arrival of these two horses into a mosquito controlled environment. Before and after experimental challenge, on days -1 and 0, and from to study completion, physical and neurological examinations were performed. Daily parameters recorded included rectal temperature, pulse rate, respiratory rate, attitude, feed intake, locomotion, recumbency, and moribund status. Signs of clinical neurologic disease consisting of changes in mentation, development of ataxia, weakness, occurrence of fasciculations and cranial nerve deficits were graded according to severity as normal (0), mild (1), moderate (2), and severe (3). Normal was defined as no change from baseline examinations. Mild abnormalities were defined as only detectable with repeated movement or stimulation depending on the site tested. Moderate abnormalities were defined as obvious with limited movement or stimulation. Severe abnormalities were those which occurred spontaneously without movement or stimulation. Fever was defined as rectal temperature $\geq$ 39.2°C (101.9°F). At the end of the study, an overdose of pentobarbital was administered to any horse which had experienced clinical WNV after experimental challenge and a full gross and histopathological evaluation was performed.

**Virulent WNV Challenge and Sample Collection**

The challenge virus, designated WNV NY99 (4132), was originally isolated from the brain of an infected crow and obtained from the Centers for Disease Control and Prevention (Fort Collins, CO). This virus was passaged once in Vero cells, once in C6/36 mosquito cells, and was passaged an additional time in BHK-21 cells cultured in 5% horse serum in order to avoid potential immune reactions to bovine serum proteins. All virus stocks were then stored in 1 ml aliquots at -80°C until use. On the day of challenge, the stock virus was thawed on ice and virus was diluted to the desired concentration in PBS immediately prior to intrathecal inoculation of

horses. The stock virus was prepared and aliquoted as stock in a single laboratory (Bowen) and all challenges utilized the same material.

Horses were anesthetized by administration of xylazine intravenously (1.0 mg/kg, IV) and, after 10 minutes, ketamine (2.2 mg/kg, IV) was administered for induction of anesthesia. The atlanto-occipital space was penetrated with an 18 gauge, 20 cm spinal needle with aseptic technique and a 1 ml volume containing a target dose of 5 $\log_{10}$ PFU/ml. Replicate virus back titrations on susceptible cells were conducted on challenge material to verify a minimum challenge dose of 1 X $10^5$ PFU/ml. Serum and EDTA and heparin plasma samples were collected by jugular venipuncture on Days 0 to 10, 14, 21, and 28 post-infection. Horse ID1 was humanely sacrificed on day 28 post-infection with intravenous injection of pentobarbital solution (Beuthanasia-D, Med-Pharmex, Pomona, CA). Tissue sections of the cerebrum, cerebellum, thalamus, midbrain, hindbrain (pons and medulla), cervical spinal cord and lumbar spinal cord were placed in 10% buffered formalin solution. Fixed tissue samples were embedded in paraffin, cut as 5 μm sections and stained with hematoxylin and eosin for microscopic viewing.

**Virus Detection**

Vero 76 (NVSL) cultures were grown at 37C, 5% $CO_2$ in Eagle's minimal essential medium (EMEM, Mediatech) supplemented with 5% inactivated fetal bovine serum (FBS), antibiotics, HEPES. Confluent T75 Vero 76 culture flasks were inoculated in duplicate with 500 uL of EDTA plasma for 1 hour at 37°C at 5% $CO_2$. After 1 hr the fluid was removed and flasks were supplemented with 5mL of culture media (EMEM with 5% inactivated FBS, antibiotics, L-glutamine), incubated, and viral cytopathic effects (CPE) were recorded for one week after.[10] The flasks were frozen overnight at -70°C, thawed, and liquid collected into 15mL conical flasks. Samples were spun at 3,000 X *g* for 15 min. Pellets were resuspended in 1mL of

Trizol®. RNA was isolated according to manufacture protocol and stored at -70C for detection of WNV. All RNA samples were treated with amplification grade DNAse I and dissolved in DEPC treated water. cDNA was synthesized from 1ug of RNA with a commercial RT-for PCR kit (Advantage®, Clontech, Mountain View, CA) by using the protocol of the manufacturer. Amplification of 2uL of cDNA was performed in a 25uL PCR reaction using TaqMan Universal PCR Mastermix (Applied Biosystems, Foster City, CA), with 0.9uM forward and reverse primers, and 0.25uM probe. The primers amplify the non-structural protein 5 (NS-5) region of the WNV genome.[16] The sequences used were 5'-GCT CCG CTG TCC CTG TGA-3' (forward) and 5'-CAG TCT CCT CCT GCA TGG ATG-3' (reverse) and 5'-6FAM-TGG GTC CCT ACC GGA ACA ACCACG T-T-TAMRA-3' (probe). Amplification and detection was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) and included 40 cycles at 94C for 45s, and 60 C for 45s with a final hold step at 4C.[144]

**WNV IgM Capture ELISA**

The IgM capture for use in horses was developed by the National Veterinary Services Laboratory (Ames, IA) and has been previously described.[100] Briefly, microtiter plate wells were coated overnight at 4°C with 100 µl goat anti-equine IgM (Bethyl Labs, Montgomery, AL) diluted at 1:400 in carbonate/bicarbonate buffer, pH 9.6. After incubation and washing, the plates were blocked with 0.5% Tween-20 diluted in PBS (PBST) with 5% milk for 2 hr at room temperature. Each serum sample, diluted 1:400 in PBST were added in duplicate to wells and incubated for 1 hr at 37ºC. After incubation and washing, bound equine IgM was reacted with WNV antigen and incubated overnight at 4ºC. After incubation and washing, a flavivirus-specific horseradish peroxidase antibody conjugate (CDC, Fort Collins, CO) was added and incubated for 1 hour at 37°C. Bound conjugate was detected after washing by incubation for 10

minutes with 3,3',5,5'-tetramethylbenzidinec (TMB, Kirkegaard & Perry Laboratories, Gaithersburg, MD) in an acidic buffer and $H_2O_2$. For control, each sample was also reacted with negative antigen. Every plate had control wells will consisting of a positive control reference sera provided by NVSL, 2 negative control horses, and negative serum control well (blank). The reactions were stopped at 10 min with 0.5 M HCl and the absorbance was read at 450 nm. The reaction was considered positive when the optical density (OD) of the sample was ≥2X the OD of the negative reference sera, and the index consisting of the OD of the sample reacted with the negative antigen (P:N ratio) ≥2.00.

**Plaque Reduction Neutralization Test**

Plaque reduction neutralization was performed to detect WNV-specific antibody post infection.[100] Briefly, serum was diluted at 1:10 and 1:100, heat inactivated at 56°C for 30 min, and incubated with an equal volume of virus (WNV, strain NY99-4132) to a final concentration of 100PFU/0.1 mL. Samples were incubated 37C for 1 h, and 0.1 mL of each was added to confluent monolayer of Vero cells in 6-well plates. After incubation for 1 h, cell monolayers were overlaid with 0.5% agarose; days after addition of the second overlay. A 90% reduction in PFU, as compared to the serum-negative control, was used as the determinant of neutralization.[20,100]

**Capture Isotype ELISA**

The isotype ELISA was based on the IgM capture for use in horses.[100] Briefly, microtiter plate wells were coated overnight at 4°C with either 100 µl goat anti-equine IgG total (heavy and light chain, H+L), IgGa, IgGb, or IgGc (Bethyl Labs, Montgomery, AL) diluted at 1:400 in carbonate/bicarbonate buffer, pH 9.6. After incubation and washing, the plates were blocked with 0.5% Tween-20 diluted in PBS (PBST) with 5% milk for 2 hr at room temperature. Each

serum sample was first screened at dilution of 1:400 (in PBST) to 1:128,000. Positive samples were diluted in PBST from 1:400 to 128,000 and negative were diluted from 1:10 to 1:400 and added in triplicate to wells and incubated for 1 hr at 37ºC. After incubation and washing, bound equine IgG was reacted with WNV antigen and incubated for 1 hr at RT. After incubation and washing, a flavivirus-specific horseradish peroxidase antibody conjugate (CDC, Fort Collins, CO) was added and incubated for 1 hour at 37°C. Bound conjugate was detected after washing by incubation for 10 minutes with 3,3',5,5'-tetramethylbenzidinec in an acidic buffer and $H_2O_2$. For control, each sample was also reacted with negative antigen. Every plate had control wells consisting serum from a positive horse (National Veterinary Services Laboratory, Ames, IA) and 2 non-infected non vaccinated horses. The reactions were stopped at 10 min with 0.5 M HCl and the absorbances was read at 450 nm and were compared against a well in which no serum was added (blank). End-point titer was defined as the highest dilution of serum that gave an OD450 value that was 2-fold greater or if the OD450 signal exceeded by more >0.1 OD unit, the signal of the horse's pre-immune serum at the same dilution.[97]

**Cytokine mRNA Expression**

Collected peripheral blood monocytes (PBMC) were separated from whole blood collected in ACD by layering leukocyte rich plasma over histopaque (1.077, Sigma-Aldrich) and centrifuging. Aliquots of PBMCs at 1x $10^7$ cells/mL in freezing media (10% DMSO in fetal bovine serum) were stored at -70ºC. The protocol for cytokine mRNA expression was based on previously described methods.[47] Briefly, PBMC at concentration of $2x10^6$ cells, were lysed in Trizol®., treated with DNAse I, and dissolved in DEPC water. cDNA from 1 ug of total RNA was synthesized with Advantage® RT-for PCR kit according to manufacturer protocol. Amplification of cDNA and Real-Time PCR was done using primers and probes established in

the literature and based on sequences for equine cytokines IL1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p35, IL-12p40, TNF-α, TNF-β, IFN-γ and glyceraldehyde-3-phosphate dehydrogenase (G3PDH).[49] Amplification of 2 μl of cDNA was performed in a 25 μl PCR reaction using a commercially available TaqMan Universal PCR Mastermix and performed with the ABI Prism 7700 Sequence Detection System. All samples were assayed in triplicates and the mean values used for comparison with all the results normalized to G3PDH expression. Relative quantitation between samples was achieved by comparing their normalized threshold cycles (Ct). Samples without cDNA were included in the amplification reactions to determine background fluorescence and check for contamination. DNase-treated RNA samples were also subjected to PCR using the G3PDH primers to confirm the absence of genomic DNA contamination.

## Results

### Clinical Disease

At D7 post infection, horse ID1 developed clinical signs consistent with WNV encephalomyelitis (Figure 3-1). Over the next 72 h, this included onset of fever 38.4 to 40.1 ºC (101.2 to 104.2°F), depression, anorexia, obtundation (grade 2) , muscle fasciculations (grade 3), hyperesthesia (grade 3), hind limb paresis (grade 3), hind limb ataxia (grade 3), and bilateral tongue paresis (grade 1). Clinical improvement was seen on D14 post infection with a return to normal temperature 37.4 to 38°C (99.3 to 100.4 °F) and mentation with no evidence of muscle fasciculations. The colt was a grade 1 for ataxia in the right hind limb, a grade 2 for paresis in the right hind limb, and had right sided tongue paresis (grade 1) on neurological examination. On D20 post infection, the colt another increase in rectal temperature to 39.8°C (103.6°F) characteristic of the biphasic fever reported with WNV infection in humans. Exacerbations of ataxia, paresis, and tongue paresis to one grade higher in severity were also noted with the fever.

The following day, however, the colt had a normal temperature of 37.5°C (99.5°F) and an improvement in neurologic deficits. Paresis, ataxia, and tongue paresis were resolved by day 28 post infection. Histopathological study revealed neural tissue lesions consistent with those previously reported for WNV encephalomyelitis in horses (glial nodules and perivascular cuffing) in the pons, medulla, and distal lumbar spinal segments.

Approximately 76 days before experimental challenge, ID2 was shipped from a farm in western Montana to Alachua, Florida. Prior to arrival the neutralizing antibody titer to WNV was <2 and the horse was clinically normal. At arrival the horse had a normal temperature, pulse, and respirations but was noted to be mildly depressed and to have a slight deviation of her nostrils consistent with facial nerve paralysis. No significant spinal abnormalities were noted. The presenting clinical signs and a positive IgM titer for WNV thirteen days after arrival it was likely that ID2 had natural exposure to WNV en route to the testing facility. The horse was maintained in a mosquito free environment until experimental challenge. After experimental challenge, from days 1 to 10 this horse was unwilling to move its neck vertically but may have been attributable to enlargement of the right submandibular lymph node. No neurological abnormalities were associated with this response and this horse did not develop signs consistent with WNV encephalomyelitis throughout the study period. On D7, there was mild depression and rectal temperature in this horse increased to 38.78°C (101.8°F). The horse was not sacrificed at the end of the trial and has remained clinically normal for over 1 year.

**Virus Detection**

Viral cytopathic effects (CPE) were observed in Vero cell cultures incubated with EDTA plasma collected from horse ID1 on D1, D2, D3 and D4 post infection. Detection of WNV RNA was confirmed by Real-Time PCR amplification of the nonstructural protein 5 (NS5) region of

WNV from culture samples. No virus induced CPE's were detected in any of the plasma collected from ID1 nor was any viral RNA detected by RT-PCR.

**Serology**

Horse ID1 had evidence of seroconversion post infection with the detection of IgM WNV antibody and a low level of neutralizing antibody (1:10) starting on D10 and these levels were detected until the end of the study. On the day of infection (two-months after initial screening), horse ID2 was negative for IgM to WNV and IgM antibodies were not detected in any of the serum samples collected post infection. Neutralizing antibody to WNV was detected at 1:100 throughout the study period.

IgG total (H+L) levels measured by capture ELISA agreed with the PRNT. WNV specific IgG total (endpoint titer of 1:400) was detected in ID1 starting on D10 post infection and remained steady until D28 (Table 3-1). IgG total was detected in ID2 starting on D0 with a measured endpoint titer of 1:64,000. The IgG total endpoint titer for ID2 decreased to 1:16,000 on D6, but rebounded to 1:32,000 on D7. IgG total antibody for ID2 returned to the starting titer of 1:64,000 by D10 and remained the same to the end of the study (Table 3-1). IgGa isotype antibody for ID1 mirrored the kinetics of IgG total, with detectable levels (endpoint titer of 1:400) starting on D10 post infection. Isotype IgGb antibody in ID1 were <1:50 throughout the study period. Isotype IgGa antibody levels in ID2 were < 1:50 on D0 but were detected on D4 (1:200) and increased to 1:16,000 by D10. Isotype IgGb antibody levels for ID2 were detected at 1:32,000 on D0, with fluctuations post infection on D1 (1:16,000) and D6 (1:8000). Levels of IgGb antibody for ID2 increased on D7 to 1:32,000 and remained steady to D28. Isotype IgGc and IgGt antibodies were not detected.

**Cytokines**

On D4 post infection a decrease in the expression from baseline of the inflammatory cytokines IL-1β, IFNγ, IL-6, and IL-8 was observed in ID2 whereas IFNγ and IL-12 expressions were increased in ID1 (Table 3-2). TNF-α was markedly decreased for ID2 compared to ID1 on D4 post infection. On D7 post-infection for ID2, increase in the expression from baseline occurred relative for the Type 1 cytokines (IFN-γ, IL-1β, IL-12) on D7 post infection. A noticeable decrease in TNF-α expression on D7 in ID2 compared with ID1 was also noted. The type 2 cytokines (IL-4, IL-5, TGF-β) were increased in ID1 from baseline on D7 post infection.

## Discussion

Apart from the major economic burden WNV has placed on the horse industry, horses provide an important spontaneous animal model for a potentially life-long debilitating and fatal human disease. Hamster and mouse models of WNV, despite convenience, do not reflect the low viremia and CNS pathology seen in horses and humans.[20,30,142, 143] The intrathecal model of WNV challenge results in reproducible and encephalomyelitis in naïve horses. This study validates its further development as a model of protective immunity to WNV.

Data obtained from naïve horse ID1 and naturally exposed horse ID2 suggests the first experimental evidence for immunity to rechallenge with WNV. Like humans, it is expected that horses are resistant to re-infection with flaviviruses and that the immunity is life-long.[100] Neutralizing antibodies to WNV have been shown to persist for a long as 7 months in horses[27], but further characterization of the immune response beyond serology has not been elaborated. In this study naturally exposed horse, ID2 was completely protected from redevelopment of WNV encephalomyelitis after intrathecal challenge with no evidence of viremia post infection. Response to challenge in naïve horse ID1 reflected previous studies using the intrathecal model,

wherein evidence of viremia, development of encephalomyelitis, and histological lesions in the pons, medulla, and distal lumbar segments was found. Further studies validating the long duration of immunity in non-naïve horses longer than one month are warranted.

In this study, IgM antibodies to WNV were detected in horse ID2 during screening, but by the time of challenge, ID2 had undergone the expected conversion from IgM to IgG, shown to be less than three months post exposure in horses. No IgM antibody was detected on the day of challenge but high levels of total IgG antibody (1:64,000) were detected. High levels of isotype IgGb antibody (1:32,000) were present on D0 in ID2 and IgGa antibody was detected at D4 and increased to 1:16,000 by D10. IgM and IgG antibodies were detected in ID1 on D10 after infection. Neither IgGc nor IgGt isotypes were detected in either horse. As been reported with other equine viral pathogens, IgGa and IgGb isotypes may play important roles with WNV infection.[23,79,120,121] IgGa and IgGb isotypes are reported to fix complement and are associated with antibody-dependent cellular cytotoxicity, whereas IgGc and IgGt are not able to fix complement and can inhibit complement fixation by other IgG isotypes.[89] A transient IgGa isotype antibody response also seen with other equine pathogens (EHV-4, *Streptococcus equi*),[89,120,121] is suggested with the high levels of IgGb antibody present in ID2 at D 0 and with IgGa induced after infection. Further studies are needed as IgGb isotype was not detected in ID1 by D28, with only IgGa antibody detected at D10-D28. It is not known whether IgGb antibody levels would have been detected at greater than 28 days in ID1 and if IgGa levels would have decreased in both ID1 and ID2.

Preliminary data characterizing the *ex vivo* PBMC cytokine expression in ID1 and ID2 using quantitative Real-Time PCR suggests that the protective immune response to WNV infection may be a Type 1 ($T_H1$) T cell profile as seen in horse ID2 on D7 PI. This is consistent

with evidence from murine studies wherein cell-mediated and $CD4^+$ T cell responses have been shown to be protective and critical for clearance of WNV from the CNS.[31,129] Furthermore with ID2, a notable decrease in TNF-α, a cytokine that has been implicated in mediating WNV entry into the CNS in murine studies was decreased on D4 and D7 PI.[29,154] Additional inflammatory and acute phase cytokines (IL-1β, IFN-γ, IL-6, and IL-8), that are typically expressed during initial viral infection were also noted to be decreased in ID2 on D4 PI. In ID1, despite an initial increase in IFN-γ and IL-12 expression levels on D4 PI that may be reflective of the induced innate immune response.[31,70] by D7 PI increased levels of Type 2 cytokines (IL-4, IL-5, TGF-β) were noted, which are normally expressed by $T_H2$ $CD4^+$ T cells.

The long-term effects of WNV infection and clearance from the CNS is becoming an area of interest as poliomyelitis and Guillain-Barre like syndromes have been described in WNV patients and also patients suffering from prolonged chronic WNV meningitis.[119] Persistent infection and recovery of virus has been reported in hamsters.[143] Interestingly horse ID1, despite resolution of clinical signs and neurological deficits by day 28 post infection had evidence of histologic lesions in the pons, medulla, and distal lumbar segments. Unfortunately, neural samples were not quantified for presence of virus, but certainly the intrathecal model would be useful for looking at the long-term effects of the virus in the CNS.

Additionally, clearance of an RNA virus with a neurotropism for neurons, requires the secretion of IFN-γ, long-term secretion of neutralizing antibody, and the presence of memory T and B cells.[53] Evidence of T cells expressing memory markers has been found in mice 56 days post intracerebral WNV infection as well as murine studies that have shown $CD8^+$ T cells and $CD4^+$ T cells as critical in the clearance of WNV in the CNS and in the control of infection.[123,129] In this study, it could be argued that horse ID2 was protected from intrathecal challenge of WNV

by the presence of neutralizing antibody in the CSF, however, duration of IgG levels for WNV in the CSF have not been quantified in the horse[104] and further characterization of the immune response in non-naïve horses looking at CNS cellular populations is needed.

This study further validates the importance of the intrathecal model in which protective immunity to WNV can be induced in the horse. The model confirms the paradigm that long-term immunity after flavivirus infection does occur in the horse allowing for more rational use of post-encephalomyelitis prophylaxis. Further development of this model of long-term immunity in the horse will allow investigation of the components of long-term protection in an outbred mammalian host. Much work is still needed to characterize the protective immune response to WNV infection. Further studies in terms of antigen specific responses, characterization of the lymphocyte populations in the periphery and CNS, and cytokine expression in the periphery and CNS are in development.

Table 3-1. IgG isotype endpoint titers post-infection

| | IgG total | | IgGa | | IgGb | |
|---|---|---|---|---|---|---|
| | ID1 | ID2 | ID1 | ID2 | ID1 | ID2 |
| D0 | <50 | 64,000 | <50 | <50 | <50 | 32,000 |
| D1 | <50 | 64,000 | <50 | <50 | <50 | 16,000 |
| D4 | <50 | 64,000 | <50 | 200 | <50 | 32,000 |
| D6 | <50 | 16,000 | <50 | 400 | <50 | 8,000 |
| D7 | <50 | 32,000 | <50 | 8000 | <50 | 32,000 |
| D10 | 400 | 64,000 | 400 | 16,000 | <50 | 32,000 |
| D14 | 400 | 64,000 | 400 | 16,000 | <50 | 32,000 |
| D21 | 400 | 64,000 | 400 | 16,000 | <50 | 32,000 |
| D28 | 400 | 64,000 | 400 | 16,000 | <50 | 32,000 |

Table 3-2. Summary of fold differences in mRNA cytokine expression in horses after WNV infection

| | D4 PI[a] | | D7 PI[a] | |
|---|---|---|---|---|
| | ID1 | ID2 | ID1 | ID2 |
| IFNγ[b] | 2.3↑ | 0.37↓ | 0.75↑ | 0.88↑ |
| IL-12p40 | 1.18↑ | 0.33↑ | 1.18↑ | 0.46↑ |
| IL-1β | 1.8↓ | 3.63↓ | 7.9↓ | 1↑ |
| IL-2 | 1.75↓ | 2↓ | 2.3↑ | 0.17↑ |
| IL-8 | 0.58↓ | 4.84↓ | 5.03↓ | 1.28↓ |
| TNFα | 1.3↓ | 10.39↓ | 1.06↓ | 6.5↓ |
| IL-4 | 5.93↓ | 0 | 8.6↑ | 0 |
| IL-5 | 1.2↑ | 0.09↑ | 1.2↑ | 1.6↑ |
| IL-6 | 1.23↓ | 7.53↓ | 1.23↓ | 0.43↑ |
| IL-10 | ND[c] | ND[c] | ND[c] | ND[c] |
| TGFβ | 1.27↑ | 1.47↑ | 1.21↑ | 2.2↑ |

[a]*ex vivo* mRNA expression from PBMC collected on D4 and D7 PI. [b]Fold increase ↑ or fold decrease ↓ relative cytokine mRNA expression from D0 baseline expression. [c]ND=not detected

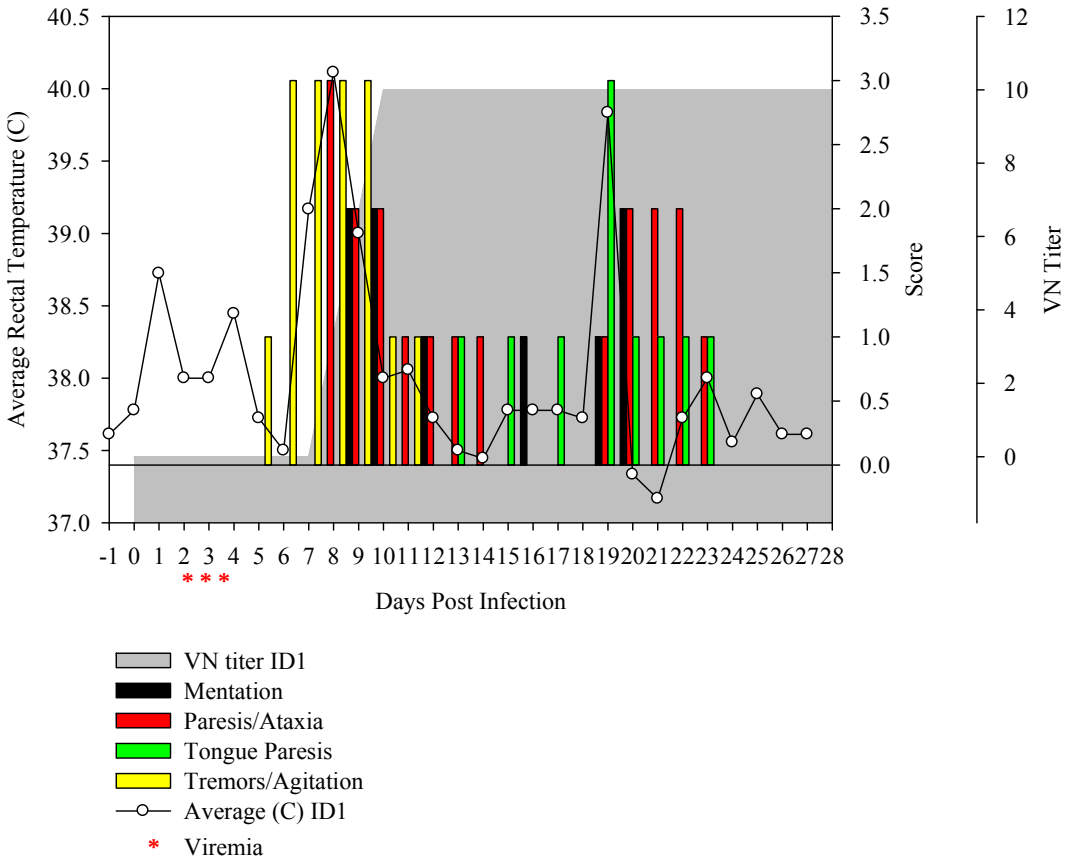


Figure 3-1. Clinical course post infection Horse ID1

# CHAPTER 4
# COMPARATIVE EFFICACIES OF THREE COMMERICALLY AVAILABLE VACCINES AGAINST WEST NILE VIRUS (WNV) IN A SHORT-DURATION CHALLENGE TRIAL INVOLVING AN EQUINE WNV ENCEPHALITIS MODEL

## Introduction

West Nile Virus (WNV) is a mosquito-borne flavivirus of the Japanese Encephalitis virus (JEV) serogroup that causes central nervous system (CNS) infection in horses and humans.[58] Since its emergence into the United States in 1999 and subsequent spread throughout the country, WNV has caused clinical disease in 23,962 humans and 24,824 horses in the U.S.,[1,2] killing approximately 928 humans, over 6,000 horses, and an estimated hundreds of thousands of birds. Although currently endemic throughout much of North America, WNV still is an important infectious pathogen with 4,085 clinical cases of disease reported in humans and 1,061 encephalomyelitis cases reported in horses in 2006.[1,2]

Early epidemiological studies in the horse indicated that vaccines were the best preventative strategy.[100] Since then, four WNV vaccines have been licensed for use in the horse by the United States Department of Agriculture (USDA) The first licensed vaccine (West Nile-Innovator®, Fort Dodge, Fort Dodge, IA) for horses has been available since 2001 and is composed of formalin-inactivated (K-WN) whole virus with adjuvant. A DNA plasmid vaccine was licensed in 2003, but has not been available commercially.[149,160] In 2004, a recombinant vaccine was licensed and consists of a canarypox virus vector (CP-WN) with insertion and expression of the membrane (prM) and envelope (E) proteins of WNV genes (Recombitek® Equine WNV Vaccine, Merial Limited, Athens, GA).[91] This preparation also contains an adjuvant. Several epidemiological studies investigating the outbreaks throughout the United States have justified the use of these commercially available vaccines as a preventative strategy.[27,46,110,117,155]

The latest equine vaccine granted licensure in September 2006 is a single-dose, attenuated West Nile Virus, live flavivirus chimera (WN-FV) vaccine (PreveNile™, Intervet, DeSoto, KS) for horses and is marketed without an adjuvant. This vaccine was based on the technology used to create an attenuated human vaccine for Japanese Encephalitis virus (Chimerivax-JE).[55] The recombinant chimera expresses the envelope (E) and membrane (prM) of WNV in a Yellow Fever vector (YF17D). The vaccine has been labeled for use in the horse for prevention of West Nile Virus viremia and as an aid in the prevention of WNV disease and encephalitis.

Two previous WNV challenge models in horses (mosquito feeding and needle inoculation) were used to substantiate the primary efficacy studies and labeling claims of the K-WN and CP-WN vaccines. These two models mirror the field infection ratio of asymptomatic:symptomatic of 11:1 and both induce viremia, but neither are able to induce significant clinical signs of WNV in horses.[20] Hence, protection in these models is defined as "prevention of viremia due to WNV." Development of a WNV challenge model of infection has been described where grave, reproducible encephalomyelitis occurred in all naïve horses when injected intrathecally with virulent WNV.[13] This model was used to establish 28-day efficacy for the CP-WN and 12-month duration-of-immunity for the WNV-FV vaccines.[76,126] This current data reports the use of the model to investigate the comparative efficacy of the three commercially available equine vaccines in a short duration challenge trial.

## Materials and Methods

### Animals

All work with animals was performed with approval and under the guidance of the University of Florida (Gainesville, FL) Institutional Animal Care and Use Committees (IACUC). Horses used were of mixed breed male and female horses, with no previous WNV exposure, as indicated by WNV neutralization titers of $\leq 5$. Standard practices for determination of health

status and preventative medicine (diet, parasite and infectious disease prevention, and podiatry) were performed under the guidance Animal Care Services, University of Florida. Rectal temperature, heart and respiratory rates, appetite, and attitude were recorded on a daily basis prior to commencement of study protocols.

**Efficacy Trials**

Horses were placed into three randomized, blinded trial groups consisting of eight horses with two horses in each group receiving (1) the inactivated K-WN vaccine, (2) the canarypox virus vectored CP-WN, (3) the chimera WN-FV vaccine, or (4) a diluent. For the K-WN and CP-WN, horses received an initial primary intramuscular (IM) injection with a 1mL volume of vaccine followed in 28 days with a second injection according to manufacturer's label. For the WN-FV, each horse received an initial injection of 1 mL diluent IM followed in 28 days by a 1 mL IM injection of the vaccine. All work with live virulent WNV was performed in accordance with Biosafety in Microbiological and Biomedical Laboratories guidelines (United States Department of Health and Human Services (HHS) Public Health Service/Centers for Disease Control and Prevention and National Institutes of Health 1999) under the approval and supervision of the University of Florida Environmental Health and Safety Office.

**Virulent Viral Challenge**

The challenge virus, designated WNV NY99 (4132), was originally isolated from the brain of an infected crow (CDC, Ft. Collins, CO). This virus was passaged once in Vero cells, once in C6/36 mosquito cells, and was passaged an additional time in BHK-21 cells and cultured in 5% horse serum in order to avoid potential immune reactions to bovine serum proteins. All virus stocks were then stored in 1 ml aliquots at -80°C until use. On the day of challenge, the stock virus was thawed on ice and virus was diluted to the desired concentration in PBS immediately

prior to intrathecal inoculation of horses. The stock virus was prepared and aliquoted as stock in a single laboratory (Bowen) and all challenges utilized the same material.

Horses were to be challenged with virulent virus greater than 28 days (or D56 of the study period) after the second vaccine injection according to the original study design. The three trial efficacy groups (CE1, CE2, and CE3) were conducted from July 2004 through April 2005 during which Gainesville, FL was affected by three major hurricanes. Due to these circumstances the actual day of challenge for each group were on D70, D56, and D70 accordingly. Horses were anesthetized by administration of xylazine intravenously (1.0 mg/kg, IV) and, after 10 minutes, ketamine (2.2 mg/kg, IV) was administered for induction of anesthesia. The atlanto-occipital space was penetrated with an 18 gauge, 20 cm spinal needle with aseptic technique and a 1 ml volume virus.

**Clinical Scoring**

Physical evaluations were performed from day -1, 0, and 1 to study completion on D21 after challenge (D91, D77, D91 of the study for CE1, CE2, and CE3). Daily parameters recorded included rectal temperature, pulse rate, and respiratory rate. Temperature was further categorized as a score of 0 if rectal temperate was 37.5 to 38.3ºC (99.9 to 100.9º F), 1 if >38.3 to 38.6ºC (100.9 to 101.5º F), 2 if >38.6 to 38.8 ºC (101.5-101.9 ºF), Grade 3 >38.6 to 39.2 ºC (101.9-102.5ºF), and Grade 4 ≥ 39.2 ºC (≥102.5ºF). Changes in attitude, feed intake hydration, and locomotion were scored as normal (0), mild (1), moderate (2), and severe (3). Injection sites were monitored daily throughout the study period. A full neurological examination was performed weekly after vaccination and daily after viral challenge until the end of the study. Signs of clinical neurologic disease consisting of changes in mentation, development of ataxia and weakness, and occurrence of fasciculations and cranial nerve paresis were scored in severity

as normal (0), mild (1), moderate (2), and severe (3). Normal was defined as no change from baseline examinations. Mild abnormalities were defined as only detectable with repeated movement or stimulation depending on the site tested. Moderate abnormalities were defined as obvious with limited movement or stimulation. Severe abnormalities were those which occurred spontaneously without movement or stimulation. Total daily clinical score was calculated with the sum of graded physical parameters and neurological signs.

**Postmortem Analysis**

At the end of the study (21 days post-challenge), an overdose of pentobarbital was administered to all remaining living horses to facilitate a full gross and histological post-mortem examination.

A full gross postmortem evaluation was performed on all study horses. Tissue samples of brain (cerebrum, thalamus, midbrain, pons, and medulla), cervical spinal cord, lumbar spinal cord, left ventricular and right atrial heart muscle, liver, lung, spleen, kidney, mesenteric lymph node, adrenal gland, thymus, and injection site muscle were fixed in 10% neutral buffered formalin and were stained with hematoxylin and eosin for histopathologic evaluation.

Histopathological grading of neural tissue (cerebrum, thalamus, midbrain, pons, medulla, cervical cord, and lumbar cord) for evidence of viral encephalitis, quantified both gliosis and perivascular cuffing. Tissues were evaluated by two independent and blinded evaluators. For scoring, ten random views were examined at 100X magnification for each location. The number of glial nodules at each site was documented and averaged, each section was reexamined, and the percentage of vessels with perivascular cuffing (vessels with accumulations of inflammatory cells) was estimated and averaged per site. Histopathologic scoring was based on methodology used by Monath et al.[94] Grade 1 was defined as "minimal" changes characterized by one to three

small focal inflammatory infiltrates, a few glial nodules. Grade 2 was defined as "moderate" changes with more extensive focal inflammatory infiltrates; glial nodules centered around one-third of the neurons. Grade 3 was defined as "severe" changes with glial nodules affecting 33 to 90% of neurons and moderate focal or diffuse inflammatory changes. Grade 4 was defined as "overwhelming" with more than 90% glial nodules and severe, widespread inflammatory infiltration.

**Case Criteria and Outcome**

The normal range of rectal temperature in the horse was defined as 37.5 to 38.3°C (99.5 to 100.9ºC, but ≥ 39.2°C (102.5 ºC) was the criteria defined for CNS disease caused by WNV in this model.[76] Failure of a protective response against clinical disease (encephalomyelitis) of WNV was defined as moderate or severe, a neurological score of ≥ 2 or signs of disease for 2 or more consecutive days with viremia, histopathologic changes consistent with encephalitis, and requirement for euthanasia. Case outcome was defined as survival to the end of the study period or nonsurvival which was euthanasia or sudden death. As defined by IACUC protocols, horses were euthanized for humane reasons due to any overall severe health condition of the animal as a result of WNV infection; persistent or acute signs of West Nile disease coupled with recumbency and/or the inability to locomote without assistance.

**Virus Isolation**

One ml of plasma from heparinized blood was incubated per well in a 24-well plate format on Vero cells as described previously and incubated at 37°C in 5% $CO_2$ for 5-7 days.[76,116] The plates were observed daily for toxicity, bacterial contamination, and cytopathic effects CPE typical of WNV infection.

**Antibody Testing—Virus Neutralization Testing**

A plaque reduction neutralization test (PRNT) was used to quantify neutralizing antibody titers in horse, as previously described.[20] Any sample that neutralized the challenge virus dose at a level ≥ 70% was confirmed by testing in duplicate and titrated by serial twofold dilutions. Neutralization at a level of ≥ 90% was considered positive for each dilution. Antibody titers were defined as the reciprocal of the highest dilution showing 90% neutralization, and a titer of 5 or greater was considered positive for antibody to WNV.

**Back Titration of Virulent West Nile Virus**

For back titrations of material used to challenge horses, a plaque forming unit (PFU) assay was performed using sample aliquots of challenge virus stock prepared for intrathecal challenge frozen at -80ºC. Samples included challenge virus frozen immediately after preparation and replicate aliquots of virus that were maintained on ice until after actual intrathecal challenge. For each back titration assay, 300 uL of 10-fold dilutions of sample challenge material was added in triplicate to 6-well cell plates containing Vero 76 monolayers. Plates were incubated for 1 hour at 37ºC and 5% $CO_2$ for virus adsorption. The cells were overlaid with 3 mL per well of 0.5% agarose in MEM medium supplemented with 350 mg/L of sodium bicarbonate, 29.2 mg/L of L-glutamine, 5% heat inactivated fetal bovine serum, and antibiotics. After 48 hours of additional incubation, a second 3 mL 0.5% agarose overlay, containing 0.004% neutral red dye, was added for plaque visualization. The plaques were scored on days 3, 4, and 5 of incubation. Dilution wells closest to 30 plaques per well were used to calculated the virus titer. Viral titer was calculated as PFU/mL=1/dilution x number of plaques x amount of inoculum/well.[10]

**Statistics**

Clinical data from all three trials were combined as 2x2 contingency tables and analyzed using $\chi 2$ and Fischer Exact test for case criteria, outcome, and viremia using MedCalc Software

(Version 8.1, Mariakerke, Belgium). Level of significance was set at $p<0.05$. Clinical scores and histological data were analyzed using Kruskal-Wallis One Way Analysis on Ranks with pairwise multiple comparison with Dunn's Method. Level of significance was set at $p<0.05$. Statistical analysis was performed by use of computer spreadsheet statistical-analysis package (Sigma Stat, V; Systat Software, Inc., Point Richmond, CA).

## Results

### Clinical Scoring

#### Post-vaccination assessment

No injection site reactions or systemic effects were observed in any of the horses post-vaccination. Four of five CP-WN horses had an increased rectal temperature ranging from 38.4 to 38.9 ºC (101.2 to 102ºF) with duration of up to five days after the second vaccination booster. Three six K-WN horses had increased rectal temperature of 38.4ºC within five days after the second vaccination.

#### Post-challenge assessment

None of the WN-FV horses developed increased rectal temperature greater than 39.2ºC on any day post-challenge, while 3/6 of the control horses, 1/5 of the CP-WN, and 1/6 of the K-WN horses had increased rectal temperature. The number of control horses with rectal temperature of >39.2ºC was significantly greater compared to all vaccinated horses ($P<0.0001$) with no significant difference detected between vaccinated horses (Figures 4-1, 4-2, 4-3, 4-4). Mild elevations in rectal temperature between 38.4 and 38.8ºC were recorded for 2-5 days post-challenge in 4/6 WN-FV horses, 3/5 CP-WN horses, one K-WN horse, and 3 control horses. Mild elevations in rectal temperature between 38.5 and 38.6ºC also occurred in two K-WN horses late in the challenge period on D17 and D18. One of the CP-WN horses also exhibited mild malaise and anorexia after challenge.

Post-challenge, all 6 control horses demonstrated moderate or severe (Grade 2 or 3) signs of WNV for at least one day and 5/6 horses demonstrated moderate to severe signs of WNV for two or more consecutive days (Figure 4-1). None of 6 WN-FV horses developed neurological abnormalities post-challenge. For the CP-WN vaccinates, 1/5 horses had consistent grade 1 neurological signs in several categories. Four of 6 K-WN horses developed mild to moderate (grade 1 to 2) neurologic signs that occurred late in the challenge period. The WN-FV and CP-WN horses had significantly fewer clinical signs than K-WN horses ($p=0.035$) or control animals ($p<0.01$) (Table 4-1).

**Histopathology**

All control horses had moderate to severe (Grade 3) microscopic changes in the midbrain, medulla, and thalamus which were significantly higher ($p<0.05$) than all of the vaccinated horses. Changes consisted of glial nodules and perivascular cuffing throughout the grey matter of the brain and spinal cord, associated primarily with the large neuronal cell bodies of the midbrain, pons, medulla, and thalamus (Tables 4-2 and 4-3). Mild inflammatory changes (Grade 1) with little to no glial nodules were found in 1/6 of the WN-FV and 1/5 CP-WN horses although these horses did not exhibit any neurologic signs. Four of six K-WN horses had mild inflammatory changes in the brain and spinal cord. Two of these horses did not exhibit mild neurologic signs, 2 exhibited mild neurological signs late in the post challenge period and one horse had an elevated temperature of 38.7ºC on D4 without progression of clinical signs.

**Case Criteria/Case Outcome**

All vaccinated horses met the criteria for protection against WNV encephalitis. In all groups irrespective of the vaccine product, this was significantly different than the control horses ($p<0.0001$). All six control horses met the case criteria for WNV encephalitis of grade 2 or more

(moderate to severe), fever, viremia, histopathological evidence of encephalomyelitis, and all were humanely euthanized accordingly before the end of the study period (Table 4-1). None of the vaccinated horses met the criteria for euthanasia and all survived to the end of the observation period. The case outcome or survivorship was significantly higher in the vaccinated horses, irrespective of product, compared to the control horses ($p<0.0001$).

**Virology**

West Nile virus was not recovered from the plasma from any vaccinated horse post-challenge and this was significantly different than the control horses ($p <0.0001$). West Nile virus was recovered from all six control horses during D1 through D5 PC. The median duration of viremia was 1.5 days and viremia ranged between 1 and 3 days.

**Serology**

All horses were screened prior to arrival at the high containment testing facility (University of Florida) for previous exposure to WNV based on negative IgM (<400) and neutralizing antibodies <5 to WNV using the IgM Capture (MAC)-ELISA and PRNT. One horse in the canarypox vaccine (CP-WN) group was found to be negative for neutralizing antibody titer of <5 at screening, but had a neutralizing antibody titer of >5 on study D0 and was dropped from the study.

At the time of the second immunization low neutralizing titers were detected in the six K-WN horses ranging from <5 to 20. One of the five CP-WN horses had a titer of 10 and none of the six WN-FV horses had detectable neutralizing antibody at second injection. At the time of challenge, the K-WN horses had a geometric mean titer (GMT) of 224 (range <5 to 320), the CP-WN horses had a GMT of 26 (range <5 to 80), and the WN-FV horses had a GMT of 5 (range <5 to 10). All control horses remained negative for neutralizing antibody after vaccination and on the day of challenge (Table 4-4).

At 7 days after challenge, all 6 control horses developed low levels of neutralizing antibody to WNV (GMT 35, range 10 to 80). The GMT of all six K-WN horses increased moderately to 320 and remained the same to the end of the study. Four of 5 CP-WN horses had a slight increase in neutralizing antibody levels (GMT 66) and all 5 horses developed a mean titer of 258 by the second week post challenge. All six WN-FV horses had rapidly and steadily increasing titers, increasing from a GMT of 122 at 7 days to 320 at 14 days and remaining at 293 during the third week after challenge (Table 4-4 and Figures 4-1, 4-2, 4-3, 4-4).

**Virus Challenge Backtitration**

The challenge material had a back titration of $2 \times 10^6$, $1 \times 10^5$, and $3 \times 10^5$ PFU/mL for trial groups CE1, CE2, and CE3, respectively.

## Discussion

This study supports the efficacy of all three commercially available equine WNV vaccines for the prevention of WNV induced encephalitis in horses. Irrespective of the vaccine administered all were protective, resulting in 100% survivorship against a severe challenge model of WNV encephalomyelitis with 100% of the controls exhibiting clinical disease. Challenge of naïve horses by this model resulted in, fever, viremia, onset of grave neurological disease, with corresponding histopathologic lesions in the CNS with 100% mortality before the end of the study.

Protection against WNV was achieved with one dose of WN-FV and a protective immune response was demonstrated as early as seven days post infection with the WN-FV vaccines with the rapid rise of neutralizing antibody, consistent with previous studies with this vaccine.[76] A protective immune response was also seen in the CP-WN horses post-infection with a more gradual increase of neutralizing antibody by 14 days post-infection. The CP-WN horses were vaccinated according to the manufacturer's label of two immunizations in this study. Whether

one dose of vaccine would induce protective immunity with the severe WNV challenge model is important to demonstrate. One study has shown protection against viremia after one dose of this vaccine utilizing the mosquito challenge model.[127] In a field study, a marked anamnestic response occurred in horses that were previously vaccinated with the K-WN and injected a year later with one dose of the CP-WN vaccine.[54] As expected, a rapid increase in neutralizing antibody occurred after the second immunization in the K-WN horses in this study.

In this study, the level of neutralizing antibody titer at the time of challenge is not predictive of protective immunity as evidenced by the range of GMT among the three vaccine groups on the day of challenge (226 (KP-WN) and 26 (CP-WN) and 5 (WN-FV)). These results mirror comparative vaccine studies done in hamsters, wherein neutralizing antibody levels as low as 5 were found to protective against WNV challenge.[142] Modified live vaccines, such as CP-WN and WN-FV, should be capable in the induction of cell-mediated immunity in addition to humoral immunity. The importance of cell-mediated immunity (T helper response and cytotoxic T cells) and memory T cells in the protection against WNV and other related flaviviruses have been demonstrated in experimental murine studies[31,123] and in field studies characterizing endemic flavivirus (Japanese Encephalitis and Dengue Fever) populations.[67,128] Characterization of the protective immune response of equine WNV vaccines has not been studied in horses due to the limitations of the previous mosquito and needle inoculation challenge models combined with the limited availability of equine specific immunologic reagents.

Information regarding the duration of immunity of vaccines against clinical disease is extremely important for Flavivirus vaccines especially in climates with year-long mosquito activity. All three vaccines (K-WN, CP-WN, and WN-FV) are able to generate a protection

against WNV infection at 28 and 56 days post vaccination. Duration of immunity of 12 months against clinical disease including encephalitis and prevention of viremia has been reported for the WN-FV.[76] Duration of immunity of 12 months with development of neutralizing antibodies and prevention of viremia, but not against development of clinical WNV disease has been shown with the CP-WN and K-WN vaccines.[91] Long-term immunity is not a feature of inactivated vaccines and the K-WN vaccinated horses in an epidemiological study of the Nebraska and Colorado outbreaks were found to have decreased neutralizing titers at five to seven months.[27] Recommendation for repeated vaccination throughout the year was the frequent conclusion made in several epidemiological studies when using the K-WN product, particularly in less temperate areas in North America with prolonged mosquito activity.[27,155] Further studies of the efficacy of all three of these vaccines against WNV induced disease >56 days post-vaccination is warranted.

The WN-FV is a novel vaccine developed from the technology used to create a live, attenuated Japanese Encephalitis human vaccine which has been studied in phase 2 clinical trials and a human West Nile vaccine currently in phase 1 clinical trials.[93,95] The vaccine does not require an adjuvant and hence would be less likely to induce vaccine reaction compared to the adjuvanted vaccines. By utilizing an attenuated flavivirus (YF-17D) genome backbone,[93] the vaccine is essentially an attenuated modified live vaccine with enhanced immunogenicity and induction of cell mediated immune responses. None of the six horses in the WN-FV had any reaction post-vaccination. While no systemic or injection site reactions were noted in any of the other vaccine groups, mild increased rectal temperatures were noted in the first five days after the second vaccination in the other two groups and may be likely to be the result of adjuvant induced inflammatory cytokines (IL-1, IL-6, TNF$\alpha$) and immune response. These horses were obtained from farms that do not practice any immunoprophylaxis in their foals, thus there had

been no prior exposure to any vaccine components which also likely decreased their overall reaction rates.

Mild elevations in rectal temperature were recorded in all four groups within the first five days post-challenge ranging from 101 to 102.5 ºF. These changes are suspected to be a response to intrathecal injection into the CNS. Whether from the actual trauma induced during the procedure, replication of the virus locally, and/or more likely infiltration of inflammatory immune mediators (cytokines and immune cells) has yet to be elucidated. Interestingly, 4/6 horses in the WN-FV had evidence of this mild elevation in temperature post-challenge and all six of these horses were completely protected from onset of WN disease and did not manifest any clinical signs during the study. Murine studies have shown the role of WNV antigen specific CD8 and CD4 T cells in clearance of WNV from the CNS along with a interplay of cytokines such as IFN-γ, TNF-α, and IFN β.[31,65,123]

Mild to moderate neurologic deficits grade 1 or 2 and less than two consecutive days duration were observed in a few of the horses in the K-WN and CP-WN groups post-challenge. Interpretation of these findings should take into account whether or not the degree of clinical disease observed would correspond to field conditions. The observations may have been biased toward detection of minimal clinical signs since the observers in this study were aware of the infection day and were presumably more able to detect neurological disease due to additional post-DVM specialty training. In addition, horses in this study were evaluated very closely and several times per day post-challenge for clinical signs, allowing for detection of slight changes from original baseline values that would not have been detected by owners or field veterinarians not intimately associated with baseline values. Epidemiological studies characterizing the outbreaks have reported a percentage of horses with appropriate vaccination history that

developed WNV disease.[27,110] Although none of the vaccinated horses in this study fit the case criteria for WNE, the percentage of horses that developed mild clinical signs mirrors the findings of these outbreak studies. None of the WN-FV vaccinates manifested any clinical signs in this study, but small sample size may be a factor as previous studies conducted by the investigators resulted in 1/20 horses vaccinated with WN-FV developing mild clinical signs post challenge at 28 days.[76]

All six control horses developed clinical signs of encephalitis had severe (Grade 3) pathological changes in the brain and spinal cord. Complete agreement between severity of clinical signs and grading of histopathologic findings was not observed among the vaccinated horses. This finding is not unexpected, as only representative samples of the brain spinal cord sections were taken from each horse and may not have included the affected tissue. In each of the vaccine groups, a few horses had evidence of mild (Grade 1) lesions changes in the brain and spinal cord. Similar residual mild focal inflammatory infiltrates were found at 30 days in the brains and spinal cords of immunized rhesus monkeys challenged intracerebrally with wild-type Japanese Encephalitis virus but were protected from clinical disease.[94] Further investigation of the resolution of these inflammatory lesions at greater than 21 days post-infection is likely warranted.

Several epidemiological outbreak studies have reported the justification for the use of vaccines as preventative strategy against WNV disease in horses. Veterinarians and owners have the unique opportunity of selecting among three effective and commercially available equine WNV vaccines to implement a suitable and personalized preventative program. Further study can be performed to elucidate the most effective protocol for each vaccine and risk groups.

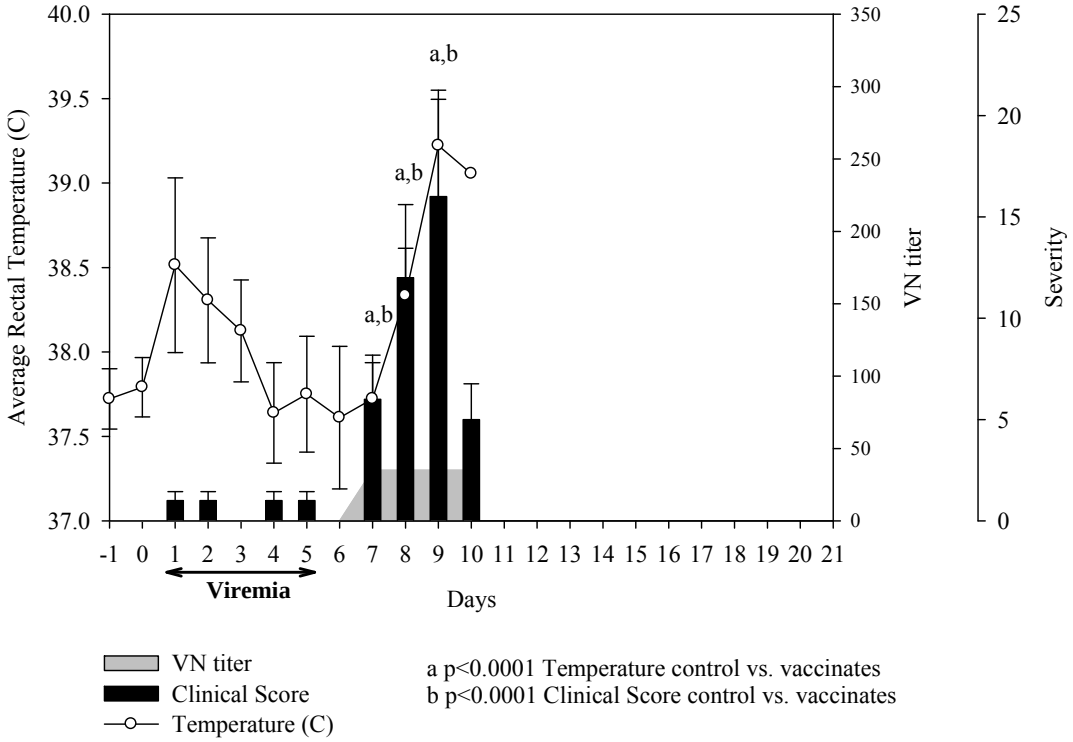


Figure 4-1. Clinical course post-challenge controls

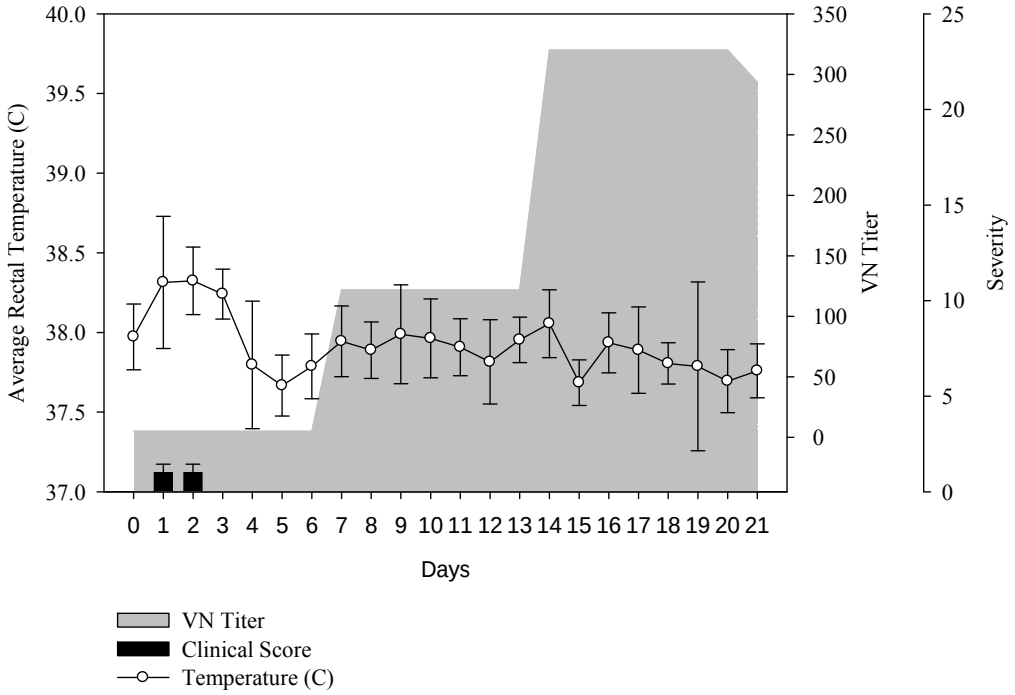


Figure 4-2. Clinical course post-challenge WN-FV

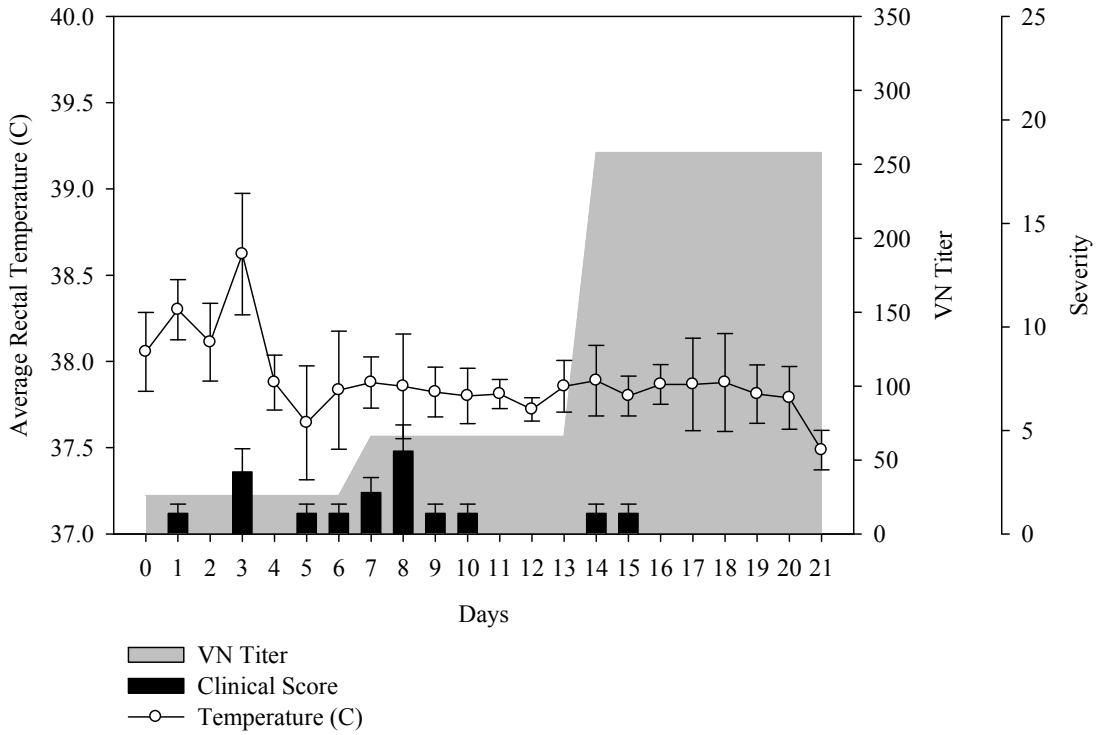


Figure 4-3. Clinical course post-challenge CP-WN

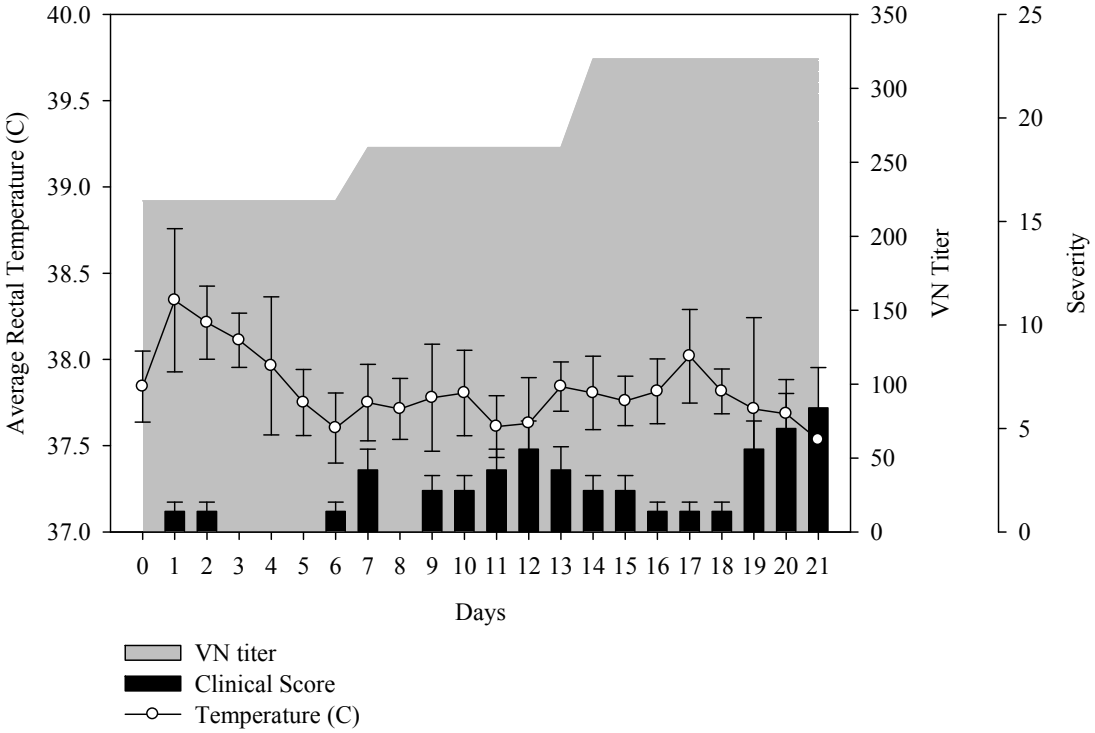


Figure 4-4. Clinical course post-challenge K-WN

Table 4-1. Case criteria for vaccinated and control horses after intrathecal WNV challenge

| Vaccine | Clinical Signs | Fever | Death | Virus Isolation | Histopath |
|---|---|---|---|---|---|
| WN-FV 6 horses | 0/6 | 0/6 | 0/6 | 0/6 | 1/6[g] |
| CP-WN 5 horses | 1/5[a] | 1/5 | 0/5 | 0/5 | 1/5[g] |
| K-WN 6 horses | 4/6[b] | 1/6 | 0/6 | 0/6 | 3/6[g] |
| Control 6 horses | 6/6[c] | 3/6 | 6/6 | 6/6 | 6/6[f] |

[a] Mild signs in several neurological categories (mentation, paresis, fasciculations, and ataxia) noted for one day. [b] Mild to moderate signs in at least one of the following categories for one to two days: mentation, paresis, fasciculations, or ataxia. [c] Moderate or severe signs in at least one of the following categories for at least two days: mentation, paresis, fasciculations, or ataxia. [d]Body temperature ≥ 39.2°C (102.5° F). [e] Death due to development of WNV disease severe enough to require euthanasia for humane reasons. [f] Encephalitic horses in the control group had moderate or severe encephalitis on histopathology. [g] Mild inflammatory histopathologic changes in neural tissues vaccinated horses.

Table 4-2. Glial nodules in vaccinates and control horses

| | Thalamus | Midbrain | Pons | Medulla | Cervical | Lumbar |
|---|---|---|---|---|---|---|
| WN-FV | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.67±1.63 | 0.58±1.20 |
| CP-WN | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.10±0.22 | 0.10±0.22 |
| K-WN | 0.00±0 | 0.00±0 | 1.17±1.83 | 0.00±0 | 0.00±0 | 0.00±0 |
| Control | 4.67±1.86* | 8.33±5.79* | 4.17±3.49 | 12.00±2.76* | 1.67±1.89 | 1.08±2.18 |

* $P<0.05$

Table 4-3. Perivascular cuffs in vaccinates and control horses

| | Thalamus | Midbrain | Pons | Medulla | Cervical | Lumbar |
|---|---|---|---|---|---|---|
| WN-FV | 0.00±0 | 1.00±2.45 | 0.17±0.41 | 0.17±0.41 | 0.42±0.66 | 2.27±5.55 |
| CP-WN | 0.10±0.22 | 0.00±0 | 2.40±5.37 | 0.00±0 | 0.10±0.22 | 0.20±0.45 |
| K-WN | 1.00±2.00 | 0.50±0.84 | 10.3±24.8 | 0.33±0.82 | 0.92±2.25 | 1.42±3.01 |
| Control | 59.4±23.3* | 60±26.5* | 47.8±28.6 | 67.7±14.9* | 9.92±12.87 | 5.33±13.06 |

* $P<0.05$

Table 4-4. WNV neutralizing antibody (GMT) titers for vaccinated and control horses post-vaccination and after WNV challenge

| | WN-FV | CP-WN | K-WN | Controls |
|---|---|---|---|---|
| 1st Injection | <5* | <5 | <5 | <5* |
| 2nd Injection | <5 | 2 | 8 | <5* |
| PC D0 | 5 | 26 | 224 | <5 |
| PC D7 | 122 | 66 | 260 | <5 |
| PC D14 | 320 | 258 | 320 | <5 |
| PC D21 | 293 | 258 | 320 | 35 |

* Horses received diluent only

CHAPTER 5
PROTECTIVE IMMUNE RESPONSES TO WEST NILE VIRUS (WNV) OF 28 DAY VACCINATED HORSESE

## Introduction

West Nile Virus (WNV) is a member of *Flaviviridae* and similar to the other viruses in this RNA virus family, it is a highly neurotropic and a causative agent of potentially fatal encephalitis in humans and in horses. Since its encroachment into the United States in 1999, the virus has caused clinical disease in 23,962 humans and 24,824 horses resulting in 928 human, over 6,000 horse, and several hundred thousand bird fatalities.[1,2] Despite its current endemic status, annual epidemics of WNV are still a concern. In 2007 there were 3510 reported cases in humans and 334 cases in horses. With specific treatment options still unavailable, vaccines are still the best preventative strategy against WNV.

Currently, human vaccines are still in development but, four equine WNV vaccines have been licensed by the USDA, with three commercially available. The first licensed vaccine (K-WN; Innovator, Fort Dodge Animal Health, Overland Park, KS), available since 2001, is composed of an adjuvanted formalin-inactivated whole virion. Licensed in 2004, a second vaccine was marketed featuring the expression of the WNV membrane (prM) and envelope (E) genes under control of the canarypox virus vector (CP-WN; Recombitek™, Merial, Duluth, GA) and packaged with an adjuvant. Since 2006 a nonadjuvanted, single-dose, attenuated West Nile Virus, live flavivirus chimera (WN-FV) vaccine (PreveNile®, Intervet, DeSoto, KS) has been also available. This vaccine is based on the technology used to create an attenuated human vaccine for Japanese Encephalitis virus (Chimerivax-JE).[55] The recombinant chimera expresses the envelope (E) and membrane (prM) of WNV in a Yellow Fever vector (YF17D). This last

vaccine has been shown experimentally in rodent and primate models to induce both humoral and cell mediated immunity (WNV specific T cell responses).[90,95]

The efficacy of these equine WNV vaccines in terms of development of neutralizing antibody, prevention of viremia, and onset of clinical disease has been tested in numerous studies.[76,90,91,127,118] In particular the K-WN and the CP-WN were tested for licensure with a 12 month duration of protection in a viremia model using systemic challenge while the WN-FV was tested in an intrathecal challenge model that induced severe clinical disease. Most recently, we compared the efficacy of the three commercially available WNV vaccines utilizing a model of severe WNV encephalitis in the horse. Irrespective of the vaccine treatment, vaccinated horses were significantly protected at 28 days post-challenge (PC) from onset of clinical disease when compared with control horses in terms of in terms of survivorship, protection from the onset of WN encephalitis and viremia..[118] However there were significant differences between these vaccines in the complete prevention of clinical signs. To date characterization of the components of protective immune responses induced with these vaccines have not been reported.

Most of the knowledge of what constitutes the successful immune response to WNV has been derived from in-bred murine infection models and appears to rely on both on humoral and cell-mediated immunity (CMI). Antibody plays a clear role in protection against WNV. Passive transfer of WNV specific IgG was shown to protect mice if administered before or shortly after lethal challenge and, in another study, B cell deficient were protected from lethality by passive transfer of immune sera.[5,30] However, recent work has also demonstrated the importance of CMI in the control and recovery from WNV infection.[31,123,129] $CD8^+$ T cells were shown to eliminate WNV infected cells via cytolytic mechanisms. $CD4^+$ T cells also have a critical role in

sustaining these WNV-specific effector $CD8^+$ T cell responses in the CNS for viral clearance and for the production of antiviral IgG.[129]

Little is still known yet of what determines secondary immunity to WNV, but is speculated to be a $CD4^+$ T helper type 1 ($T_H1$) biased response based on related flavivirus studies. In one study, a $CD4^+$ $T_H1$ response was shown in immunized mice challenged with Yellow Fever virus to be critical for survival.[73] A $CD4^+$ $T_H1$ response was present in the peripheral blood mononuclear cells (PBMC) in people subclinically infected with Dengue virus while those that developed clinical disease had a $T_H2$ response.[57,125]

This data reports our objective to characterize the immune responses in twenty-eight day vaccinated horses challenged intrathecally with virulent WNV. Our hypothesis was that vaccinated horses have a WNV $CD4^+$ $T_H1$ response. Lymphocyte subsets, lymphoproliferative responses of equine peripheral blood monocytes (PBMC), *ex vivo* and *in vitro* cytokine mRNA profiles, and immunoglobulin isotypes were studied to characterize the immune response.

## Materials and Methods

### Animals.

All work with animals was performed with approval and under the guidance of the University of Florida (Gainesville, FL) Institutional Animal Care and Use Committees (IACUC). Horses used were of mixed breed male and female horses, with no previous WNV exposure, as indicated by WNV neutralization titers of $\leq 5$. Standard practices for determination of health status and preventative medicine (diet, parasite and infectious disease prevention, and podiatry) were performed under the guidance Animal Care Services, University of Florida. Rectal temperature, heart and respiratory rates, appetite, and attitude were recorded on a daily basis prior to commencement of study protocols.

**Challenge Trials.**

Horses were placed into three randomized, blinded trial groups consisting of eight horses with two horses in each group receiving (1) the inactivated K-WN vaccine, (2) the canarypox virus vectored CP-WN, (3) the chimera WN-FV vaccine, or (4) a diluent. For the K-WN and CP-WN, horses received an initial primary intramuscular (IM) injection with a 1mL volume of vaccine followed in 28 days with a second injection according to manufacturer's label. For the WN-FV, each horse received an initial injection of 1 mL diluent IM followed in 28 days by a 1 mL IM injection of the vaccine. The challenge virus, designated WNV NY99 (4132), was prepared as describe previously.[118] Horses were challenged intrathecal with WNV as previously described.[118] In order to characterize the immune response after vaccination and after virulent challenge whole blood and serum was taken via venipuncture from horses at the start of the study period and day of the first vaccination (D0), day of the second vaccine injection (D28), and day of virulent challenge (D0PC). After challenge, horses were bled on third (D3PC), seventh (D7PC) and tenth days (D10PC) and then weekly (D14PC and D21PC) until the end of the challenge period. Collected PBMC were separated from whole blood collected in ACD by layering leukocyte rich plasma over histopaque (1.077, Sigma-Aldrich) and centrifuging. PBMC were stored at -70°C in concentrations of at 1x 107 cells/mL in freezing media (10% DMSO in fetal bovine serum) until use. Whole blood was also collected on these days in serum separator tubes, allowed to clot, separated and stored until use at -70 ºC

**Flow Cytometry for Lymphocyte Phenotyping.**

Immunophenotyping was performed on PBMC to determine lymphocyte subsets as previously described.[59] Aliquots of 100uL containing 1 x 106 cells were stained for 30 minutes with murine monoclonal antibodies binding to equine T lymphocytes (HB19A;VMRD, Inc., Pullman, WA), $CD4^+$ T lymphocytes (HB61A; VMRD), $CD8^+$ T lymphocytes (MCA2385;

Serotec, Raleigh, NC), and B lymphocytes (B29A, VMRD). A monoclonal antibody of the same isotype but not reactive with equine cells was used as the negative control (MCA928;Serotec). Analyses were performed with a FACSort flow cytometer equipped with Cell Quest software (BD Biosciences, Rockville, MD). Data were collected from 10,000 events for each sample, with forward scatter and side scatter parameters used to gate the lymphocyte populations. Data were expressed as percentages of each subset with the gated population.

**Lymphocyte Proliferation Assay.**

Immediately after thawing, PBMC were placed in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2mM glutamine, 25mM HEPES, and penicillin-streptomycin (100U and 100ug/mL, respectively). More than 70% of the cells were viable based on trypan blue exclusion. Proliferative responses were conducted in replicate wells of round-bottom 96-well plates (Costar) for 5 days at 37ºC and 5% CO2. PMBC (1x $10^6$) were cultured for 5 days in duplicate wells with 10ug/mL phytohemagglutinin (PHA-P, Sigma-Aldrich, St. Louis, MO) (positive control), 10ug/mL ChimeraVax WNV antigen (Acambis, Int., Cambridge, MA), 10ug/mL Vero-cell antigen (negative control). The ChimeraVax WNV antigen was used because of its biosafety classification status. The PBMC were radiolabeled for the last 18h of culture with 0.25uCi of 3[H] thymidine (DuPont, New England Nuclear) and harvested onto glass filters, and radionucleotide incorporation was determined using a Betaplate 1205 liquid scintillation counter (Wallac). Results are presented as the stimulation index (SI)=mean cpm of the stimulated cells / mean cpm of cells with negative control.

**Ex vivo Cytokine mRNA Expression.**

The protocol for cytokine mRNA expression was based on previously described methods.[47] Briefly, PBMC at concentration of 2x$10^6$ cells, were lysed in Trizol®., treated with DNAse I, and dissolved in DEPC water. cDNA from 1 ug of total RNA was synthesized with

Advantage® RT-for PCR kit according to manufacturer protocol. Amplification of cDNA and Real-Time PCR was done using primers and probes established in the literature and based on sequences for equine cytokines IL1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p35, IL-12p40, IL-13, TNF-α, TGF-β, IFN-γ and glyceraldehyde-3-phosphate dehydrogenase (G3PDH).[49] Amplification of 2 μl of cDNA was performed in a 25 μl PCR reaction using a commercially available kit (TaqMan Universal PCR Mastermix, ABI, Foster City, CA) and performed with a commercial real-time PCR thermocycler (ABI 7500 Sequence Detection System, ABI). All samples were assayed in triplicates. The mean values used for comparison with all the results were normalized to G3PDH expression and relative quantitation between samples was achieved by the ΔΔCt Relative Comparative CT method using the ABI 7500 Fast Sequence Detection Software version 1.4.0.25. Prior to using the comparative CT method amplification efficiencies of the target genes and housekeeping gene were validated. Samples without cDNA were included in the amplification reactions to determine background fluorescence and check for contamination. DNase-treated RNA samples were also subjected to PCR using the G3PDH primers to confirm the absence of genomic DNA contamination.

**In Vitro Cytokine mRNA expression in Antigen Stimulated PBMC.**

PBMC used for quantification of mRNA expression of cytokines that reflect $T_H$ cytokine responses were prepared exactly as described above with exception that the cells were stimulated with antigen for 48h. The time selection was based on a time-response curve for IL-4 and IFN-γ mRNA expression. Briefly, replicate wells of PBMC plated at a dilution of 1x $10^6$ cells were cultured in duplicate with 10ug/mL phytohemagglutinin (PHA-P) (positive control), 10ug/mL ChimeraVax WNV antigen, 10ug/mL Vero-cell antigen (negative control). At the end of 48 hours, cells were removed and RNA and corresponding cDNA was prepared as described

previously. For this assay IL-4 and IFN-γ were evaluated for detection of $T_H$ cytokine bias. The macrophage derived cytokines IL-12 p35 and TNF-α were also measured to examine overall Type 1 and inflammatory cytokine status in stimulated PBMC.

**Capture Isotype ELISA**

The isotype ELISA was based on the IgM capture for use in horses. Briefly, microtiter plate wells were coated overnight at 4°C with either 100 µl goat anti-equine IgG total (heavy and light chain, H+L), IgGa, IgGb, or IgGc (Bethyl Labs, Montgomery, AL) diluted at 1:400 in carbonate/bicarbonate buffer, pH 9.6. After incubation and washing, the plates were blocked with 0.5% Tween-20 diluted in PBS (PBST) with 5% milk for 2 hr at room temperature. Each serum sample was first screened at dilution of 1:400 (in PBST) to 1:128,000. Positive samples were diluted in PBST from 1:400 to 128,000 and negative were diluted from 1:10 to 1:400 and added in triplicate to wells and incubated for 1 hr at 37ºC. After incubation and washing, bound equine IgG was reacted with WNV antigen and incubated for 1 hr at RT. After incubation and washing, a flavivirus-specific horseradish peroxidase antibody conjugate (CDC, Fort Collins, CO) was added and incubated for 1 hour at 37°C. Bound conjugate was detected after washing by incubation for 10 minutes with 3,3',5,5'-tetramethylbenzidinec (SureBlue™ TMB Microwell Peroxidase Substrate, KPL, Gaithersburg, MD) in an acidic buffer and H2O2. For control, each sample was also reacted with negative antigen. Every plate had control wells consisting serum from a positive horse (National Veterinary Services Laboratory, Ames, IA) and 2 non-infected non vaccinated horses. The reactions were stopped at 10 min with 0.5 M HCl and the absorbances was read at 450 nm and were compared against a well in which no serum was added (blank). All values with an OD >0.1 were considered positive. A sample had a positive titer if it was at least 2-fold over its OD when reacted against the control antigen. End-point titer was

defined as the highest dilution of serum that gave an OD450 value that was 2-fold greater than the signal of the horse's pre-immune serum at the same dilution.[22]

**Statistical Analysis**

Statistical analysis was performed using SigmaStat® version 3.0 (SPSS, CA) software. A one-way analysis of variance (ANOVA) was used to compare the lymphoproliferative responses, cytokine mRNA expression, antibody concentrations, and percentages of lymphocyte subsets between experimental groups. Data that did not meet the assumptions for parametric testing were analyzed with Kruskal-Wallis ANOVA on ranks with multiple pairwise comparisons with Dunn's Method or Holm-Sidak method when appropriate. A two-way ANOVA for repeated measures was used to determine the effects of time (post-vaccination and post-challenge), the vaccination treatment group, and the interaction between time and group on lymphoproliferative responses, cytokine mRNA expression, antibody concentrations, and percentages of lymphocyte subsets. Variables did not meet the assumptions of the ANOVA were rank transformed prior to analysis. Multiple pairwise comparisons were done with Dunnet's test, when appropriate. For each test, significance was set at a P value of $<0.05$.

## Results

The clinical results of these horses have been previously reported and are abstracted in this paper for context. Challenge in horses at 28 days post primary vaccination of any of the vaccines resulted in 100% survivorship, protection from the onset of WN encephalitis and viremia. Horses vaccinated with WN-FV showed significantly fewer clinical signs than horses vaccinated with K-WN ($p \leq 0.035$) and the control horses ($p \leq 0.01$). Mild residual inflammatory lesions were seen in a few horses from all of the vaccine groups. Challenge by this model caused grave neurological signs, viremia, moderate to severe histopathologic lesions in the brain and spinal cord, and an outcome of 0% survivorship in all six control horses.[118]

**PBMC Lymphocyte Subsets**

Immunophenotyping to determine the subsets of PBMC was performed on cells collected prior to immunization at the start of the study (D0) and after immunization (D0PC) and after virulent WNV challenge at D3PC and D21PC. After immunization at D0PC, K-WN and WN-FV vaccinated horses had a significantly higher percentage of $CD4^+$ T cells than either the CP-WN vaccinated or control horses (Figure 5-1). A significant decrease in the percentage of B lymphocytes cells was also noted in the K-WN and WN-FV vaccinates compared with the control horses.

At D3PC, K-WN and CP-WN vaccinates were significantly decreased in $CD4^+$ T lymphocyte percentage from values on D0 and D0PC (Figure 5-1). An apparent but not statistically significant increase in the percentage of $CD8^+$ T lymphocytes was also measured in the K-WN vaccinated horses on D3PC compared to the other vaccine groups on this day. At the end of the study on D21PC, the percentage of $CD4^+$ T lymphocytes in the K-WN group was significantly increased compare to their values before and on D3PC (Figure 5-1). On D21PC, the T cell population and $CD4^+$ T lymphocytes percentages in the WN-FV vaccinates was increased but not statistically significant compared to the other days of measurement.

**WNV Antigen Specific Lymphocyte Proliferation Responses**

**Post-vaccination responses.** WNV antigen specific proliferative responses and stimulated cytokine profiles were evaluated in PBMC collected prior to immunization, D0 and after immunization, D0PC. On D0, there was no significant difference in the levels of proliferation between the control and vaccinated horses (Figure 5-2). After immunization and prior to challenge, D0PC, only the CP-WN had a significantly increased proliferative response to WNV antigen.

**Post-challenge responses.** At D3PC, no significant proliferative response to WNV antigen was measured in any of the vaccinated horses. At D21 PC, PBMC collected from K-WN and WN-FV horses proliferated in response to WNV antigen, and these responses were significantly greater than responses compared to their indices for D0, D0PC, and D3PC. The WNV antigen responses for these groups on D21PC were also significantly higher their respective PHA mitogen indices. The PBMC from all horses and samples proliferated when stimulated with mitogen and there was no proliferative response of PBMC to stimulation with Vero cell antigen (negative control; data not shown).

**Ex vivo mRNA Cytokine Expression of Equine PBMC.**

The regulation of cytokine responses were measured *ex vivo* to examine the status of overall innate and adaptive responses in horses undergoing virulent WNV challenge. Tumor necrosis factor-α (TNF-α) was elevated in the control horses an average of 15-fold over that of pre-infection values in all horses and the vaccinated horses on D3PC and D7PC ($p \leq 0.01$) (Figure 5-3). IL-5 demonstrated consistent increases on D3PC and D7PC in the vaccinated horses with significant increases on D3PC in the K-WN and WN-FV ($p \leq 0.01$) and on D7PC for the WN-FV ($p \leq 0.01$) Interferon-γ (IFN-γ) was significantly increased on D3PC in both the K-WN and WN-FV horses. Responses for all of the remaining cytokines were variable. Specifically, the K-WN horses had significant elevations in IL-6, TGF-β, IL-12 and IL-2 on D3PC and IL-12 on D7PC.

**In vitro Cytokine mRNA Expression in Antigen Stimulated PBMC**

Regulation WNV-antigen-specific IL-4 ($T_H2$) and IFN-γ ($T_H1$) expression was measured to confirm antigen specificity of the immune response. To detect any additional biasing of T cells responses in PBMC, IL-12p35 and TNF-α expression were also measured.

After immunization on D0PC the K-WN and CP-WN horses had significantly higher IL-4 mRNA expression in response to *in vitro* stimulation with WNV antigen than control or WN-FV horses. (Figure 4a) After challenge expression of both IL-4 and IFN-γ mRNA in stimulated PBMC collected from K-WN vaccinates on D3PC and D21PC were significantly higher than in any of the other vaccinated groups (Figure5-4). Stimulated PBMC from control horses collected on D3PC also had a significant expression of IFN-γ compared to all other groups. Expression of IL-4 and IFN-γ in response to *in vitro* stimulation was notably decreased from levels on D0 and D0PC in PMBC from CP-WN and WN-FV.

Groups were compared as a ratio of $T_H1$ cytokine (IFN-γ) to $T_H2$ cytokine (IL-4) response with results summarized in Table 5-1. On D3PC the IFN-γ/IL-4 ratio was significantly higher in the control horses when compared to the other groups. The ratio was also increased in the K-WN horses (0.83) compared to the CP-WN and WN-FV groups. On D21PC, however, although not statistically significant, the mean IFN-γ/IL-4 ratio of WN-FV (1.67) horses was higher than K-WN (0.53) or CP-WN groups (0.09).

Similar to the trend seen *ex vivo* in PBMC with TNF-α, WNV antigen stimulated PBMC collected on D3PC from control horses had a 3 fold significant increase in the mRNA expression when compared with the CP-WN and WN-FV horses (Figure 5-4). Stimulated PBMC collected on D3PC from K-WN horses also had a significant elevation in TNF-α expression compared with the other vaccinates. Additionally CP-WN and WN-FV groups had significant decreases in TNF-α expression in stimulated PBMC collected on D3PC compared to levels on other days. .

Interleukin 12p35 expression in response to *in vitro* stimulation of PBMC collected from control horses was increased on D0PC and significantly increased on D3PC when compared to

other groups. Expression of IL-12p35 was decreased in response to stimulation in PBMC collected from the other vaccinated horse (data not shown).

**WNV IgG Isotype Antibody Profiles**

Antigen specific IgG isotypes were tested as an in vivo measurement of cytokine bias of $T_H1$ or $T_H2$ profile as cytokines regulate isotype switching.

**Post-vaccination responses.** The kinetics of the WNV total IgG antibody titers mirrored the virus neutralizing titers previously reported. On the day of the second immunization (D28), low to moderate anti-WNV total IgG titers, ranging from <50 to 1000 were detected in the all six K-WN horses (Table 5-2). Anti-WNV total IgG was not detected in the CP-WN horses with one dose of vaccine and, as expected the WN-FV horses were negative having only received diluent. At the time of the second immunization, all six K-WN horses had low levels of WNV IgGa antibody (ranging from 50 to 1,000), but anti-WNV IgGb antibody was not detected. Anti-WNV IgGa and IgGb were not detected in any of the CP-WN or WN-FV horses after immunization. All control horses remained negative for anti-WNV total IgG, isotypes IgGa and IgGb after vaccination and on the day of challenge (D0PC) (Tables 5-2 and 5-3).

On D0PC, the K-WN horses had the highest mean endpoint titer (MET) of anti-WNV total IgG (ranging from 1000 to 64,000) (Table 5-3). Four out of five CP-WN horses had WNV total IgG ranging from ≤ 50 to 200, and all six WN-FV horses had a low total titer of 50. On D0PC, the K-WN horses had a mean endpoint anti-WNV IgGa titer of 20,000 (range, 400 to 64,000) and five out of six horses had measurable WNV IgGb antibody (MET of 11,000) (Table 5-3). One out the five CP-WN horses had a low WNV IgGa antibody titer of 400 but three out of the five had WNV IgGb antibody titers (MET of 260). Low levels of WNV IgGa antibody (MET of 50) but not WNV IgGb were detected in the six WN-FV horses at the time of challenge.

**Post-challenge responses.** After challenge total IgG levels rose in the K-WN stayed level throughout infection. Similarly, the CP-WN horses rose after infection with titers ranging between 16,000 and 128,000. Although the WN-FV vaccinates had initially the lowest levels, WNV total IgG titers rose the most rapidly after challenge (ranging between 16,000 and 128,000).

After challenge and similar to the WNV total IgG, the IgGa isotype expanded in the K-WN horses, ranging from 400 to 64,000. WNV IgGb antibody in the K-WN horses rose slower but were 48,000 (range 16,000 to 128,000) by the second week postchallenge. After challenge, WNV IgGa and IgGb antibody titers rose rapidly in the CP-WN horses. In the WN-FV vaccinates, rapid increases in WNV IgGa and IgGb antibody response was measured by D7PC, peaking at 91,000 and 97,000 respectively by the second week.

On D7PC, all six control horses developed low levels of total IgG to WNV (MET of 683, range 50 to 1,600) (Table 5-3). Only one out the six control horses had a detectable WNV IgGa antibody titer of 400 and no WNV IgGb antibody was detected in any of the control horses.

WNV IgG c and IgG (T) antibodies were not detected in any of the horses. Using various WNV antigens (inactivated whole virus, expression vector derived WNV E protein from Focus Diagnostics (Cypress, CA), ChimeraVax WNV antigen) IgGc and IgG(T) antibodies in was not detected (data not shown). A direct IgG (T) ELISA was developed using tetanus toxoid (Fort Dodge, Overland Park, KS) as the antigen at 1:400. IgG (T) antibodies (mean titer of 400) were detected with this method in samples obtained from horses with a known history of tetanus vaccination (data not shown). Anti-tetanus IgG (T) antibodies were not detected in any of the horses that were in the comparative West Nile Virus vaccine study using the direct IgG (T)ELISA.

## Discussion

This is the first study examining the basic immunology of WNV in a primary host which develops encephalitis. Since licensed vaccines against WNV are available for horses, this host also can provide important data regarding what constitutes a protective immune response. To date, several vaccine studies have demonstrated the efficacy of the three commercially available equine WNV vaccines in the protection against onset of viremia, and neutralizing antibody response. In a previous study we demonstrated that these vaccines were all significantly protective against clinical disease when WNV was given intrathecally at 28 days after primary vaccination. Here, we have further characterized the antigen specific humoral and cell-mediated immune responses to WNV infection in vaccinated horses in this study.

The killed West Nile vaccine consists of a formalin-inactivated whole virion and is formulated with MetaStim™ (a lipidic, non-aluminum, dual phase adjuvant).[90] Inactivated virus vaccines induce antibodies but induce poor cell-mediated and mucosal immunity as they present antigen exogenously and through a MHC class II pathway. Lipidic based adjuvants mainly stimulate antibody responses and may induce $T_H2$ responses and few cytotoxic T-lymphocyte (CTL) responses.[138] In this study, the humoral immune response in the K-WN was the predominant response with the earliest development of WNV antigen specific IgG, and isotypes IgGa and IgGb. In the K-WN, total IgG and IgGa were detected starting on D28 with isotype IgGb appearing shortly thereafter. K-WN had increased WNV antigen specific lymphocyte proliferative responses in PBMC collected after immunization on D0PC with significant increase in stimulated IL-4 ($T_H2$) expression and a correlating significant increased $CD4^+$T cell subset. A $T_H2$ profile was also present *ex vivo* in the PBMC at D3PC with the increased expression of IL-4, IL-5, and IL-10. With WNV challenge, a shift from $T_H2$ to $T_H1$ profile is suggested with the

increased *ex vivo* expression of IFN-γ and IL-12 in PBMC collected on D3PC and D7PC respectively. Furthermore on D21PC, K-WN vaccinates had significant WNV proliferative lymphocyte responses with an increased IFN-γ/IL-4 ($T_H1$) ratio in stimulated PBMC. A significant increase in the $CD4^+$ T cell subset was also seen in the K-WN horses on D21PC.

The CP-WNV vaccine is a recombinant DNA vaccine consisting of the cDNA segments of the prM and E WNV genes inserted so that expression of the proteins are under control of the canarypox virus vector. It is expected that the immune response to this construct would be similar to that of a live attenuated vaccine. The WNV E and M antigens should be presented via the MHC class I restricted pathway to T cells, allowing for induction of cell mediated immunity as well as neutralizing antibody. The CP-WN vaccine is also formulated with Carbopol™(a proprietary polymer based adjuvant) that stimulates humoral and T cell responses.[90] In this study, low levels of IgG and isotypes of IgGa and IgGb were detected after immunization (D0PC) with induction of humoral response after WNV challenge. Total IgG and IgG isotype levels rose rapidly by the second week after challenge. After WNV challenge, cell-mediated immune responses were expected but significant T cell responses were not measured in the CP-WN horses in terms of WNV lymphoproliferative responses, stimulated in vitro cytokine expression, or lymphocyte subset populations. Explanation for the diminished T cell response is unclear. One possibility may be that the viability of the PBMC for the CP-WN was decreased, but viability of PBMC was reflective of other vaccinate groups on corresponding days of collection (>70% viability by trypan blue exclusion). The CP-WN PBMC proliferated in the presence of mitogen also indicating viability.

The WN-FV is a non-adjuvanted, single dose modified live Chimeric vaccine. This vaccine was based on the technology used to create an attenuated human vaccine for Japanese

Encephalitis virus (Chimerivax-JE).[93] The recombinant chimera expresses the envelope (E) and membrane (prM) of WNV in a Yellow Fever vector (YF17D). This last vaccine has been shown experimentally in rodent and primate models to induce both humoral and cell mediated immunity (WNV specific T cell responses).[55,94] Additionally in a human clinical trial, high levels of neutralizing antibody and WNV responsive $CD4^+$ and $CD8^+$ T cells and IFN-γ secreting responsive T cells were developed in human WN ChimeraVax vaccinates.[95] In this study, the WN-FV had little to no detectable IgG antibodies at the time of challenge. Significant production of total IgG and IgGa and IgGb isotype antibodies were induced rapidly after WNV challenge. After vaccination on D0PC, WN-FV vaccinated horses had a significantly higher percentage of $CD4^+$T cells than control or CP-WN horses. On D7PC *ex vivo* expression of IL-5 was significantly increased in WN-FV and may reflect the marked induction of IgG antibody production (via stimulation of B cell growth and immunoglobulin secretion). On D21PC, WN-FV horses had PBMC that had significant WNV proliferative responses with an increased IFN-γ/IL-4 ($T_H1$) ratio and correlative increase in the $CD4^+$ T cell populations.

In this study, a primary immune response to WNV infection was seen in the naïve, non-immunized control horses. IgG total, IgGa, and IgGb antibodies were not detected in the control horses until Day 7PC and at a low endpoint titer. WNV antigen proliferative responses were not detected in PBMC from the control horses. Tumor necrosis factor-α expression was markedly increased in these susceptible horses while, although present, was limited in the protected horses. There was also increased ex vivo expression of IFN-γ and IL-12 on D3PC and D7PC in susceptible horses but this response was similar to the protected horses. Although TNF-α is a potent anti-viral cytokine, increased levels of TNF-α in murine studies have also been implicated in the breakdown of the blood-brain-barrier (BBB) and increased entry of WNV into the

CNS.[29,154] This study may be the first to confirm the important of TNF-α in the pathogenesis of WNV disease in a natural host. There was also a nonsignificant increased TGF-β (data not shown) and this has been associated hepatocyte apoptosis in humans infected with Yellow Fever Virus.[106]

The induction of antibody and IL-5 seen in the K-WN and WN-FV vaccinated horses were similar *in vivo* immune responses reported in a recent murine study for a candidate human WNV vaccine. The adjuvanted recombinant WNV subunit vaccine contains a truncated WNV envelope (80E) protein and WNV non-structural protein (NS1). The vaccine was found to induce high levels of neutralizing antibody as well as significant induction of antigen specific cytokine expression of IFN-γ, IL-4, IL-5, and IL-10 in splenocytes collected from immunized mice.[72] Based on these results researchers speculated that a successful WNV vaccine would elicit high levels of specific viral neutralizing antibodies and cellular immune responses produced from a "balanced" $T_H1/T_H2$ immune response.

Subisotype IgGa and IgGb titers followed the IgG total responses in the vaccinated horses. As has been reported with other equine viral pathogens, IgGa and IgGb isotypes likely are important in generating neutralizing antibody in WNV infection. IgGa and IgGb isotypes are reported to fix complement and are associated with antibody-dependent cellular cytotoxicity, whereas subisotypes IgGc and IgG(T) are not able to fix complement and can inhibit complement fixation by other IgG isotypes.[101]

As previously reported with WNV intrathecal challenge, virus is detected in the peripheral blood of the naïve controls by D3PC. This event is associated immunologically with (1) decreased levels of WNV proliferative response of PBMC collected in all groups. (2) decreases in $CD4^+$ T cell subsets in the vaccinated horses and an increase in $CD8^+$ T cell subset in the K-

WN. (3) differing *ex vivo* expressions of inflammatory cytokines (such as TNF-α, IFN-γ, IL-6, IL-1β) in the control, K-WN, and WN-FV horses. Decreased WNV proliferative PBMC and alterations in the $CD4^+$ and $CD8^+$ subsets may be due to trafficking of T cells to infected cells (intrathecal endothelial cells). In the non-primed animal, this response is not successful in control infection, while in the primed animal, viremia (and encephalitis) is prevented. In naturally infected immunologically naïve horses, lymphopenia is a common finding in horses with encephalitis.[105] In murine studies, WNV specific $CD8^+$ and $CD4^+$ T cells into the CNS were critical for the clearance and recovery from WNV infection.[123,129] Further studies of the immune response in the CNS and CSF in vaccinated horses are warranted.

In summary, protective recall responses were seen in vaccinated horses with WNV challenge and data was supportive of the hypothesis that vaccinated horses have WNV antigen specific $T_H1$ $CD4^+$ T cell response. WNV proliferative responses were most significantly increased in the K-WN and WN-FV vaccinates at 21 days after challenge, with an increased IFN-γ/IL-4 ratio and both groups had increased $CD4^+$ T cell populations. Although Type 1 cytokines are present, a protective response is also characterized by the rapid induction of an antibody response mediated at least in two of the vaccines by IL-5. WNV antibodies for IgG total, IgGa, IgGb were detected after vaccination in the K-WN and CP-WN horses and there were marked increases in antibodies in the CP-WN and WN-FV horses by D14PC. The third component of a successful response also appears to be correlated with low expression levels of TNF-α. In contrast, naïve control horses had significant expression of TNF-α after virulent challenge and developed a primary immune response to WNV infection characterized by detectable IgG total, IgGa, IgGb at D7, and no WNV antigen specific lymphocyte proliferation. Definitive confirmation that the protective immune response in vaccinated horses as WNV

antigen specific CD $4^+$ $T_H1$ would require: (1) measurement of T cell responses in PBMC samples depleted of T cells or T cell subsets with immunomagnetic sorting or intracellular cytokine staining for IL-4 and IFNγ of lymphocytes, and (2) demonstration of the protective response of WNV specific cells to naïve recipients

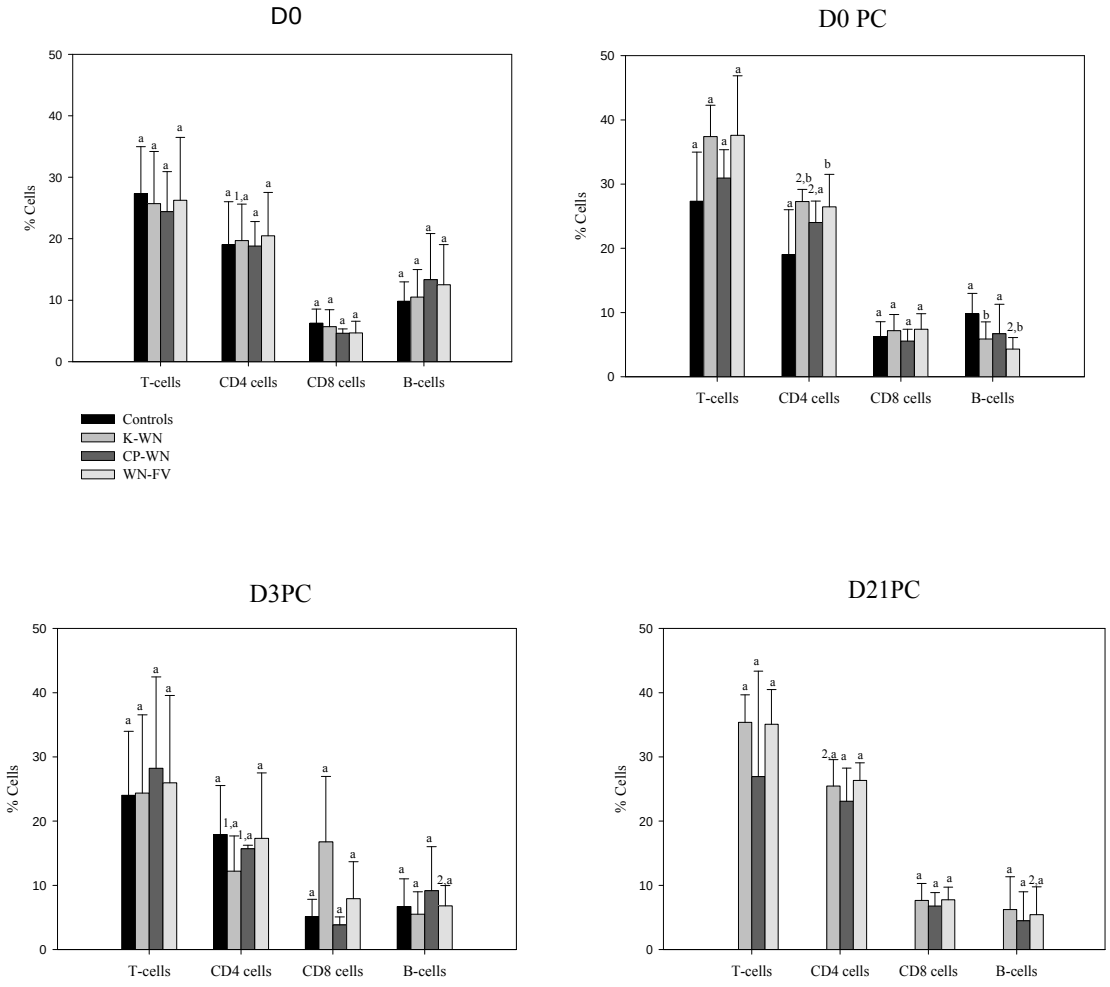


FIGURE 5-1. PBMC lymphocyte subsets of vaccinated horse and controls after WNV challenge. Bars represent mean % of cells with SD. Differences in letters and numbers are significant ($P<0.05$) between group and between days of challenge respectively.

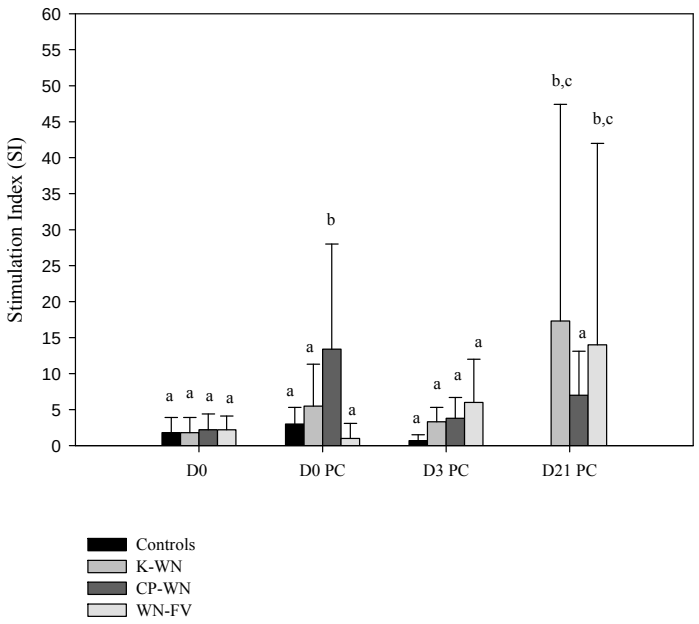


FIGURE 5-2. WNV antigen specific lymphocyte proliferation responses of equine PBMC from vaccinated and control horses. Stimulation Index (SI) expressed in means, bars are SD, groups with different letters are significant ($P<0.05$).

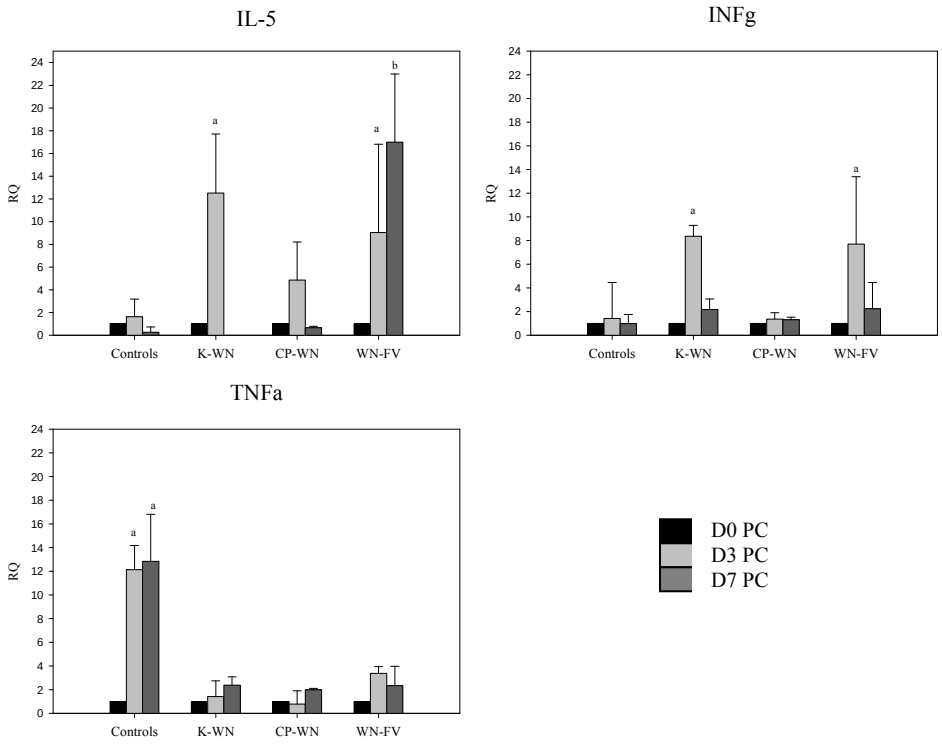


FIGURE 5-3. *Ex Vivo* mRNA Cytokine expression of equine PBMC D3 and D7 post-challenge. Results are shown as mean Relative Quantitation (RQ) with error bars indicating standard deviation (SD). a and b, $P<0.05$, groups with different letters are significantly different.

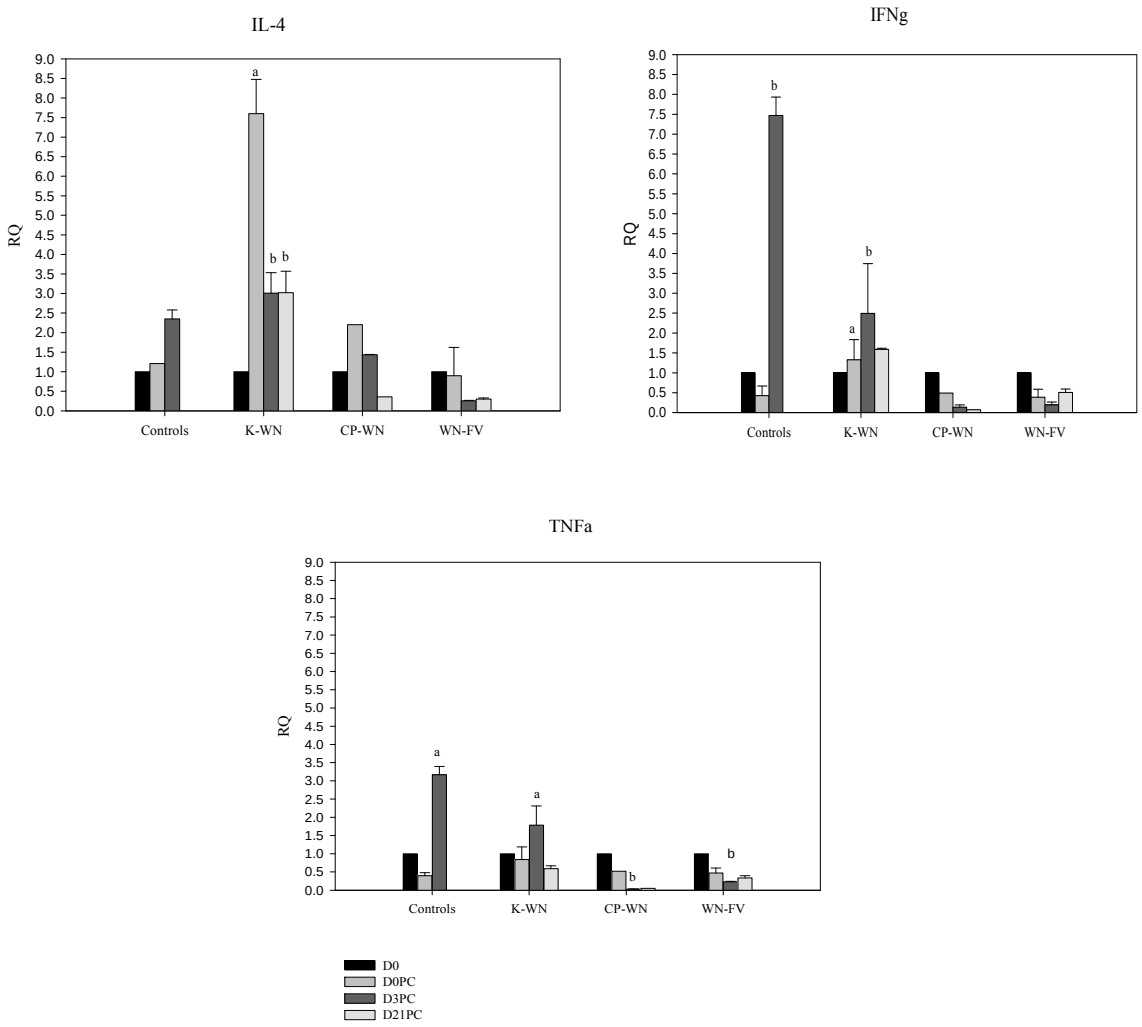


Figure5-4. In vitro relative quantitative cytokine mRNA expression of WNV antigen stimulated PBMC. Results are shown as mean Relative Quantitation (RQ) with error bars indicating standard deviation (SD). a and b, $P<0.05$, groups with different letters are significantly different.

Table 5-1. Mean IFNγ/IL-4 ratio of WNV antigen stimulated PBMC

| Groups | D0PC | D3PC | D21PC |
|---|---|---|---|
| K-WN | 0.17 | 0.83 | 0.53 |
| CP-WN | 0.22 | 0.20 | 0.09 |
| WN-FV | 0.43 | 0.28 | 1.67 |
| Controls | 0.35 | 3.17* | ND |

*P<0.05

Table 5-2. Post-vaccination WNV IgG isotypes mean endpoint titers of vaccinated horses and controls

| IgG total | K-WN | CP-WN | WN-FV | Controls |
|---|---|---|---|---|
| D0 | <50±0 | <50±0 | <50±0* | <50±0* |
| D28 | 250 ± 371 | <50±0 | <50±0 | <50±0* |
| IgGa | K-WN | CP-WN | WN-FV | Controls |
| D0 | <50±0 | <50±0 | <50±0* | <50±0* |
| D28 | 292 ± 374 | <50±0 | <50±0 | <50±0* |
| IgGb | K-WN | CP-WN | WN-FV | Controls |
| D0 | <50±0 | <50±0 | <50±0* | <50±0* |
| D28 | <50±0 | <50±0 | <50±0 | <50±0* |

* Horses received diluent only, Mean ± SD.

Table 5-3. Post-challenge WNV IgG isotypes mean endpoint titers of vaccinated horses and controls

| IgG total | K-WN | CP-WN | WN-FV | Controls |
|---|---|---|---|---|
| D0PC | 26K± 30K | 150 ± 71 | 50±0 | <50±0 |
| D7PC | 16K± 14K | 6K± 14K | 11K± 26K | 683 ± 727 |
| D14PC | 60K± 40K | 54K± 21K | 117K± 26K | NA |
| D21PC | 56K± 40K | 96K± 45K | 99K± 48K | NA |
| IgGa | K-WN | CP-WN | WN-FV | Controls |
| D0PC | 20K ± 25K | 120 ± 157 | 50±0 | <50±0 |
| D7PC | 17K± 24K | 7K± 8K | 10K± 12K | 108 ± 143 |
| D14PC | 28K± 28K | 52K± 53K | 91K± 43K | NA |
| D21PC | 36K± 31K | 45K± 53K | 71K± 49K | NA |
| IgGb | K-WN | CP-WN | WN-FV | Controls |
| D0PC | 11K ± 8K | 260 ± 192 | <50±0 | <50±0 |
| D7PC | 26K± 24K | 12K± 14K | 1K± 3K | <50±0 |
| D14PC | 48K± 43K | 67K± 40K | 97K± 51K | NA |
| D21PC | 48K± 42K | 72K± 55K | 78K± 58K | NA |

PC= Post-challenge

# CHAPTER 6
# SUMMARY AND CONCLUSION

In the first part of this dissertation, the model of equine WNV encephalitis was further developed to examine whether it could be used to characterize the protective immune response to WNV infection. In a pilot study, a naturally exposed horse was completely protected from clinical disease in the face of intrathecal rechallenge with virulent WN virus. WNV IgM antibodies were not detected at the time of challenge but WNV IgG antibody was present. WNV IgG total antibody and IgGa and IgGb isotype antibody endpoint titer levels of 1:64,000 were measured. This study demonstrated the first data to support of long-term immunity to WNV in naturally exposed horses.

The data presented in the second part of this dissertation compared the efficacy of three commercially available equine WNV vaccines at 28days using the model of equine WNV encephalitis. Irrespective of the vaccine treatment, all vaccinated horses were significantly protected from onset of WN encephalitis when compared to naïve, unvaccinated horses. Some of the horses vaccinated with the killed and recombinant canarypox WNV vaccines had some mild clinical signs after viral challenge, whereas the horses vaccinated with the chimera WNV vaccine did not manifest any clinical signs.

Further characterization of the immune responses of the vaccinated horses was presented in the third and final part of this dissertation. A protective recall response was seen in the vaccinated horses with WNV challenge. Horses vaccinated with killed and chimera WNV vaccines had significant WNV proliferative responses at 21 days after challenge that were increased in $CD4^+$ T cell population and $T_H1$ cytokine profile. Rapid antibody response of total IgG, IgGa, and IgGb isotypes was also induced after viral challenge and in the killed and chimera horses associated with increased IL-5 cytokine levels. Vaccinated horses had

significantly decreased expression of inflammatory cytokine, TNF-α, compared with the non-vaccinated control horses.

In conclusion, the findings of the present studies supports the hypothesis that a protective immune response consists of a WNV antigen specific $T_H1$ $CD4^+$ T cell response in horses, but furthermore relies on an a robust humoral antibody response. The significant difference of TNF-α levels between the naïve horses versus the vaccinated horses, suggests that the protective immune response relies also on the innate immune response and is likely to be a complex and balanced interplay of the innate and adaptive immune responses. Characterization of the protective immune response of these vaccinated horses in the CNS is also needed. Non-vaccinated horses had significant degree of severe histopathologic lesions due to WN encephalitis compared to the vaccinated horses. WN virus at $10^2$ PFU and viral RNA was recovered from CNS tissues (thalamus, midbrain, hindbrain, cerebrum, spinal cord) from the non-vaccinated horses, but no virus or viral RNA was obtained in any of vaccinated CNS tissues (data not shown). Preliminary cytokine expression profiles of CNS tissues using Real-Time PCR showed increased levels of IFNγ and TNFα expression in the chimera and killed vaccinated horses, but not in the recombinant canarypox vaccinates and nor in the control horses. Further work is required to elucidate this unexpected finding as it implies that our understanding of the CNS immune response to WN infection is far from complete.

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