ABSTRACT

JULIO C. MONTERO: Emergence of the West Nile virus in the United States: Veterinary and Human Public Health Implications.
(Under the direction of Dr. Louise M. Ball)

West Nile virus (WNV) (Flaviviridae), an arbovirus (arthropod-borne) isolated in the Western Hemisphere for the first time in New York in 1999, has become endemic in the United States, affecting wild birds, horses, humans and other mammals. Mortality rates associated with the North American variant of WNV among corvid birds (American crow), horses and humans in 2000 was 97%, 40%, and 10%, respectively.

The current geographic distribution of WNV in North America includes 31 states, the District of Columbia, and the Canadian Province of Ontario. U.S. Public Health authorities estimate that WNV will continue to expand its range towards the west and eventually become a coast-to-coast arboviral pathogen.

The goal of this study was to investigate vaccination as a means of controlling the spread of infection in equine hosts. In August 2001, the United States Department of Agriculture (USDA) conditionally approved a whole cell killed vaccine intended to protect horses from WNV infection. Thirty-five US Army-owned horses at Fort Bragg were inoculated with the WNV vaccine in the months of April and May 2002. Determination of antibody titers against WNV antigens was assessed using the Plaque Reduction Neutralization Test (PRNT). Results indicate that the WN virus vaccine is capable of stimulating the production of antibodies at 28 and 63 days after vaccination. Twenty five percent of the horses in the population sample (3 out of 12 animals) had detectable titers after the first immunization while 75% (9 of 12) had detectable antibody titers after the second administration of the vaccine.

I dedicate this manuscript to my wife, Mary Ann, who has supported and nurtured our marriage throughout veterinary school, graduate school and several military deployments.

To my children, Julio Manuel, and Alejandro, all my love and admiration.

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Last but not least, I want to express my sincere gratitude to Dr. Louise M. Ball for being an exceptional teacher and mentor during my education experience at the School of Public Health.

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LIST OF ABBREVIATIONS

APHIS	Animal Plant Health Inspection Service
CDC	Centers for Disease Control and Prevention
CINDI	Center for Integration of Natural Disaster Information
EPA	Environmental Protection Agency
JE	Japanese Encephalitis virus
k-DA	kilo-Dalton
ml	milliliter
nm	nanometer
NVSL	National Veterinary Service Laboratories
PRNT	Plaque Reduction neutralization Test
SLE	Saint Louis Encephalitis virus
ss RNA	single stranded Ribonucleic Acid
USAMRIID	United States Army Medical Research Institute of Infectious Diseases
USDA	United States Department of Agriculture
USGS	United States Geological Survey
WNV	West Nile virus

I. INTRODUCTION

A. Purpose

The first purpose of this investigation was the examination of basic veterinary and human public health and epidemiologic characteristics of the West Nile virus (WNV) in the United States from its isolation in late summer 1999 throughout the month of July 2002. A member of the family Flaviviridae (ssRNA), WNV is rapidly expanding its range in the United States and has become an endemic viral agent for animals and humans. WNV is one of the fastest-growing threats to horses nationwide. The incidence of equine WNV has more than doubled in just over 3 years. Up to 40% of horses diagnosed with WNV have died or been euthanized.

In August 2001, the United States Department of Agriculture (USDA) conditionally licensed a killed vaccine intended to protect horses against WNV. This study evaluated the efficacy of the only commercially available vaccine in a population of horses in Cumberland County, North Carolina. The main goal of the vaccine evaluation was to determine whether or not the vaccine was able to stimulate the equine immune system, which is a prerequisite from clinical WNV infection.

B. Background

1. West Nile Virus Overview

West Nile virus (WNV) (genus *Flavivirus*) is a member of the Japanese encephalitis (JE) virus serological complex (Table 1), which contains a number of viruses associated with human encephalitis (1,2).

Table 1. Members of the Japanese encephalitis (JE) virus serocomplex viruses (Source: CDC, 2001)

Virus	Abbreviation	Geographic location
Cacipacore	CPC	South America
Koutango	KOU	Africa
Japanese encephalitis	JE	Asia, Oceania, Australia[a]
Murray Valley encephalitis	MVE	Australia
Alfuy	ALF	Australia
St. Louis encephalitis	SLE	North America, South America
West Nile encephalitis	WN	Africa, Asia, Europe, North America
Kunjin	KUN	Australia
Yaounde	YAO	Africa

Structurally WNV has a 30- to-35 nm icosahedral core composed of multiple copies of a 12-kDa capsid protein (Figure 1).

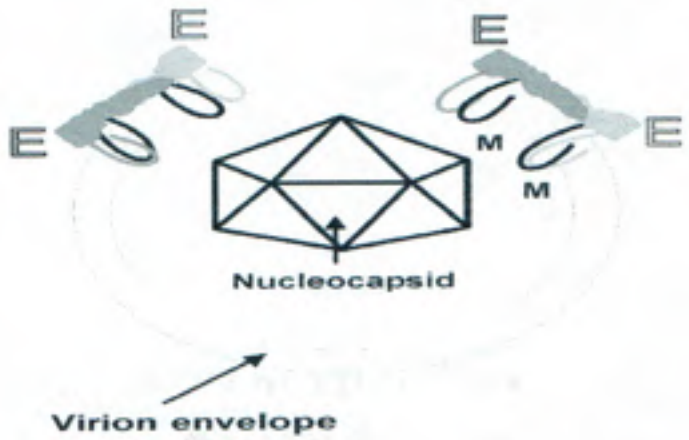

Figure 1. Structure of the flavivirus virion. The virion has an envelope derived from the host cell membranes. E-glycoprotein (E), an integral membrane protein associates with the other integral membrane proteins prM protein (in immature virions)
(Source: CDC,2001)

The genetic material enclosed inside the capsid consists of a single-stranded, positive-sense RNA of approximately 12,000 nucleotides (1,2). The capsid is enclosed in a host cell-derived envelope that has been modified by the insertion of two integral membrane glycoproteins, E (53kDa) and prM (18-20 kDa). The prM protein is cleaved to the M protein (8 kDa) late in virus maturation by a cellular protease. The genome also encodes seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) that are responsible for the intracellular machinery of the virus (Figure 2). The E-glycoprotein is the most immunologically important structural protein. It mediates virus-host cell binding and elicits most of the neutralizing antibodies during clinical infection. (1,2,4).

Figure 2. Genomic structure of flaviviruses. The flavivirus genome is 11,000 to 12,000 nucleotides long. Both the 5'- and 3'- ends contain noncoding (NC) regions. The genome encodes 10 proteins, 3 of which are structural proteins (C, M, and E), and 7 of which are nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). The M protein is synthesized as a precursor (prM) protein. The prM protein is processed to pr + M protein late in the virus maturation by a convertase enzyme (furin). (Source CDC, 2001)

The first isolation of WNV in a human patient took place in Uganda's West Nile district in 1937 from a febrile adult female (3). WNV is endemic to Africa, the Middle East, Southwest Asia, Europe, Oceania and most recently North America. (1,2,3) (Figure 3). According to the Centers for Disease Control and Prevention (CDC), recent outbreaks of WN virus encephalitis in humans have occurred in Algeria in 1994,

Romania in 1996-1997, the Czech Republic in 1997, the Democratic Republic of the Congo in 1998, Russia in 1999, the United States in 1999-2002, and Israel in 2000. Epizootics of disease in horses occurred in Morocco in 1996, Italy in 1998, the United States in 1999-2002, and France in 2000. In the U.S. through mid-July, 2002 WN virus has been documented in Alabama, Arkansas, Connecticut, Delaware, the District of Columbia, Florida, Georgia, Illinois, Indiana, Iowa, Kentucky, Louisiana, Maine, Maryland, Massachusetts, Michigan, Mississippi, Missouri, Nebraska, New Hampshire, New Jersey, New York, North Carolina, North Dakota, Ohio, Oklahoma, Pennsylvania, Rhode Island, Tennessee, Texas, Virginia and Wisconsin. As of July 21, 2002, there have been 152 total human cases of West Nile virus illness reported to CDC and confirmed, including 18 fatalities (28).

Figure 3. Geographic distribution of the JE viruses serocomplex. (Source: CDC, 2001)

The first recorded cases of WNV in the western hemisphere occurred in humans,

horses and wild birds in New York City in 1999 (1,2,6,7-10, 11-15). Originally, these clinical cases of encephalitis or meningitis were diagnosed as St. Louis Encephalitis virus infections (SLE) (2,4,6,9). Surprised by the virulence and high mortality associated with the avian infections, veterinarians at the Bronx zoo requested the CDC to do more specific testing arguing that cases of SLE in avian species are rarely fatal. Results of further genomic analysis and sequencing of the viral specimens isolated from clinically infected humans and animals revealed WNV as the causative agent of infection (2,5,11,12). A possible explanation for the misdiagnosis of SLE was the similar genetic constitution of members of the family Flaviviridae. All flaviviruses are closely related antigenically, which accounts for the serologic cross-reactions observed in the initial diagnostic laboratory (1,4).

Although members of the JE serocomplex share some antigenic determinants, genomic characteristics at the genus level are significant enough to require individual vaccine development. The existing need for accurate identification of WNV infections for both treatment and surveillance, has resulted in an increased interest in the development of serological and biochemical diagnostic tests and vaccine development. Currently, there are no approved vaccines available to protect humans from WNV or any other flaviviruses. Due to the close antigenic relationships between the flaviviruses, acute- and convalescent-phase serum specimens from patients are required to assess antibody response to infection. Virologists have divided WNV genetically into two lineages: WN1 and WN2 (1). Only members of the lineage WN1 viruses have been associated with

clinical encephalitis or meningitis. Lineage WN2 viruses are maintained in enzootic foci in Africa and have not been associated with clinical disease. According to the Centers of Disease and Prevention (CDC), the closest relative of NY99 WNV is a virus circulating in Israel (Isr98) from 1997 to 2000 (1). The close genetic relationship between WNV isolates from Israel and New York suggests that the virus was imported into North America from the Middle East (1,2,8,11).

The exact mechanism of WNV introduction into the western hemisphere (e.g. infected bird, mosquito, human or another vertebrate host) is not known at the present time (1,2). A striking feature of the WNV variant found in the United States is the high number of avian deaths during outbreaks, particularly in the family Corvidae (common crow, fish crow, blue jay) (1,2) (Table 2).

Avian pathogenesis is characterized by involvement of visceral organs, with deaths from myocarditis and encephalitis. WNV is shed in cloacal fluids, and bird-to-bird transmission may occur by the oral-fecal route or ingestion during predation. Mortality of experimentally infected American Crows with the NY99 WNV variant in 2000 was 97% (1), while in horses and humans it was 38% and 10%, respectively (13,14). Infection of one laboratory technician has been documented suggesting that virus can be aerosolized (15). WNV is classified by the CDC at Biohazard Level 3 (24).

Natural WNV transmission cycle involves the interaction of *Culex* mosquitoes, wild birds and possibly bats as viremic hosts (Figure 4). To this date it has not been demonstrated by experimentation that incidental hosts (e.g., humans, horses) play a significant role in horse-to-horse or human-to-human infections. Viremic state achieved

in incidental hosts are not considered high enough to infect mosquitoes and to perpetuate transmission (1,4).

Table 2. Species found positive for the WNV in surveillance efforts. (Source:USGS-NWHC)

Bittern, Least	*Ixobrychus sinensis*	Jay, Blue	*Cyanocitta cristata*
Blackbird, Red-winged	*Agelaius phoeniceus*	Kestrel, American	*Falco sparverius*
Bluebird, Eastern	*Sialia sialis*	Killdeer	*Charadrius vociferus*
Cardinal, Northern	*Cardinalis cardinalis*	Kingfisher, Belted	*Ceryle alcyon*
Catbird, Gray	*Dumetella carolinensis*	Merlin	*Falco columbarius*
Chickadee, Black-capped	*Poecile atricapillus*	Mockingbird, Northern	*Mimus, polyglottos*
Cormorant, Double-crested	*Phalacrocorax auritus*	Titmouse, Tufted	*Chordeiles minor*
Cowbird, Brown-headed	*Molothrus ater*	Ovenbird	*Seiurus aurocapillus*
Crow, American	*Corvus brachyrhynchos*	Owl, Great Horned	*Bubo virginianus*
Crow, Fish	*Corvus ossifragus*	Phoebe, Eastern	*Sayornis phoebe*
Dove, Mourning	*Zenaida macroura*	Rail, Virginia	*Rallus limicola*
Duck, Mallard	*Anas platyrhynchos*	Raven, Common	*Corvus corax*
Finch, House	*Carpodacus mexicanus*	Robin, American	*Turdus migratorius*
Flicker, Northern	*Colaptes auroatus*	Sanderling	*Calidris alba*
Goldfinch, American	*Carduelis tristis*	Skimmer, Black	*Rynchops niger*
Goose, Canada	*Branta canadensis*	Sparrow, Song	*Melospiza melodia*
Grackle, Common	*Quiscalus quiscula*		
Grouse, Ruffed	*Bonasa umbellus*	Thrush, Hermit	*Catharus guttatus*
Gull, Great Black-backed	*Larus marinus*	Thrush, Wood	*Hylocichla mustelina*
Gull, Herring	*Larus argentatus*	Turkey, Wild	*Meleagris gallopavo*
Gull, Ring-billed	*Larus delawarensis*	Turnstone, Ruddy	*Arenaria interpres*
Hawk, Broad-winged	*Buteo platypterus*	Veery	*Catharus fuscescens*
Hawk, Cooper's	*Accipiter cooperii*	Vulture, Black	*Coragyps atratus*
Hawk, Red-tailed	*Buteo jamaicensis*	Warbler, Blackpoll	*Dendroica striata*
Hawk, Sharp-shinned	*Accipter striatus*	Warbler, Canada	*Wilsonia canadensis*
Heron, Great Blue	*Ardea herodias*	Warbler, Yellow-rumped	*Dendroica coronata*
Heron, Green	*Butorides virescens*	Warbler, Black-throated Blue	*Dendroica caerulescens*
Hummingbird, Ruby-throated	*Archilochus colubris*	Waxwing, Cedar	*Bombycilla cedrorum*

Captive North American Bird Species

Crane, Sandhill	*Grus canadensis*	Magpie, Black-billed	*Pica pica*
Eagle, Bald	*Haliaeetus leucocephalus*	Night-Heron, Black-crowned	*Nycticorax nycticorax*
Gull, Laughing	*Larus atricilla*	Owl, Snowy	*Nyctea scandiaca*

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Gull, Laughing	*Larus atricilla*	Owl, Snowy	*Nyctea scandiaca*

Other Free-Ranging Bird Species

Dove, Rock (pigeon)	*Columba livia*	Starling, European	*Sturnus vulgaris*
Pheasant, Ring-necked	*Phasianus colchicus*	Swan, Mute	*Cygnus olor*
Sparrow, House	*Passer domesticus*		

Free-Ranging Mammal Species

Bat, Big brown	*Eptesicus fuscus*	Raccoon	*Procyon lotor*
Bat, Little brown	*Myotis lucifugus*	Skunk, Striped	*Mephitis mephitis*
Chipmunk, Eastern	*Tamias striatus*		

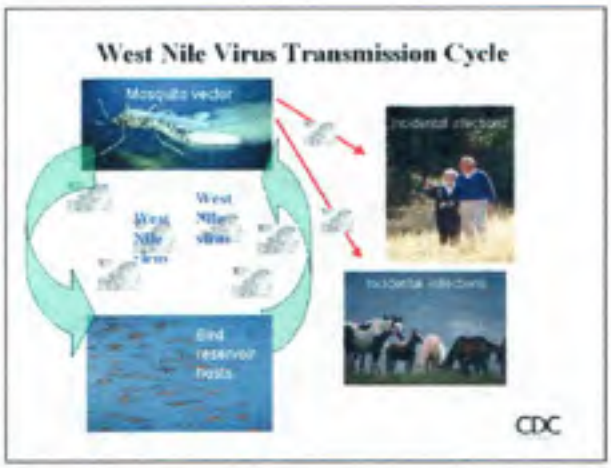
Pet and Other Domesticated Species

Cat	Horse
Chicken	Macaw
Cockatiel	Parakeet
Cockatoo	Peacock
Dog	Rabbit, domestic
Finch, Zebra	Turkey, domestic
Goose, domestic	

Exotic Species housed in Zoos

Cormorant, Guanay	Pheasant, Himalayan Impeyan
Duck, Bronze-winged	Tragopan, Blythe's
Flamingo, Chilean	

Figure 4. WNV transmission cycle. (Source: CDC, 2002)



Over wintering is by vertical transmission through mosquito ova and infected, hibernating adult mosquitoes (4,25). The CDC and the USDA have suggested that WNV survival during the winter can be achieved by infection of urban-adapted *Culex* mosquitoes. Mild environmental conditions during winter could also promote survival. A mid-winter isolate from a hawk suggests that chronic infection and vertebrate-to-vertebrate transmission contributes to winter survival. In North America the National Wildlife Health Center of the U.S. Geological Survey reports that 80 species of wild and domestic birds have been found positive for WNV during surveillance efforts. (Table 2). The CDC in coordination with other Federal, state and local public health agencies have documented 27 species of mosquitoes that are able to transmit WNV to birds, humans, horses and other mammals (17-18,25). The number of human infections with WNV in the United States for the years 1999-2001 was 62, 21, and 31, respectively (11-15). The first human cases (3) of the year 2002 were reported in Louisiana on July

10^{th}.

Table 3. Clinical infections with WNV in the United States-1999-2002 (Source CDC,2001)
*( ) = fatal # of cases Ω - As of 22 July 2002

Infection cases	1999	2000	2001	2002Ω
Humans	62 (7)*	21 (2)	66 (9)	3
Horses	25 (9)	58 (23)	733 (210)	23(6)
Wild birds	194	4323	7333	437
Mosquito pools	67	481	322	26
Sentinel chickens	0	6	5	10

The combined 1999-2002 fatalities are 18 out of 152 clinical cases. In serological surveys done in 1999 by the CDC, up to 2.6% of the population of the City of New York shows seroconversion to WNV (4). The interpretation of this fact is that only a few patients show clinical symptoms of active infection. WNV infection ranges from no apparent signs, flu-like symptoms, to encephalitis, and death. Elderly individuals older than 75 years of age are more susceptible to develop active infection (11,12,19).

Most common mosquito vectors in the United States are *Culex quinquefasciatus, Culex pipiens, Culex salinarius, and Culex restuans* (12,18). Other genera of vectors include *Aedes, Anopheles, Coquilletidia, Ochlerotatus, and Psorophora*. An important factor in the epidemiology of WNV is that mosquitoes of the *Culex* genus are well adapted to survive and multiply in urban settings in discarded tires, containers, ponds and other man-made structures (2,18, 24). Natural hosts also play an important epidemiologic role in disease dispersion. Migratory birds using the eastern flyway have been implicated in the distribution of the virus to southern United States. In 2000, surveillance showed

expansion of viral activity to 12 states extending from the Canadian border to North Carolina, a distance of 900 kilometers (5,13,17-18). In 2001, a large outbreak of equine cases (n=551) in Florida, Georgia and Alabama suggested WNV reached those states because of migratory bird activity. The number of reported equine cases in 2001 represents a twelve-fold increase from 2000 levels (Table 3). Surveillance indicates that intense vector activity increases the risks of infection in horses and humans. In humans WNV infection is characterized by self-limiting symptoms of weakness, malaise, and poor coordination. Mortality is about 10%, especially in people older than seventy-five years of age.

WNV-induced mortality in horses is significant, approximately 38%. In relation to veterinary public health, the horse is the most commonly infected domestic livestock species. In serological surveillance efforts dogs, cats, squirrels, raccoons, mice and rats have been found positive but they appear to be more resistant to infection that horses. Horses are incidental hosts of the virus, becoming infected by the bite of mosquitoes, primarily members of the Culex genus (13,17). As WNV becomes an endemic agent in the United States it may have an economic impact in the trading of racehorses. Currently, the European Union (EU) exercises strict oversight on the importation of horses from counties in the United States that had positive cases of WNV or any horse that has positive titers against WNV. In two occasions last year the EU refused entry to horses that showed positive titers for WNV. In both cases, the horses had been immunized for WNV. The implication for owners of highly priced Thoroughbreds is that the use of the vaccine should be based on risk analysis and science. States with important equine

industries (e.g. KY, FL, NY) regularly allocate significant amounts of resources to vector control program and public education to minimize economic losses. The first documented cases of WNV infection in horses were reported in Egypt and France in the early1960s (1,2). Epidemics have occurred in Israel during 1950-1954 and in 1957. Other epidemics have been reported in the Rhone delta of France in 1962 and 2000, in Romania and Russia in 1996-1999 (13,14). The largest recorded epidemics occurred in South Africa in 1974 and in Russia in 1996. The Russian epidemic was characterized by a large number of human clinical cases, close to 900 (1,4).

Other vertebrate hosts that can become infected with WNV include the big brown bat (*Eptesicus fuscus*), little brown bat (*Myotis lucifugus*), eastern chipmunk (*Tamias striatus*), raccoon (*Procyon lotor*), domestic cat (*Felis domesticus*) and rabbits (18). In North Carolina, the agency responsible for monitoring WNV is the Division of Environmental Health. The agency reported that in 2001 2 horses and 11 birds tested positive for WNV in the state (Table 4).

During the early part of the year 2000, Federal authorities developed a surveillance system to monitor active cases of WNV in the United States. The system tracks all types of infections (e.g. humans, birds, horses, mosquito-pools) and it involves the cooperation of the following Federal departments and agencies: USDA, CDC, USGS, EPA with state and local public health agencies. The CDC and the USGS manage the database CINDI (www.usgs.gov) that monitors occurrence of WNV and other natural and environmental disasters. At the present time, WNV surveillance by public health agencies continues. Potential approaches to limiting the spread of infection include vector control,

vaccination of susceptible host as well as education of the public. Implementing personal protective measures to reduce exposure to mosquitoes is an effective way of limiting the spread of the disease. Some of these include the use of insect repellants, avoiding outdoor activities in times where vectors are more active and the elimination of their potential breeding areas (e.g. discarded tires).

2. Vaccine Overview

The USDA regulates veterinary vaccines, and requires that all fully licensed vaccines must be pure, safe, potent and efficacious. Conditional licenses may be granted by USDA under expedited procedures "in order to meet an emergency condition, limited product, local situation, or other special circumstance." Conditionally licensed products must have proven purity, safety and a reasonable expectation of efficacy. USDA usually also requires progress reports from the manufacturer concerning efforts to complete full license requirements.

Conditionally licensed products frequently do not have full vaccination/challenge studies complete at the time of conditional licensure, but in order to expedite product availability, USDA accepts more indirect evidence to establish a reasonable expectation of efficacy. Also, since the manufacturer's batch release potency test is normally considered by USDA to be final and complete only after the potency test method is proven to correlate to the vaccination challenge study, USDA almost always considers potency test development studies incomplete and ongoing under conditional licensure.

North Carolina West Nile Virus Cases Laboratory Confirmed

County	Species	Date Collected
Cabarrus	Blue Jay	8/8/2001
Camden	American Crow	9/15/2001
Camden	American Crow	9/16/2001
Currituck	American Crow	9/20/2001
Currituck	American Crow	9/27/2001
Mecklenburg	American Crow	9/24/2001
Chowan	American Crow	10/3/01
Cabarrus	Blue Jay	10/3/01
Hyde	Horse	09/26/01
Hyde	Sentinel Hen	9/17/01
Hyde	Sentinel Hen	9/17/01
Pasquotank	Horse	9/27/01
Pitt	Sentinel Hen	10/23/01

Table 4. Cases of WNV infections in North Carolina in 2001. (Source: NC Department of Health,2002)
Source: http://deh.enr.state.nc.us/phpm/pages/html/confirmed_positive.html

On August 1, 2001 the Animal and Plant Health Inspection Service (APHIS,USDA) issued a conditional license to Fort Dodge Laboratories, Inc, a division of American Home Products, Inc for a vaccine intended to aid in the prevention of disease in horses caused by West Nile virus. Product use is restricted to a licensed veterinarian with approval from state regulatory activity. The vaccine is for intramuscular use in horses. Two doses are required, given 3 to 6 weeks apart. Annual revaccinations are recommended after the initial set of immunizations. The cost of the vaccine for the consumer is $16.00 per dose.

Development of the equine vaccine utilized genetic material from the original WNV isolate from New York (NY99) derived from human and avian clinical cases. The material was inoculated into horses. Those horses that developed clinical encephalitis were euthanized and viral material was obtained from their central nervous system to create the vaccine "seed". Vero 76 cells were then inoculated with the virus to verify infectivity and to isolate and purify the vaccine antigen. The WNV vaccine type is killed and adjuvanted with MetaStim™, an exclusive synthetic agent in Fort Dodges' equine vaccines. It contains easy-to-metabolize lipids that stimulate the immune system and reduces the potential for injection site reactions.

According to Fort Dodge technical vaccine literature the adjuvant works in two phases. Phase 1 is the lipid-based. The lipid component disperses and forms droplets in an aqueous environment. Surface tension and the oil/water interface distribute the droplets uniformly. Phase 2 is the surfactant phase in which the lipid is broken down into

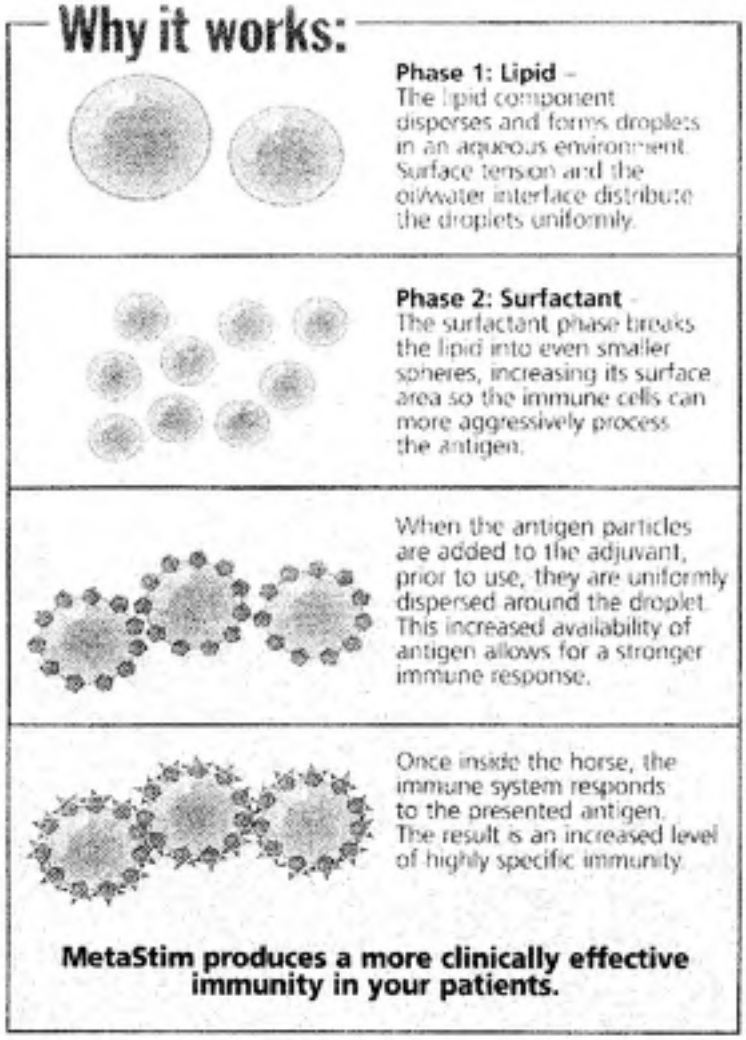
smaller spheres, increasing its surface area so the immune cells can more easily process the antigen. When the antigen particles are added to the adjuvant prior to use, they are uniformly dispersed around the droplet. The increased availability of the antigen allows for a stronger immune response.

After administration, the immune system responds to the presented antigen resulting in specific immunity. Figure 5 depicts the basic components of the adjuvant MetaStim™. During clinical trials the vaccine was tested in 649 horses in five states. It was shown to be 96.28% free of local or systemic reactions.

A horse that has been immunized for WNV with the commercially available vaccine will develop antibody titers against the virus. Since it is impossible to distinguish antibodies generated by natural clinical infection and those produced by active immunity (vaccine), problems of economic nature might arise if a vaccinated horse is to be transported to some countries of the European Union that are free from WNV. The same is also true for horses from Europe used in international competitions. A country may not want to participate in equine events held in the United States if the state in which the competition takes place has active cases of WNV. The epidemiology of WNV in the United States during the last three and a half years suggests that it will soon be a coast-to-coast arboviral pathogen.

Fort Dodges' WNV equine vaccine is the only product available for conditional use in this country. Considerable research is being done at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), the CDC, and the USDA to develop vaccines against WNV infection in humans, poultry and potentially other animals.

Figure 5. Metastim™ adjuvant of the WNV vaccine.



Israeli researchers have developed and received approval for a vaccine to be used in Israel to protect geese in commercial operations (4). WNV is able to infect 80 species of wild and domestic birds and several species of wild and domestic mammals.

II. METHODS AND MATERIALS

A. Animal Selection and Study Design

Mares and geldings of varying ages and breeds were used in the investigation. (Table 5). Each animal was given a physical examination that included determinations of temperature, pulse and respiratory rates and general behavioral status. The horses of this investigation were stabled at the Fort Bragg equine facility in Cumberland County, NC. West Nile vaccinations were administered on April 11 and May 9, 2002. Vaccine lot and serial number were Lot 16663C and 1666109A, respectively. Expiration date on the label is 01 MAR 2004. Each dose consisted of a 1-ml intramuscular injection administered in the muscles of the neck. All horses in the study were current in their immunizations (e.g., rabies, equine rhinopneumonitis, equine influenza, eastern and western equine encephalitis) and current and negative for the equine infectious anemia virus (Coggins test).

B. Specimen collection and storage

Blood was collected for serum on April 11, May 9, and June 13, 2002 to assess the immune response after vaccinations. Collection was done by jugular venipuncture via a Vacutainer® using a 20gauge needle. Blood was allowed to coagulate for 1 hour. After coagulation the serum component was separated by centrifugation and transferred to 3-ml red top tubes for storage. Each tube was labeled with the date of collection and the animals' identification. Samples were kept under normal freezing conditions in a commercial refrigerator until they were shipped by overnight commercial courier to the

National Veterinary Service Laboratories (NVSL) of the USDA in Ames, Iowa.

Table 5. Animal identification, breed, age, sex, and days of vaccination, April–May 2002

Identification	Breed	Age (yr)	Sex	Vaccination Dates
1. SPOTTY	Appaloosa	14	Male	11 April 02, 9 May 02
2. SAM	Thoroughbred	12	Male	11 April 02, 9 May 02
3. MIDNIGHT	Tennessee Walker	10	Male	11 April 02, 9 May 02
4. JACK	Quarter Horse	19	Male	11 April 02, 9 May 02
5. FANCY	Quarter Horse	9	Female	11 April 02, 9 May 02
6. CRIMSON	Quarter Horse	19	Female	11 April 02, 9 May 02
7. CLARENCE	Quarter Horse	11	Male	11 April 02, 9 May 02
8. BUCK	Arabian	17	Male	11 April 02, 9 May 02
9. BARON	Quarter Horse	18	Male	11 April 02, 9 May 02
10. HOMER	Quarter Horse	19	Male	11 April 02, 9 May 02
11. TERRIE	Quarter Horse	26	Male	11 April 02, 9 May 02
12. SHILOH	Quarter Horse	7	Male	11 April 02, 9 May 02

C. Antibody titer assessment

At the NVSL, assessment of antibody titers for WNV in the samples was performed according to testing laboratory protocol #EOPRO1401.01 which was approved on May 5th, 2001. The plaque reduction virus neutralization (PRNT) test is used to detect and quantitate specific serum antibodies directed against WNV. The test is performed by

assaying serum dilutions against a constant concentration of virus. In the first step, serum dilutions are mixed with equal volumes of known virus concentration and incubated. Then, the virus-serum mixture is inoculated onto sensitive cell cultures in 25 cm^2 flasks, or 6 well plates. Cells are overlaid with an agar solution, allowed to incubate for several days and then overlaid with a second agar solution containing a neutral red dye. Following an overnight incubation, the flasks/plates are observed for the formation of virus-produced plaques. Serum that reduces the numbers of plaques by 90% or greater, as compared with the virus control flasks, are considered positive for virus neutralizing antibody. Titers are reported as < 1:10, 1:10, 1:100, or $\geq$ 1:100.

Samples were tested for virus-neutralizing antibodies to WNV (NY99-4132 strain, 1 Vero cell passage) by the plaque-reduction neutralization test in Vero 76 cell cultures.

D. Data analysis

Analysis of data related to the efficacy of the WNV vaccine in the population sample was performed using the ©SAS Institute Inc JMPIN statistical software, version 4. The population sample of this study consisted of 12 animals randomly selected from the entire population of 35 animals. The alpha level of significance was set at 0.05.

III. RESULTS

A. Horses

Basic information on the twelve animals that constitute the sample population in this study is listed in Table 4. The mean age of horses in the population sample is 15.17 years with a standard deviation of 5.44. The sample median and range for the variable of age were 15.5 years and 19 (26-7), respectively (Table 6). None of the horses developed anaphylactic shock after vaccine administration or local injection site inflammation in the form of granuloma or abscess. Table 6 lists the serological data for the population sample on days 0, 28, and 63 after initial vaccination for WNV.

On day 0 (April 11, 2002) all horses of this study received the first dose of the WNV vaccine. Serological analysis revealed they did not have detectable titers against WNV on that particular date. On day 28 (May 9, 2002) the horses were immunized again with 1-ml of the WNV vaccine intramuscularly. Plaque reduction neutralization tests (PRNT) revealed that three horses out of 12 (25%) did have positive antibody titers for WNV at the 1:100 level (2) and at the 1:10 level (1). The ages of these geldings were 12, 12, and 19 years.

On day 63 (June 13, 2002) 75% of the horses (9 out of 12) were found with detectable antibody titers for WNV at the 1:100 level (5) and at the 1:10 level (4). At the present time none of the horses has shown any clinical signs of WNV or any other arboviral infection.

Animal identification	Day 0 Titers	Day 28 Titers	Day 63 Titers	Age (years)
SPOTTY	NEG (1:10)	NEG (1:10)	POS (1:10)	14
SAM	NEG (1:10)	POS (1:100)	POS (≥ 1:100)	12
MIDNIGHT	NEG (1:10)	NEG (1:10)	NEG (1:10)	10
JACK	NEG (1:10)	POS (1:100)	POS (≥ 1:100)	19
FANCY	NEG (1:10)	NEG (1:10)	POS (1:100)	9
CRIMSON	NEG (1:10)	NEG (1:10)	POS (≥ 1:100)	19
CLARENCE	NEG (1:10)	POS (1:10)	POS (≥ 1:100)	12
BUCK	NEG (1:10)	NEG (1:10)	NEG (1:10)	17
BARON	NEG (1:10)	NEG (1:10)	POS (1:10)	18
HOMER	NEG (1:10)	NEG (1:10)	POS (1:10)	19
TERRIE	NEG (1:10)	NEG (1:10)	NEG (1:10)	26
SHILOH	NEG (1:10)	NEG (1:10)	POS (1:10)	7

Table 6. Antibody titers against WNV in the sample population.

B. Statistical analysis

Risk factors of age, sex, and breed were not analyzed in this investigation due to small sub-sample sizes and lack of independence of the two vaccination events, which would render hypothesis testing non-significant. Therefore, presentation of all data should be interpreted with caution.

Due to time and budget considerations the population sample was set at 12 out of a total equine population of 35. Detectable levels of antibodies in the sample population after the first and second vaccinations were 3 (25%) and 9 (75%), respectively. The statistical analysis utilized for this investigation was the McNemar chi-squared (χ^2) test for before-after measurements. This test is a marginal homogeneity of proportions, which determines whether the proportion responding under two conditions is the same. Adjustments are made for the fact that the data involve pairs (each pair is one observation under one condition and one observation under the other condition). The McNemar chi-squared (χ^2) test formula is listed in Table 7, which illustrates the cell categories comparing two criterion of classification that the test formula is based on, follows:

$$\chi^2 = (b\text{-}c)^2 / b + c$$

Table 7

2 x 2 Contingency Table

a	**b**
c	**d**

Statistical analysis was performed on the initial (day 0), first vaccination (day 28) and second vaccination (day 63) titers to determine if there was similar significance in titer levels between them. Tables 8 and 9 are the contingency tables of initial by first vaccination and initial by second vaccination. Each table is followed by the McNemar chi-squared test, which is compared to the critical chi-squared with one degree of freedom at the 0.05 significance level (95% confidence). One degree of freedom is based on the number of rows minus one multiplied by the number of columns minus one [(r-1)(c-1)] rule for finding degrees of freedom to a 2 x 2 contingency table (42).

Table 8

Initial Titer Levels by First-Vaccination Titer Levels

	First Vaccination Titers	
Initial Titers	+	–
+	0	3
–	12	9
Total	12	12

McNemar $\chi^2 = (3-12)^2/3 + 12 = 5.40$

Critical χ^2 (1df) at 0.05 significance level = 3.84

McNemar χ^2 (5.40) > Critical χ^2 (3.84), so reject H_0

Table 9

Initial Titer Levels by Second-Vaccination Titer Levels

	Second Vaccination Titers	
Initial Titers	+	–
+	0	9
–	12	3
Total	12	12

McNemar $\chi^2 = (9\text{-}12)^2 / 9 + 12 = 0.4286$

Critical χ^2 (1df) at 0.05 significance level = 3.84

McNemar χ^2 (0.4286) < Critical χ^2 (3.84), so can not reject H_0

A limited interpretation of the McNemar chi-squared tests is that there is a difference between the observed titer levels seen between the initial vaccination group and the first and second vaccination group. Further refinement of the study design is necessary to compare the immune response between a negative control group with an experimental one.

IV. DISCUSSION OF RESULTS

It is generally accepted that WNV is now a well-established endemic arbovirus in the United States. Almost four years after its initial appearance in New York City in late summer 1999, WNV is present in 30 states in the USA, the Canadian Province of Ontario and the Caribbean island of Grand Cayman. As of July 7, 2002 the westernmost documented distribution of WNV in continental United States (CONUS) is Harris County in Southeastern Texas (Houston). Three human cases have been reported in July 2002 in the state of Louisiana.

In 1999 WNV outbreaks were recorded in 4 states (NY, NJ, CT, MD). Sixty-two humans developed clinical disease and 7 died. Equine infections were 25 with 9 deaths and 194 wild birds tested positive for WNV. In the year 2000 WNV expanded to 8 new states (RI, DE, MA, PA, NH, NC, VT, VA) and the District of Columbia. During the 2000 outbreak we observed less human infections and more veterinary infections. Twenty-one human clinical infections were recorded and only 2 fatalities. The number of equine clinical infections almost tripled from 1999 levels to 58 active cases with 23 fatalities (39.7% mortality rate). Infections of wild birds increased almost 23 times from 194 (1999) to 4323 animals in 2000. In terms of geographical distribution, WNV dispersed about 900 km from the Canadian border to North Carolina.

In 2001 WNV could be found in 27 states and the District of Columbia. WNV reached southern United States (Florida, Georgia, Alabama) and extended westward to Louisiana and Arkansas. Infection levels in all affected species were higher than the ones

observed in 2000. Sixty-six humans were clinically infected and 9 people died (MR 13.6%). The number of equine cases experienced a 12-fold increase to 733 cases while the number of wild birds diagnosed with WNV rose to 7,333 cases. Five hundred and fifty one of the equine infections (75% of 2001 cases) occurred in a concentrated focus of infection in northern Florida, southern Georgia and eastern Alabama that suggests a possible role of migratory birds in WNV dispersal.

Most probably, the health impacts of WNV will be observed in elderly people and susceptible equine and wild bird populations. Sporadic human mortality to the virus will occur in the future as the virus spreads west in the United States and south to Central and South America with migratory birds. Human mortality might be higher than 10% in some Latin American countries where funding for virus surveillance and vector control might not exist. WNV is one of several infectious diseases transmitted by biological vectors emerging at an accelerated pace throughout the world (1-2,6-9). Some investigators suggest that the emergence of various infectious pathogens throughout the world is partly due to anthropogenic activities that induce environmental degradation (i.e. deforestation, habitat fragmentation), global trade and travel and global warming (20). Without a doubt, WNV has become endemic in NA and is expanding its range in the United States and Canada (1,2,4,5,23). At the present time, 31 states, the District of Columbia and the Canadian province of Ontario have had active cases of WNV. An active surveillance system established by federal, state and local governments is in place to monitor the distribution of the virus. It appears the WNV has been able to survive the winter seasons in North America in mosquito and wild bird populations. Unusual benign winter

conditions in 1998-2001 have allowed an increased survival rate of the vector and have probably favored WNV range expansion south and west in the United States (21,23,25).

Future protection of both humans and animals from WNV infection will require the implementation of public health measures that include vector and pathogen monitoring, vector control and development of WNV vaccines intended for humans and other domestic animals (host immunization). The use of pesticides by communities to control vector populations is effective an effective approach but it can be environmentally taxing. Use and monitoring of a web-based surveillance system could be advantageous to communities. Detection of early biological activity at the county level of community organization can lower pesticide use significantly. This web-based reporting and surveillance systems effectively links and promotes epidemiological training among Federal, state and local public health organizations. Examples of these web-based nets are ArboNet, CINDI and ProMed. The system utilizes mapping resources of the United States Geological Survey, human epidemiological tools from the CDC and veterinary epidemiological data from USDA to generate national, state and county maps for the entire country.

States (e.g. NY, NJ, FL) are beginning to initiate fumigation and other vector control practices based on data generated by mosquito-pool serological surveys and bird mortality rates. The net effect of these efforts is to reduce the use of pesticides and manpower and allocate resources when risks of transmission and infection are higher. During the last four years, WNV infections predominantly peak in late summer throughout the fall.

The development of vaccines to protect humans and other domestic animals will directly help to reduce the morbidity and the mortality of WNV infections in North America and throughout the world. WNV may constitute an arbovirus that will trigger a plethora of research and vaccine development that can be adapted for use in other members of the viral family Flaviviridae. With mortality rates in some avian species of over 97%, 38-40% mortality in horses and up to 10% mortality in humans, WNV is an important emerging public health threat in both human and veterinary medicine.

The results of this study indicate that the WNV vaccine was capable of inducing protective antibody titers in 25% of vaccinated as early as 28 days after a first dose administration. After completion of the recommended vaccine regimen (two doses 3-6 weeks apart), 75% of the animals produced protective antibody titers. The WNV vaccine appears to offer an acceptable level of protection in locations that exhibit high densities of mosquitoes. This protection is enhanced when steps are taken to control mosquito populations in particular vicinity. Increased hygiene and sanitation practices (e.g., proper tire and water container disposal, prompt removal of organic wastes) do reduce the available habitat for mosquito reproduction. Vector control with approved pesticides is also effective, especially during explosive expansion of mosquito populations during the rainy season.

WNV vaccine development intended for human use and other animal species (e.g. poultry, companion animals) is currently taking place in government research laboratories (USAMRIID, CDC, USDA) and biotechnology firms (Fort Dodge, Merial, Novartis). Refinement of molecular techniques in the isolation and identification of

flaviviruses will allow for the manufacturing of purer and more efficacious vaccines. WNV is the first arbovirus for which a unified surveillance system has been developed to track its dispersion and to inform and educate the public about this emerging pathogen.

V. CONCLUSION

After reviewing the patterns of infection in humans and animals and its aggressive dispersal throughout North America we have to conclude that WNV is now endemic to the region and will become a coast-to-coast arboviral pathogen. It is very possible that the virus will reach the southern hemisphere within this decade via migratory birds. Although mortality in humans is approximately 10%, WNV has the potential of affecting a large number of people if the virus is maintained in the sylvatic cycle in mosquitoes and wild birds.

WNV is probably the first flavivirus for which a web-based, multi-agency monitoring and surveillance system has been designed to educate the public and to reduce its morbidity and mortality. Development of a vaccine against WNV in horses marks the first commercially available immunogen against a flavivirus and has triggered and intense biomedical research activity with the ultimate goal of developing vaccines to protect humans and other domestic animals.

The study in a limited way confirms the manufacturer's claim that the equine WNV vaccine is able to stimulate the production of antibodies when used according to the recommended protocol.

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