THE EPIDEMIOLOGICAL RISK ASSESSMENT OF WEST NILE VIRUS IN NEW YORK STATE

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Epidemiology is a holistic investigation of factors affecting the health and illness of populations. The goal of this study was to assess different risk factors related to West Nile virus (WNV) in New York State (NYS). WNV first appeared here in 1999 and is now considered endemic in NYS. The factors that play a role in the perpetuation of the virus are not fully understood. We studied a known host, a possible vector, and two other possible hosts. Our study encompassed the risk factors of crows in Tompkins County, the role of non-*Culex* species mosquitoes in Tompkins, Nassau, and Suffolk Counties, the possible component of dairy cattle in the New York City Watershed (Delaware County), and the element of mesopredators in Nassau and Suffolk Counties.

BIOGRAPHICAL SKETCH

Christine is a native of California. Her education within the California State University system prepared her for the work done at Cornell. This included a Master of Science degree at Cal Poly Pomona in equine reproductive immunology and a Bachelor of Arts degree at California State University, Northridge, in women's studies. The rigor of the work here primed her to be offered a policy fellowship with the California Council on Science and Technology in Sacramento.

I dedicate this to my wonderful husband, Steven Gabriel Meza:

Your support made this happen.

And also to the memory of Mrs. Nancy Streeter, my high school English teacher:

Your belief and preparation also made this happen.

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LIST OF ABBREVIATIONS

C	Celsius
CI	confidence interval
F	Fahrenheit
G	acceleration of gravity
MIR	minimum infection rate
mL	milliliter
NAb	neutralizing antibody
NYCW	New York City Watershed
NYS	New York State
PCR	polymerase chain reaction
pfu	plaque forming unit
PRNT	plaque reduction neutralization test
RT-PCR	reverse transcription-polymerase chain reaction
US	United States
WNV	West Nile virus
µL	micro liter

PREFACE

West Nile virus (WNV) is transmitted mainly between avian hosts in enzootic cycles by a mosquito vector. The virus has significant disease effects on humans and equines when it bridges into an epizootic cycle. Since the initial epidemic of WNV in 1999, perennial outbreaks in New York State suggest the local establishment of natural foci with perpetuation of the virus among susceptible hosts rather than reintroduction of the virus. The factors that play a role in the perpetuation of the virus are not fully understood. American crows (*Corvus brachyrhynchos*) are known to be highly susceptible to infection with the virus. We investigate the factors that put crows at risk of infection in Tompkins County, New York, during the period of 2000 through 2008 in a case-control study. Cases were crow carcasses that were found dead and tested positive for WNV using real-time reverse transcription (RT-PCR) or VecTest®. Data on putative risk factors were collected and assessed for significance of association with the presence of WNV using logistic regression analysis to evaluate the significance of each factor while simultaneously controlling for the effect of others. The risk of a crow carcass testing WNV-positive varied with age, season of the year, and ecological area where the carcass was found. Crows that were more than one year old were 4 times more likely to be WNV positive in comparison to birds that were less than one year of age. It was three times more likely to find WNV-positive carcasses in urban areas in comparison to rural areas. The risk of testing WNV-positive did not vary by sex of the crow carcass.

Aedes (Finlaya) japonicus japonicus (Theobald) (*Ae. japonicus*) mosquitoes have the potential to be a vector of at least three kinds of encephalitis, including WNV. *Ae. japonicus* has successfully established itself in New York. Detection of WNV in pools of this field collected invasive species, combined with their ability to feed on humans,

make these mosquitoes of public health significance. In 2008 we baited traps to collect *Ae. japonicus* mosquitoes and test them for WNV. We utilized a targeted approach and set up traps at known sites where crows had tested positive for WNV. In 2009 we used a similar approach in Nassau and Suffolk County. Tompkins County did have WNV-positive *Ae. japonicus* pools. The minimum infection rate (MIR) was 25.91. There were no positive pools found in Nassau or Suffolk County. There was a greater likelihood to have a positive pool in the fall versus the summer. There was also a greater likelihood to have a positive pool in a rural rather than a suburban neighborhood.

Additionally, this research project investigated whether dairy farms have the potential to serve as sites where WNV has evolved to become an endemic disease in the state of New York. It was carried out on farms within the New York City Watershed (NYCW) where we have a well-established research program collaborating with local dairy farmers. An additional focus of this research centered on endemic locations with human cases. This was executed in areas within New York State where we have a disease control and research program that includes collaboration with local stakeholders and data collection examining the risk of zoonotic diseases that are associated with wildlife in Nassau and Suffolk counties on Long Island. None of the dairy cattle tested positive for WNV in the NYCW. On Long Island, 50% of the raccoons were seropositive for WNV. Risk factors for these mesopredators included age, sex, and season.

CHAPTER 1:

INTRODUCTION TO WEST NILE VIRUS

Origins

West Nile virus (WNV) was originally isolated in 1937 from the blood of a sick patient in the West Nile District of Uganda (Blitvich, 2008; Komar, 2003). Over the next several decades, the virus spread from Africa, to Europe, Asia, and Australia. Since 1994, the virus has caused frequent outbreaks of severe encephalitic disease in humans and horses in Europe, and is an example of a zoonotic pathogen that has caused disease in humans and other vertebrates.

Induction in the Americas

In 1999, WNV was discovered for the first time in the United States (US) in New York City (NYC). WNV reached Southern California in 2003 and Northern California in 2004. WNV has dispersed across the North American continent. It is now found throughout the Americas. WNV is the leading cause of human arboviral encephalitis in the US (Kramer *et al.*, 2007, Trevejo & Eidson, 2008).

The prototype WNV in North America, the NY strain (NY99), is similar to WNV isolates from Israel obtained from dead Israeli geese in 1998, suggesting that WNV was introduced to New York from the Middle East. The method of introduction is unknown.

Avian

In North America, a wide range of bird species has been infected with WNV. It is the passerines (i.e., songbirds) that are important reservoirs (Trevejo & Eidson, 2008). A hallmark of WNV epidemics in North America has been the large number dead birds found from the Corvidae family, especially American crows (*Corvus brachyrhynchos*)

(Nielsen & Reisen, 2007). In North America, the NY99 strain of WNV was originally isolated from an American crow (Komar, 2003). Because of their high mortality and proximity to humans, American crows have been used as an indicator of WNV transmission. These birds are competent amplifying hosts able to infect blood-feeding mosquitoes efficiently. Crows come together each night at communal roosts and provide a concentrated source of blood for the host-seeking mosquitoes that feed at dusk (Brault *et al.*, 2004, Nielsen & Reisen, 2007). It is possible that crows can transmit the virus to other crows, even though this transmission has been proven only under experimental conditions but not in natural settings. Although residential housing areas with large crow populations have experienced higher incidences of mosquito and human infections, wild birds are considered the principal hosts of WNV. (Eidson *et al.*, 2001, Nielsen & Reisen, 2007). Mosquitoes, particularly the *Culex* and *Aedes* species, are the primary vectors.

Pathogenesis

Virions are assembled in the lumen of the endoplasmic reticulum and then transported within vesicles to the cell surface, where they are released by exocytosis. The pathogenesis of WNV is similar to that of other flaviviruses (Samuel & Diamond, 2006). Following peripheral inoculation, initial WNV replication is believed to occur in the Langerhans dendritic cells of the skin. The virus then spreads to the lymph nodes and bloodstream, followed by peripheral tissues such as the spleen and kidney. The virus may then penetrate the central nervous system (CNS), resulting in inflammation of the medulla, brain stem, and spinal cord (Guarner *et al.*, 2004, Kleinschmidt-DeMasters *et al.*, 2004, Samuel & Diamond, 2006).

Prevalence in the United States

WNV has been responsible for more than 27,000 human cases, over 25,000 equine cases, and hundreds of thousands of avian deaths in the US, with incidences of human and horse infection usually peaking in late summer and early fall (Roehrig *et al.*, 2002). WNV has been detected in over 30 species of animals (Blitvich, 2008). Tree squirrels are susceptible to WNV-associated neurologic disease at an infection rate comparable to that found in dead birds. Positive species include the fox squirrel (*Sciurus niger*), the Western gray squirrel (*Sciurus griseus*), and the Eastern gray squirrel (*Sciurus carolinensis*). Between 2004 and 2006, these three species of squirrels were found dead by the public and tested positive for WNV with a prevalence mirroring that of dead birds for each of the following years: 2004 = 64.5% (56.4% birds), 2005 = 32.2% (32.9% birds), 2006 = 23.2% (24.2% birds).

Tree squirrels in particular may be highly susceptible to WNV neurologic disease (Padgett *et al.*, 2007). Of 36 living tree squirrels tested in California, 25% had a detectable viremia, and 3 (8%) had titers high enough to infect mosquitoes. Three different tree squirrel species were found to be susceptible to WNV throughout California. Testing dead squirrels has been implemented as part of the WNV surveillance strategy in California. Concern has arisen that if these squirrels can serve as reservoir hosts, they might infect mammalophagic mosquito species, a possibility that could markedly increase risk of transmission to humans and horses.

Golden hamsters, Eastern cottontail rabbits, Eastern chipmunks, and fox squirrels can develop a level of WNV sufficient to infect mosquitoes (Tesh *et al.*, 2005, Tonry *et al.*, 2005, Root *et al.*, 2006, Platt *et al.*, 2008). Some species of reptiles can contribute to the WNV amplification cycle (Blitvich, 2008). American alligators can develop a

viremia that exceeds 10^5 pfu ml^{-1} (Klenk *et al.*, 2004). Reptiles might be potential amplifying hosts because they are known to develop a viremia of long duration that can last over the winter.

Symptoms

Humans

In humans, WNV can be asymptomatic, can appear as a mild febrile illness, or can be fatal. The majority of WNV infections in humans are asymptomatic, but during recent outbreaks in Europe, Israel, and the US, approximately 20% of infections have resulted in a mild flu-like illness termed West Nile fever (WNF) (Hayes *et al.*, 2005, Davis *et al.*, 2006). Symptoms include an abrupt onset of fever, headache, myalgia, nausea, fatigue, weakness, vomiting, and diarrhea (Davis et al., 2006, Sejvar & Marfin, 2006, Sejvar, 2007), which develop 2 to 14 days after virus infection. The illness typically lasts 2 to 5 days but in some cases can persist for over a month. Approximately one in 150 WNV infections in humans leads to severe neuroinvasive disease (WNND) characterized by encephalitis. The fatality rate in patients with WNND is approximately 10%. Long-term neurological sequelae occur in more than 50% of WNND patients. WNND is common in elderly and immunocompromised patients and is rarely reported in patients less than 30 years of age.

Horses

In horses, signs of WNV infection include depression, abnormal gait, ataxia (primarily hind limb), muscle tremors, knuckling over, and recumbency (Trevejo & Eidson, 2008). While treatment for horses includes anti-inflammatory drugs, vitamins, and fluids, prevention and environmental control of vectors (mosquitoes) and vaccination are recommended.

A transient, low-level viremia has been documented in naturally and experimentally infected horses and donkeys (Snook *et al.*, 2001), a level thought to be insufficient to infect mosquitoes. It is unlikely that a horse infected with WNV would transmit the virus to humans or to other horses under normal circumstances. Of all horses exposed to WNV, a 10 to 12% morbidity and mortality rate has been seen in horses in Europe, Israel, and the US (Blitvich, 2008). In clinically ill horses, the mortality rate in the US is approximately 1/3 for horses with WNV, yet vaccination can reduce the risk of death by 44%. Clinical cases of WNV in horses do not usually precede human cases in the same area.

Birds

In birds, WNV is characterized by various neurologic signs involving ataxia, paralysis, and incoordination (Blitvich, 2008) as well as non-neurologic signs such as depression, lethargy, ruffled feathers, weight loss, and myocarditis. Crows, jays, and other members of the family Corvidae suffer extremely high mortality rates following WNV infection. Peak viremia for American crows is $10^{9.4}$ pfu ml^{-1}. The American crow population in the US has declined by an estimated 45% since the introduction of WNV in 1999 (LaDeau *et al.*, 2007, Blitvich, 2008).

Transmission

WNV in the Western Hemisphere is maintained in a bird–mosquito transmission cycle (Blitvich, 2008). Disease associated with WNV infections has been primarily recognized in the US in birds, humans, and horses (Salazar *et al.*, 2004). Birds are the primary reservoir host for WNV. The principal vectors are *Culex* species mosquitoes that are responsible for transmission to birds, as well as from birds to incidental hosts such as humans and horses. The virus does not amplify in infected horses and humans

sufficiently to infect mosquitoes and allow spreading to other susceptible hosts. Equines generate a viremia level of $10^{3.5}$ pfu ml^{-1}, yet the viremia level needed for transmission is 10^5 pfu ml^{-1} (Turell *et al.*, 2000, Sardelis *et al.*, 2001, Blitvich, 2008). Humans, horses, and most other mammals are therefore considered to be incidental or "dead-end" hosts because they do not produce significant viremia and thus do not contribute to the transmission cycle.

WNV epidemics can occur in both rural and urban areas (Dauphin *et al.*, 2004). *Culex* is one of the most efficient genera for spreading the virus among birds and from birds to humans and other mammals. Birds will sustain an infectious viremia for 1 to 4 days after exposure. If they survive, they have lifelong passive immunity. WNV, like many arboviruses, has two distinct transmission cycles:

1) A primary enzootic or amplification cycle involving one set of vectors and avian hosts.
2) Secondary cycles involving potentially different arthropods and transmission to other hosts such as humans and horses (Turell *et al.*, 2001).

Opportunistic feeders are bridge vectors that feed on infected birds and then transmit the virus to a susceptible vertebrate host.

Although WNV is primarily transmitted to vertebrates by an arthropod vector, various non-vector-borne modes of transmission have been documented. WNV has been transmitted to humans as a result of organ transplantation, blood transfusion, breastfeeding, intrauterine transmission, and needle-stick injury. Oral transmission of

WNV has been reported for the American crow, common grackle, house finch, and house sparrow. Direct transmission has been documented in the American crow, blue jay, magpie, and gull. American crows can shed over $10^{8.8}$ pfu ml^{-1} in their feces, which suggests that exposure to contaminated fecal matter is a potential source of WNV transmission (Kipp *et al.*, 2006). Furthermore, WNV-infected horse meat was implicated as the source of a WNV outbreak at alligator farms in Georgia (Miller *et al.*, 2003). Alligators can become infected via direct contact with infected tank-mates (Klenk *et al.*, 2004), and cats have become infected via ingestion of infected mice (Austgen *et al.*, 2004).

The shedding of WNV infection by infected Eastern chipmunks and tree squirrels suggests the potential for zoonotic transmission (Padgett *et al.*, 2007, Platt *et al.*, 2007). Infection with WNV via the oral route has been reported in golden hamsters, alligators, cats, and raptors (Miller *et al.*, 2003, Austgen *et al.*, 2004, Klenk *et al.*, 2004, Sbrana *et al.*, 2005, Nemeth *et al.*, 2006, Trevejo & Eidson, 2008). In the wild, fecal shedding of WNV was detected among birds in New York State (NYS) during the winter, suggesting lateral transmission (Dawson *et al.*, 2007, Trevejo & Eidson, 2008). Finally, a 2002 outbreak of WNV among turkey-farm workers may have resulted from aerosol exposure (CDC, 2003).

Environment

Environmental factors, particularly temperature and rainfall, have long been known to influence the transmission cycles of arboviruses. Mosquitoes are more efficient vectors of flaviviruses when temperatures are above average, from April to June, particularly when combined with rainfall (Epstein, 2001). The 2002–2004 US outbreaks took place during periods of above-average summer temperatures (Reisen *et*

al., 2006). Drought, which brings avian hosts and mosquito vectors into close contact, can also increase WNV transmission (Shaman *et al.*, 2005).

Risk Factors

In humans, advanced age is the greatest risk factor for severe neurologic disease and death from WNV (Huhn *et al.*, 2003). Persons over the age of 50 are twenty times more likely to contract the disease.

The risk of WNV infection rises with an increase in the length of time spent outdoors, failure to apply mosquito repellent, inhabiting a building with a flooded basement, or the presence of mosquitoes in the home.

In a New York study of the original strain of NY99 virus, a horse's age was found to have no effect on the risk of contracting WNV (Trock *et al.*, 2001). However, in Texas in 2002, age was indeed a risk factor for horses (Ward, 2006). Such a risk factor for equines may have changed with the new, WN02 strain of WNV.

Outbreak patterns in the New World have not mirrored that in the Old World (Rappole *et al.*, 2006). The pattern of WNV outbreaks that has emerged over the years since the first Western Hemisphere appearance in 1999 suggests over-winter persistence of the virus (Nasci *et al.*, 2001, Rappole *et al.*, 2006). The ability of WNV to persist over the winter has reinitiated the enzootic transmission of the disease every year in the Northeastern US and may be responsible for its endemic status in NYS. Successfully predicting the distribution of arboviruses or the intensity with which they will be transmitted in a particular environment depends on developing in-depth knowledge of the relevant arbovirus transmission cycle (Ebel *et al.*, 2005).

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CHAPTER 2:

FACTORS ASSOCIATED WITH THE RISK OF WEST NILE VIRUS AMONG CROWS IN NEW YORK STATE[*]

Abstract

West Nile virus (WNV) is transmitted between avian hosts in enzootic cycles by a mosquito vector. The virus has significant disease effects on humans and equines when it bridges into an epizootic cycle. Since the initial epidemic of WNV in 1999, perennial outbreaks in New York State suggest the local establishment of natural foci with perpetuation of the virus among susceptible hosts rather than reintroduction of the virus. The factors that play a role in the perpetuation of the virus are not fully understood. American crows (*Corvus brachyrhynchos*) are known to be highly susceptible to infection with the virus. We investigated the factors that put crows at risk of infection in Tompkins County, New York, during the period of 2000 through 2008 in a case-control study. Cases were crow carcasses that were found dead and tested positive for WNV using real-time reverse transcription (RT-PCR) or VecTest®. Data on putative risk factors were collected and assessed for significance of association with the presence of WNV using logistic regression analysis to evaluate the significance of each factor while simultaneously controlling for the effect of

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others. The risk of a crow carcass testing WNV-positive varied with age, season of the year, and ecological area where the carcass was found. Crows that were more than one year old were four times more likely to be WNV-positive in comparison to birds that were less than one year of age. It was three times more likely to find WNV-positive carcasses in urban areas in comparison to rural areas. The risk of testing WNV-positive did not vary by sex of the crow carcass.

Keywords

West Nile virus, epidemiology, case-control, American crow, risk factors

Introduction

West Nile virus (WNV) has been of public health concern in North America since it first appeared in the northeastern region of the United States (US) in 1999 (Komar *et al.*, 2003). The emergence of this zoonotic pathogen poses a great challenge because of its complex epidemiologic characteristics. WNV infects multiple host species, and this ability to infect and cause viremia in a wide variety of host species coupled with the involvement of multiple arthropod vectors in the transmission of the disease may have hindered efforts to control the spread of the disease (Bowen & Nemeth, 2007, Andreadis *et al.*, 2004, Anderson *et al.*, 2006). The adaptation of a generalist strategy (i.e., WNV capacity to infect multiple hosts) is a likely process by which the pathogen persists in the environment, and could be affected by a combination of agent, host, and environmental factors.

The endemicity of WNV in the northeastern US is believed to be sustained by an urban cycle of transmission involving birds as reservoir hosts and mosquito species as vectors (Brown *et al.*, 2008, Brault, 2009). There is a wide range of susceptibilities of

WNV infection among birds, and corvids (particularly American crows [*Corvus brachyrhynchos*]) have the highest mortality rates among North American birds (Salazar *et al.*, 2004). WNV-infected crows develop high viral titers in blood and other tissues (Komar et al., 2003). Because of their high mortality and proximity to humans, American crows have been used as an indicator or sentinel for WNV activity (Eidson *et al.*, 2001, McLean *et al.*, 2001). Crows have been useful as sentinels because they are easier to spot than smaller birds. As the study of factors affecting the health and illness of populations, epidemiology can serve as the foundation of interventions made in the interest of public health and preventive medicine.

Transmission factors that exacerbate the risk of WNV-associated disease in humans in the Northeast remain a focus of investigation. Tompkins County in New York State has been the center of a long-term study of crow behavior and ecology (McGowan, 1998, McGowan, 2001, Clark *et al.*, 2006). Looking at cases of crow mortality within the county could lead to identifying potential risk factors associated with WNV that strengthen disease surveillance and efforts to control future outbreaks. Our knowledge of the ecology of the disease remains limited. Therefore, epidemiologic studies focusing on ecological factors that play a role in perpetuating the risk of WNV in ecological niches will contribute to our understanding of the disease and the implementation of cost-effective mitigation strategies.

Material and Methods

Study concept—A case-control study was undertaken to identify factors that put crows at risk of WNV infection and that might enhance or detract from the perpetuation of the virus among crows within an ecological niche.

Target and study populations—The target population consisted of sick and dying American crows in Tompkins County in central New York State during the period of January 2000 through December 2008. Crows were collected and submitted to either the Animal Health Diagnostic Laboratory (AHDL) at Cornell University or the New York State Department of Health Wadsworth Laboratory (NYSDH) to be tested for WNV.

Definition of a case: Cases consisted of dead birds that tested positive for WNV by VecTest® (Medical Analysis Systems, Inc., Fremont, CA) or real-time reverse-transcriptase polymerase chain reaction (RT-PCR) method targeting the envelope (E) coding region of the gene. Birds that tested positive by RT-PCR in the initial assay were confirmed using standard RT-PCR targeting the nucleocapsid region (Lanciotti *et al.*, 2000, Shi *et al.*, 2001).

Definition of controls: Controls were defined as all crow carcasses within the study areas that tested negative for WNV by real-time RT-PCR.

Virus detections

5´ Nuclease real-time RT-PCR—The real-time and standard RT-PCR assays were run as previously described with some cycling modifications (Lanciotti et al., 2000, Shi et al., 2001). The SmartCycler (Cepheid, Sunnyvale, CA) thermal cycling conditions consisted of 42ºC for 15 minutes, 95Cº for 10 minutes, and 36 cycles of 95ºC for 15 seconds, 50ºC for 10 seconds, and 60ºC for 100 seconds.

Standard RT-PCR—The Perkin Elmer Cetus (PerkinElmer, Waltham, MA) thermal cycling conditions consisted of 50ºC for 30 min, 95ºC for 15 min, and 50 cycles of 95ºC for 45 sec, 62ºC for 45 sec, and 70ºC for 1 min.

Table 2.1. Crow carcasses found in various types of land cover in Tompkins County, New York, 2000–2008

Land Cover Classification (NLCD 2001)	Number of Cases	Number of Controls	Odds Ratio (95% Confidence Interval)
Type of development			
Low, medium intensity	31	79	
No development	10	61	2.4 (1.1, 5.3)
Development/Type of intensity			
Low intensity	13	42	
Medium intensity	18	37	0.63 (0.3, 1.5)
No development/Land coverage			
Deciduous, evergreen, forest	1	15	
Pasture hay, cultivated crops	9	46	0.34 (0.03, 2.91)

Note. 20 cases and 27 controls were not included because of missing coordinates

Risk factors—Data collected for each crow carcass included date of carcass recovery, host factors (age and sex), and ecological factors (development and habitat). Sex determination was based on necropsy, and age was a two-tier aging classification based on tail shape (Clark *et al.*, 1991, Yaremych *et al.*, 2004a). The age of the bird was classified as adult if older than one year and as juvenile if less than one year. The location where the bird was found was recorded as GPS coordinates. This location was later used in the Arc Manifold 8.3 (Manifold Net, Ltd, Carson City, NV) to be translated into land cover classification using National Land Cover Data 2001 (Table 2.1). These ecological factors included intensity of development (no development, low, and medium), land coverage (forest, deciduous, and evergreen), and type of

pasture (hay, cultivated crops). Medium density refers to apartments, townhomes, and condominiums and two-story single family homes on small lots. Low density refers to single-family homes on at least a half-acre of land. The geographical locations of the cases and control birds were then grouped into rural (no development) and residential areas (low and medium density), and were analyzed according to location and population density.

Statistical Analysis—All putative risk factors were screened initially for their significance of association with the likelihood of cases using the chi-square test (all the factors were categorical). If the expected number of observations per a specific level of the putative factor and disease status was less than five, a Fisher Exact test was used to evaluate the significance of association. Factors that were significant in this bivariate analysis were considered further in a multivariate analysis using the logistic regression approach to assess the effect of each factor while simultaneously controlling for the effect of other factors. In the multivariate analysis, season was grouped as either summer/spring or fall/winter. The magnitude of risk or the strength of the association was quantified using the odds ratio (odds of being a case if the animal had the factors). Confidence interval estimates were computed at an α of 0.05. The statistical analysis was performed using Egret, Cytel Statistical Software, Cytel Software Corporation, Cambridge, MA.

Results

A total of 228 crow carcasses (61 cases and 167 controls) met the inclusion criteria and were included in the analysis. Figure 2.1 is an ecological map showing the location where WNV-positive crow carcasses were found. We were unable to pinpoint

the coordinates for 20 cases and 27 control crow carcasses; hence, they were omitted from further analysis.

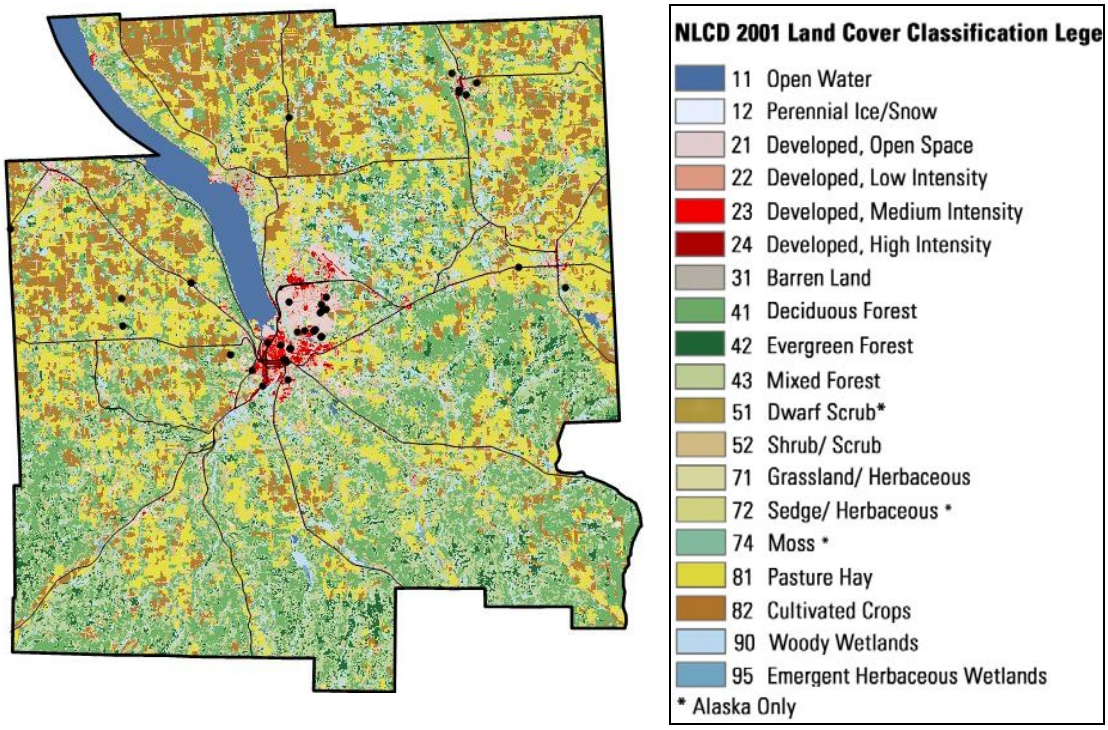

Figure 2.1. National Land Cover Data (NLCD) map of West Nile virus cases (N=61) in Tompkins County, New York (2000–2008). Black dots indicate location of where West Nile virus–positive crow carcasses were found.

Table 2.1 shows the results of the analysis between different land cover types and the likelihood of WNV. A WNV-positive crow was twice as likely to be located in a developed area than in a non-developed area [odds ratio (OR) = 2.4 and 95% CI (1.1, 5.3)] (Table 2.1). We further examined the association between the likelihood of WNV and the intensity of development. Type of development (medium versus low density of human population) was not significantly associated with the likelihood of WNV in dead crows [OR= 0.6 (0.3, 1.5)]. Furthermore, we examined the land coverage within no-development areas. There was no significant association between

pasture or forested land (regardless of tree type) and the likelihood of encountering a WNV case [OR = 0.34 (0.03, 2.91)].

Table 2.2. Risk factors examined for associations with West Nile virus in crow carcasses tested in Tompkins County, New York, 2000–2008

Factor	Number of Cases	Number of Controls	Odds Ratio (95% Confidence Interval)
Age			
< 1 year	24	91	
≥ 1 year	34	46	0.3 (0.2, 0.6)
Season of the year*			
Summer	57	25	1.0
Fall	3	11	0.2 (0.03, 0.5)
Winter	0	54	0.01 (0.001, 0.06)
Spring	1	77	0.01 (0.001, 0.04)
Geographic location (Development)			
Low-medium intensity	31	79	
No development	10	61	2.4 (1.1, 5.3)
Sex			
Male	25	42	
Female	23	47	1.2 (0.6, 2.4)

* Winter = January–April; Spring = May–June; Summer = July–September; Fall = October–December

Table 2.2 shows the results of the bivariate analysis of the putative risk factors that were considered in the analysis. Carcasses of crows that were more than a year old were three times more likely to be WNV-positive than younger crow carcasses [95% CI (0.2, 0.6)]. It was more likely to find dead crows that tested positive for WNV in the summer than any other season of the year (OR for fall was 0.2, for winter was 0.01, and for spring was 0.01). Carcasses that were recovered in low- to medium-intensity development areas were twice as likely to be WNV-positive than crow carcasses that were found in no-development (rural) areas [OR = 2.4, 95% CI (1.1, 5.3)]. There was no association between the likelihood of testing WNV-positive and the sex of the crow carcasses (Table 2.2).

Table 2.3. Results of logistic regression analysis of factors associated with the likelihood of West Nile virus in American crows in Tompkins County, New York, 2000–2008

Factors	Regression Coefficient	Standard Error	Odds Ratio (95% CI Lower)
Age			
< 1 year	0		1.0
≥ 1 Year	1.446	0.437	4.3 (1.8, 10.0)
Season*			
Fall and Winter	0		1.0
Spring and Summer	2.604	0.798	13.5 (2.8, 64.6)
Geographical location			
Low-medium intensity	1.146	04.60	1.0 3.4 (1.3, 7.8)
No development			
Constant	-4.666	0.955	

* Fall and Winter = October–April; Spring and Summer = May–September

The multivariate analysis showed that the risk of WNV infection in crows was associated with crow age and with season and geographic location where the crow was found (Table 2.3). The results show the odds ratios, adjusted for the presence of other variables in the model. Crows that were one year or older were four times more likely to be WNV-positive that crows that were less than one year old (95% CI was 1.8, 10). Crow carcasses that were found during spring and summer seasons were at greater risk of being WNV-positive than carcasses that were discovered during fall or winter seasons (OR = 13.4; 95% CI 2.8, 64.6). Crow carcasses that were located in low-medium development (residential) areas were three times more likely to be WNV-positive than carcasses that were located in rural areas (95% CI was 1.3, 7.8). The likelihood that a crow carcass was positive for WNV was not influenced by the sex of the bird.

Discussion

We carried out a case-control epidemiologic study to address our stated objectives. The case definition included crows that were found dead and submitted to the AHDL at Cornell for WNV testing (Lanciotti *et al.*, 2000, Shi *et al.*, 2001). Although these birds had been submitted to the AHDL through an active surveillance program that included media awareness and bird-watcher solicitation, there is a chance that we missed some of the birds that were scavenged by wild animals. Furthermore, while these crows are non-migratory, it is difficult to know whether crows moved from where they were infected to other sites before death. There is also a possible bias to the location of where the birds were found as it is more likely for birds to cluster in urban areas and also for their carcasses to be found because of their size and because of increased foot traffic.

By virtue of their design, data on putative risk factors in case-control studies are collected retrospectively; hence, there is a potential for confounding effects among these factors (Schlesselman, 1982). We controlled for the potential confounding effect among the hypothesized risk factors by developing a systematic approach to data analysis. First, we screened variables for potential association with crow mortality from WNV, and second, we considered only risk factors that were significant in the multivariate analysis. Variables that were considered to have a biological role in the risk of mortality but were not significant in the bivariate analysis were also included in the multivariate analysis.

Since WNV was first introduced to the US in 1999, season, and specifically summer, has been the greatest risk factor for crow morbidity and mortality (CDC, 2000, Caffrey *et al.*, 2005). The most obvious cause is that vector abundance and activity is greatest

during the summer season in temperate latitudes, such as much of the US (Andreadis *et al.*, 2004). Therefore, the finding of crow carcasses corresponding to this time of known mosquito activity is not surprising.

The increased risk of WNV exposure linked to older age biologically makes sense since the first year of life for crows has many risk factors for death beyond disease exposure (McGowan & Caffrey, 1994, Ludwig *et al.*, 2010). The greater exposure risk in residential areas supports previously published data. Urbanization is one of the ecological risk factors associated with WNV transmission in the northeastern United States (Brown *et al.*, 2008). This may be due in part to the fact that crows, like the pigeon and the gull, are adapted to human habits and therefore often select peri-domestic habitats (Yaremych *et al.*, 2004b). A distinct feature of WNV in North America has been large numbers of corvids dying in peri-urban areas (Nielsen & Reisen, 2007). One outcome of high densities of crows and some other birds in peri-urban versus rural habitats is the increased likelihood of being found by the public and submitted for testing.

In our study, carcasses of younger crows were less likely to test positive for WNV. This does not align with the findings of one study that had been previously observed and published (Clark *et al.*, 2006). However, our study differs in several ways. First, our study was of longer duration. Second, ours is a case-control study, while the Clark *et al*., 2006 study was population-based. Furthermore, our controls and cases were gathered concurrently. A recent paper regarding a 2005 WNV study of crows in Quebec, Canada, found that younger crow carcasses were less likely to be WNV-positive (Ludwig *et al.*, 2010). One hypothesis for higher WNV-positive results in older animals is simply a greater likelihood of being exposed or infected over time.

The observed association between residence, pasture, and forest is that birds were more likely to be found along major roads in Tompkins County. There was no significance of association between the classes of ecological niches, possibly because the areas are not mutually exclusive between either the crows inhabiting the niche or the vector species co-inhabiting the same area.

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CHAPTER 3:

POTENTIAL VECTOR FOR WEST NILE VIRUS AMONG INVASIVE MOSQUITO SPECIES IN NEW YORK*

Abstract

Identifying the spectrum of vectors that play a role in perpetuating West Nile virus (WNV) infection in endemic foci will help in controlling the spread of the disease. *Aedes (Finlaya) japonicus japonicus (Theobald)* (*Ae. japonicus*) mosquitoes have the potential to be a vector of at least three kinds of encephalitis, including WNV. *Ae. japonicus* has successfully established itself in New York State. Detection of WNV in pools of this field-collected invasive species, combined with their ability to feed on humans, make these mosquitoes of public health significance. In 2008 we baited traps to collect *Ae. japonicus* mosquitoes and test them for WNV. We used a targeted sampling approach and set up traps at known sites in Tompkins County where crows had tested positive for WNV. In 2009 we used a similar approach in Nassau and Suffolk County. We found WNV-positive *Ae. japonicus* pools in Tompkins County. The minimum infection rate (MIR) was 25.91. We found that positive pools were more likely in the fall than in the summer, and that positive pools were more likely to be found in rural than in suburban neighborhoods. We found no positive pools in Nassau or Suffolk County.

Keywords

Aedes japonicus japonicus, West Nile virus, zoonosis, arbovirus, bridge vector

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Introduction

Since the initial epidemic of West Nile virus (WNV) in 1999, the presence of perennial outbreaks in New York State (NYS) suggests the local establishment of natural foci as a result of perpetuation of the virus among susceptible hosts rather than reintroduction of the virus. The factors that play a role in its perpetuation, including the spectrum of hosts and vectors, are not fully understood. Although species of *Culex* mosquitoes are identified as the most important vectors, WNV has been detected in more than 60 species of mosquitoes worldwide (Blitvich, 2008). Non-indigenous vectors that arrive, establish, and spread in new areas have instigated epidemics of human diseases (Lounibos, 2002). *Aedes japonicus japonicus* (*Ae. japonicus*) is a recent and widespread invasive mosquito species and has become established in the United States (US) (Armistead *et al.*, 2008). *Ae. japonicus* has some characteristics of a bridge vector for WNV because of its vector competence, ability to feed on mammals and birds, and potential abundance near sites of known WNV transmission (Morris *et al.*, 2007, Molaei *et al.*, 2009). WNV has been detected in field-collected *Ae. japonicus* from at least nine different states, including NYS (CDC, 2009).

According to the State of New York Department of Health West Nile Surveillance Summary, WNV has become endemic in NYS since its first outbreak in 2000 (http://www.health.state.ny.us, 2008). Several factors may play a role in perpetuating the endemicity of the virus within these areas from which the vector could not be ignored. From 2000 to 2008 our lab investigated the factors that put crows at risk of infection in Tompkins County, New York (DeCarlo *et al.*, 2010). In 2008 we used a targeted approach and set up mosquito traps at known sites where crows that tested positive for WNV had been found (see Figure 3.1) in the hope of understanding the

dynamic of the disease in these endemic foci. The effort in the current paper was focused on the potential vectors to this possibly debilitating disease in these foci.

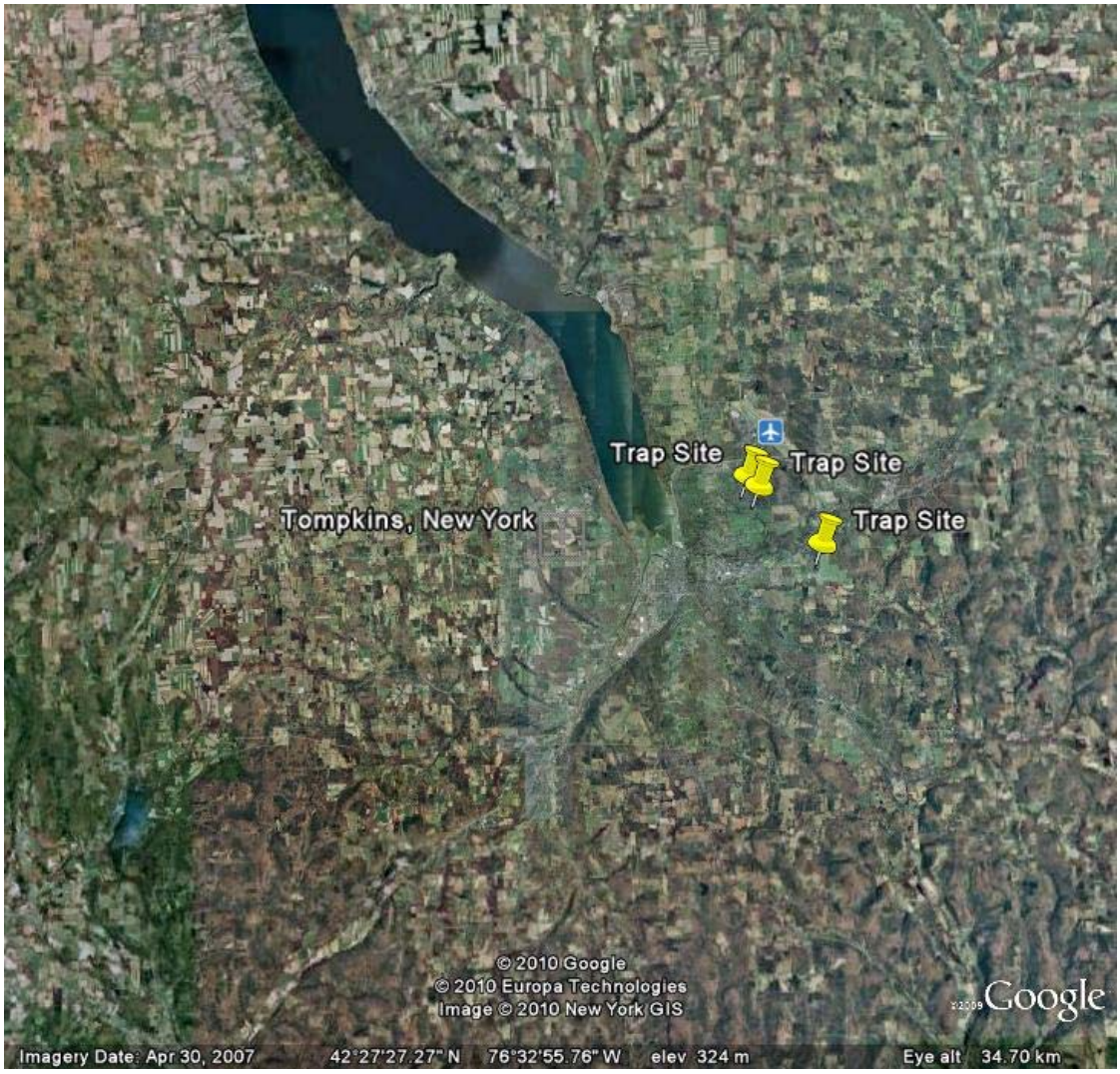

Figure 3.1. Mosquito trap sites in Tompkins County, New York.

The objective of this study was to investigate the potential spectrum of invasive mosquito vectors in two endemic foci in NYS and to shed light on the factors among them that might have led to the likelihood of testing positive for WNV. *Ae. japonicus* was first reported in the US in 1998 from collections in September and October made in Suffolk County, Long Island, New York (Peyton *et al.*, 1999). The species

continued to be a problem on Long Island through 2008 (NYSDOH, 2010). In 2009, in collaboration with the Nassau and Suffolk County health departments, we used a targeted sampling approach to investigate the infection rate among mosquitoes in these endemic foci (see Figure 3.2 and Figure 3.3).

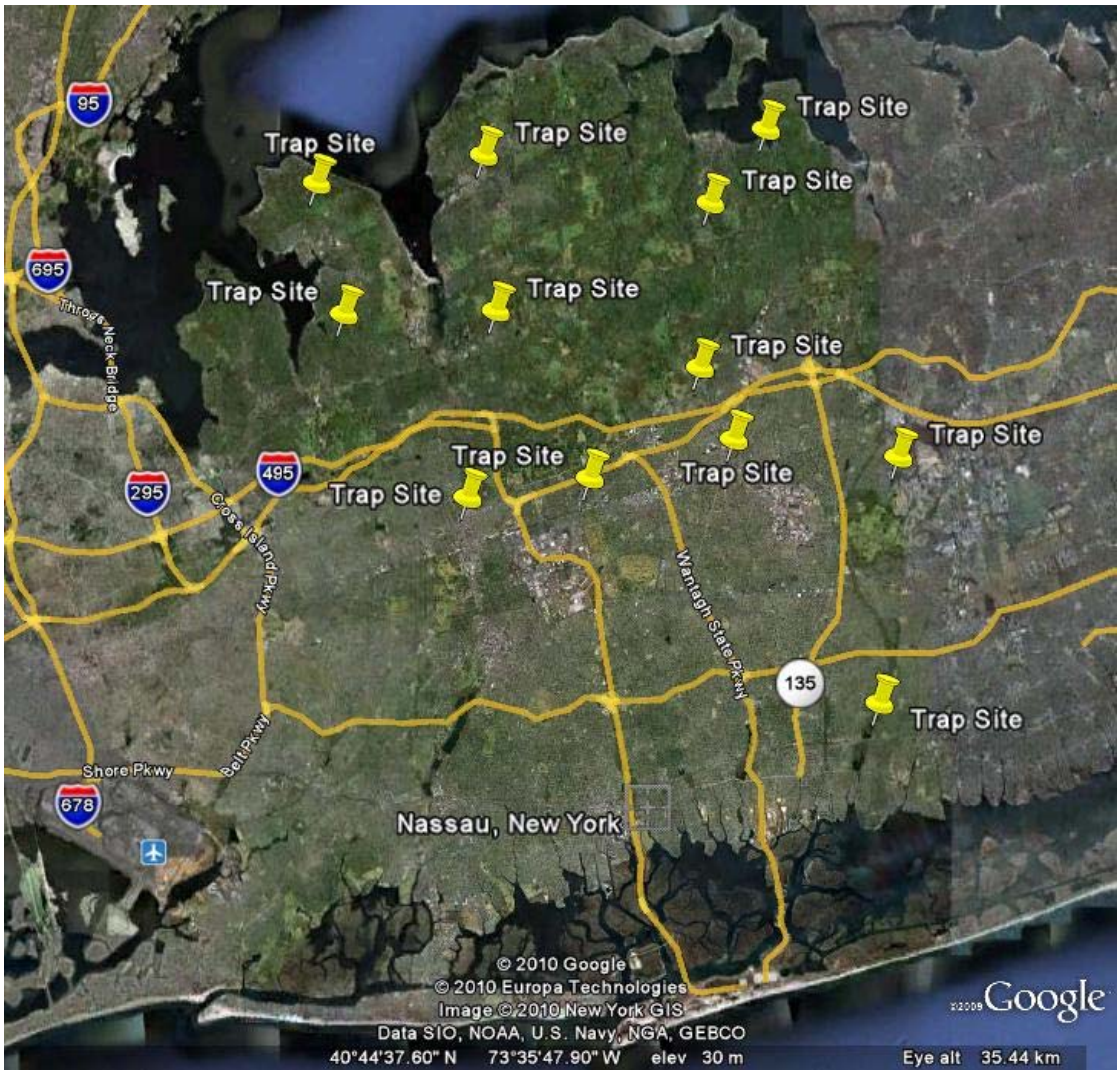

Figure 3.2. Mosquito trap sites in Nassau County, New York.

Figure 3.3. Trap sites in Suffolk County, New York.

Material and Methods

***Mosquito Collection*—**Gravid traps were used to capture ovipositing *Ae. japonicus* females in Tompkins County, New York (BioQuip Products, Inc., Rancho Dominguez, CA). Traps were set from July 10, 2008, through October 10, 2008, for two trap days per week at 3 sites. CDC light traps baited with dry ice and gravid traps were used to capture host-seeking and ovipositing *Ae. japonicus* females in Nassau County, New York. Traps were set from July 22, 2009, through September 30, 2009,

for three trap days per week at 11 sites. CDC light traps baited with dry ice, BG-Sentinel™ traps, and gravid traps were used to capture host-seeking and ovipositing *Ae. japonicus* females in Suffolk County, New York. Traps were set from August 25, 2009, through September 29, 2009, for two trap days per week in August and one trap day per week in September at 5 sites.

Mosquitoes were identified to species using a chill plate (BioQuip Products, Inc., Rancho Dominguez, CA) with the aid of an optical microscope and descriptive keys (Andreadis *et al.*, 2005). All Nassau and Suffolk County mosquitoes were sorted by their respective laboratory personnel and sent on dry ice to Cornell University.

Individual mosquitoes were dissected using sterilized disposable #10 scalpels (BD Biosciences, Franklin Lakes, NJ) and forceps (BioQuip Products, Inc., Rancho Dominguez, CA). Heads and thoraxes were removed by cutting between the second and third leg. Only the heads and thoraxes were tested.

Disruption and homogenization

Disruption of the samples was done in sterile Eppendorf 1.5 mL conical tubes (Eppendorf North America, Hauppauge, NY) using sterile plastic pestles (Life Science Products, Inc, Chestertown, MD). Homogenization was accomplished through the use of Qiashredder according to manufacturer's instructions (Qiagen, Valencia, CA).

RNA

RNA isolation was achieved through the utilization of the RNeasy mini kit according to manufacturer's instructions (Qiagen, Valencia, CA). RNA integrity was tested by a

method developed in Hawaii (Hoffmann et al., 2004) using primer pair 18S417/920c (GenBank).

Virus detections

5´ Nuclease real-time RT-PCR—The TaqMan real-time and standard RT-PCR assays were run using previously established procedures with some cycling modifications (Lanciotti *et al.*, 2000, Shi *et al.*, 2001). The ABI 7500 Fast Real-Time PCR system (Applied Biosystems™ by Life Technologies™, Carlsbad, CA) and AgPath-ID™ One Step RT-PCR kit (Ambion® Applied Biosystems™ by Life Technologies™, Carlsbad, CA) were used with thermal cycling conditions of 1 cycle at 45ºC for 10 minutes, 1 cycle at 95Cº for 10 minutes, and then 36 cycles of 95ºC for 15 seconds, and 60ºC for 45 seconds.

Standard RT-PCR—The Bio-Rad® iCycler® (Bio-Rad Laboratories, Hercules, CA) thermal cycling conditions consisted of 1 cycle at 50ºC for 30 minutes, 1 cycle at 95ºC for 15 minutes, and then 50 cycles of 95ºC for 45 seconds, 62ºC for 45 seconds, and 70ºC for 1 minute.

MIR—The minimum infection rate (MIR) was calculated using the formula (number of positive pools / total specimens tested) x 1000, with the data representing a single species collected over a single time period and from a single geographic area. The tool used for this was PooledInfRate, version 3.0, a Microsoft® Excel™ add-in (Biggerstaff, 2006). This program also includes calculation of a 95% confidence interval (CI), which reflects, in part, the sample sizes used in the calculations. The time period was concurrent with the mosquito-collection time frame, July 10, 2008, through October 10, 2008. The data were stratified by county.

Data analysis—Estimates of the infection rate among mosquitoes (the proportion that tested positive for the presence of the virus out of all the mosquitoes of that species tested) are reported as interval estimates. In situations where no viruses were detected, the 95% confidence interval for zero events was calculated using the formula $p \approx 3 / n$ (Hanley & Lippman-Hand, 1983). The significance of association between the putative factors (season of collection and geographic locality) was evaluated using the chi-square test in Statistix 9.0 (Analytical Software, Tallahassee, FL). The risk of detecting the virus in mosquitoes associated with a particular factor was quantified using the odds ratio as an approximate estimate of the relative risk.

Results

Mosquitoes were trapped in three endemic areas: Tompkins County, Nassau County, and Suffolk County. The data were stratified by county. All the samples were tested in pools. In Tompkins County, the mean pools were 8 *Ae. japonicus* per trap day using the gravid trap. In Nassau County, mean pools were 3 *Ae. japonicus* per trap day using the CDC light trap and 2 per trap day using the gravid trap. In Suffolk County mean pools were 11 *Ae. japonicus* per trap day using the gravid trap, 23 using the CDC light trap, and 58 using the BG-Sentinel™ trap.

Tompkins County—In Tompkins County we trapped 193 *Ae. japonicus* mosquitoes yielding a total of 24 pools that ranged from 1 to 19 mosquitoes per pool. Five pools tested positive for WNV. The MIR was 25.91 (see Table 3.1). Although we trapped and tested other species of mosquitoes in Tompkins County (see Table 3.1), our species of interest was *Ae. japonicus*.

Table 3.1. Minimum infection rate (MIR) for West Nile virus among mosquitoes collected from different geographical locations in New York State

	Tompkins County (TC)	Nassau County (NC)	Suffolk County (SC)
Species	**MIR (95% Confidence Interval)**	**MIR (95% Confidence Interval)**	**MIR (95% Confidence Interval)**
Ae. japonicus N = 193 (TC) N = 174 (NC) N = 1437 (SC)	25.9 (3.5–48.3)	0.0 (0.0–1.7)	0.0 (0.0–0.2)
Culex spp. N = 59	33.9 (0.0–80.1)		
Ae. triseriatus N = 79	0.0 (0.0–3.8)		
Anopheles punctipennis N = 2	0.0 (0.0–150)		

There was a significant association between the season of the year and the likelihood of the virus in the *Ae. japonicus* mosquitoes. There was a greater likelihood (10 times greater) of having a positive pool in the fall than in the summer (OR = 0.1). Most of the mosquitoes that tested positive were from rural areas. Positive pools were also twice more likely to be found in a rural than in a suburban neighborhood (see Table 3.2). The only *Culex* species (*spp*.) in Tompkins County that we trapped that were positive were collected in summer, on one day (July 14, 2008), at one locale (suburban); thus no odds ratio was calculated for this species. No other species in Tompkins County that we trapped tested positive for WNV.

Table 3.2. Factors associated with the likelihood of WNV in *Ae. japonicus* mosquitoes trapped in Tompkins County in New York State

Factor	Odds Ratio	95% Confidence Interval
Season		
Summer	0	
Fall	0.1	0.01–0.8
Geographic location		
Rural	0	
Residential	1.6	0.2–12.4

Long Island—The study focused on the invasive species *Ae. japonicus* on Long Island. In Nassau County we trapped 174 *Ae. japonicus* mosquitoes yielding a total of 71 pools with a range of 1 to 8 mosquitoes per pool. None of the mosquito pools tested positive for WNV. The 95% confidence interval for zero events in Nassau County was 0.017 (see Table 3.1). In Suffolk County we trapped 1,437 *Ae. japonicus* mosquitoes yielding a total of 47 pools with a range of 1 to 50 mosquitoes per pool. None of the mosquito pools tested positive for WNV. The 95% confidence interval for zero events in Suffolk County was 0.002 (see Table 3.1).

Discussion

The primary objective of the study was to identify the spectrum of non-*Culex* species. The Department of Health in NYS carries out a routine surveillance for the presence of the virus in *Culex* species trapped in NYS. *Ae. japonicus* has the potential to be an enzootic or epizootic vector of at least three kinds of encephalitis: WNV, eastern equine encephalitis, and St. Louis encephalitis (Sardelis & Turell, 2001, Sardelis *et al.*, 2002, Sardelis *et al.*, 2003). The species could serve as a bridge vector for WNV since it displays an aggressive opportunistic daytime feeding habit, taking blood meals from

avian and mammalian hosts, including humans (Andreadis *et al.*, 2001, Sardelis & Turell, 2001). Analysis of vertebrate blood meal sources for *Ae. japonicus* in New Jersey (NJ) revealed that humans were the second most frequent host, at 36% (Molaei *et al.*, 2009). White-tailed deer in that study was the favorite blood meal. WNV has also been detected in field-collected female mosquitoes in NJ.

A positive sample in our study was indication of infection within the salivary gland. This denotes escape of the virus from the midgut. Results will be lower overall positive rates of infection, but positives indicated that the virus had disseminated within the mosquito. This means there could have been a possibility of transmitting the virus by bite. The lack of any WNV-positive *Ae. japonicus* in either of the counties on Long Island could mean that this species is not a risk factor for WNV in that area. The presence of WNV-positive *Ae. japonicus* in Tompkins County is a reason to continue testing this species in the area. One confounding factor is that the testing in all three counties did not happen in the same year (2008 for Tompkins County versus 2009 for Nassau and Suffolk County on Long Island). However, the greater numbers of *Ae. japonicus* caught in Suffolk County may be attributable to the use of the BG-Sentinel™ trap. This trap was originally designed to capture *Aedes aegypti* species of mosquito; it has recently been shown to be effective in trapping *Aedes albopictus* species of mosquito as well (Krockel *et al.*, 2006, Farajollahi *et al.*, 2009). Overall, the greatest mean per trap night was achieved in Suffolk County using the BG-Sentinel™ trap. Since Suffolk County was the only county employing this trap type, the comparison can only be within that county for that species of interest. Means were 5 times greater for the BG-Sentinel™ trap than for the gravid trap and 2.5 times greater for the BG-Sentinel™ trap than for the CDC light trap.

Of crucial consideration for any differences in the numbers collected of a particular species of mosquito is habitat. *Ae. japonicus* is a container-breeding species. This includes both artificial and natural containers (Andreadis *et al.*, 2001). East Coast scientists have found that *Ae. japonicus* is attracted to discarded tires as breeding sites (Kutz *et al.*, 2003, Kaufman *et al.*, 2005). A NYS study showed that tires on dairy farms were important breeding sites for this species (Kaufman *et al.*, 2005). Both Tompkins County and Suffolk County in New York still maintain dairy farm operations and yielded the largest numbers of *Ae. japonicus* collected.

Source of preferred blood meals is another consideration for *Ae. japonicus* collection. Studies done in the Northeast have shown that *Ae. japonicus* is mammalophagic and that its preference tends toward large mammals such as deer, horses, and humans (Apperson *et al.*, 2004, Molaei *et al.*, 2009). Both Tompkins County and Suffolk County still maintain a suburban and agriculture mix in their domain. Nassau County is more densely populated, with a mix of urban and suburban residents.

The confidence interval for zero events represents the maximum risk of infection. The maximum risk of WNV infection for *Ae. japonicus* in Nassau County is 1.7% and in Suffolk County is 0.2%.

Finding *Ae. japonicus* in NYS does make biological sense, as this species of mosquito is cold-weather tolerant. *Ae. japonicus* mosquitoes can over-winter at temperatures of -18ºC (-39ºF) (Bevins, 2007). The average minimum temperature in Tompkins County is -10.6ºC (-12.9F), in Suffolk County is -3ºC (26.6ºF), and in Nassau County is -5.9ºC (21.4ºF) (WorldClimate, 2010). These mosquitoes can breed in artificial containers and in natural habitats (Andreadis *et al.*, 2001, Kaufman *et al.*, 2005). They thrive in a

range of habitats including rural, suburban, and urban settings (Andreadis et al., 2001). In a recent study conducted in Tompkins County, *Ae. japonicus* was the most abundant species in 77% of positive containers (buckets, flower pots, and birdbaths) (Tuiten *et al.*, 2009).

Ae. japonicus have a long active period (June through October) (Andreadis et al., 2005, Oliver & Howard, 2005). Their seasonal abundance period in the northeastern US is mid-September (Andreadis *et al.*, 2005). A laboratory study showed that they laid fewer eggs in October than in September (Oliver & Howard, 2005).

Detection of WNV in pools of this field-collected invasive species, combined with their ability to feed on humans, make these mosquitoes of public health significance (Andreadis *et al.*, 2001, Turell *et al.*, 2001). Their daytime biting habits make this information worth disseminating to the public so that personal protection methods can be observed.

The introduction of non-indigenous species of mosquitoes happens throughout the world (Bevins, 2007). The interactions of these invasive species are not the same, however, in all areas. (Juliano & Lounibos, 2005) WNV was introduced after the invasive mosquito species became endemic. Evidence suggesting established, propagating populations of *Ae. japonicus* in NJ and NYS was reported in 1998 (Peyton *et al.*, 1999). WNV was introduced to North America (on Long Island) in 1999 (Bernard & Kramer, 2001). *Ae. japonicus* successfully established itself and has become invasive. Separate introduction of vectors (i.e., *Ae. japonicus* mosquitoes) and pathogens (i.e., WNV) can produce disease outbreaks and may have been a contributing factor in New York.

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CHAPTER 4:

THE ROLE OF DAIRY CATTLE AND RACCOONS IN THE EVOLUTION OF ENDEMIC FOCI FOR WEST NILE VIRUS IN NEW YORK STATE[*]

Abstract

The factors that promote the evolution of epidemic foci of the West Nile virus (WNV) into enzootic niches of disease are not fully known. As a part of the effort to shed light on these factors we investigated the role of a couple of potential reservoir/hosts that might perpetuate the disease in New York State (NYS). The hosts of interest in this study were dairy cattle (domestic animals) and raccoons (wildlife). We carried out a cross-sectional study to explore the role of cattle and raccoons in the risk of perpetuating the circulation of the virus in endemic foci in NYS. It was carried out on farms within the New York City Watershed (NYCW) where we have a well-established research program collaborating with local dairy farmers. An additional focus of this research centered on endemic locations with human cases. This was executed in areas within NYS where we have a disease control and research program which includes collaboration with local stakeholders and data collection examining the risk of zoonotic diseases that are associated with wildlife in Nassau and Suffolk counties on Long Island. None of the dairy cattle tested positive for WNV in the NYCW. On Long Island, 50% of the raccoons were seropositive for WNV. Risk factors for these mesopredators included age, sex, and season.

Keywords

West Nile virus, dairy cattle, raccoons, endemic

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Introduction

Agricultural foci are sites of interaction between humans, domestic livestock, wildlife, and arthropod vectors; as such they have the potential to function as ecological nidi for many arboviral diseases, including West Nile virus (WNV). This research project investigated whether dairy farms have the potential to serve as sites where WNV has evolved to become an endemic disease in the state of New York. It was carried out on farms within the New York City Watershed (NYCW), where we have a well-established research program collaborating with local stakeholders collecting data on dairy cattle (*Bos taurus*) (Ziegler *et al.*, 2007, Barwick *et al.*, 2003, Starkey *et al.*, 2006). Published data are lacking for confirmation of WNV infection in ruminants in North America (Callan & Van Metre, 2004). However, seroprevalence studies in endemic areas of Europe and Africa indicate that WNV exposure in ruminants is common (McLean *et al.*, 2002).

An additional focus of this research centered on endemic locations with human cases. This was executed in areas within New York State (NYS) where we have a well-established disease control and research program. This includes collaboration with local stakeholders and data collection to examine the risk of zoonotic diseases that are associated with wildlife in Nassau and Suffolk counties on Long Island (Boulanger *et al.*, 2008). Perennial outbreaks in the state suggest the local establishment of natural foci with perpetuation of the disease among susceptible hosts, rather than reintroduction via migratory birds. Our emphasis was on raccoons (*Procyon lotor*) as a component of this ecosystem. Among the mesopredators in North America, raccoons are known to have a high seroconversion rate (45.6%), which suggests that they are one of the candidate reservoirs for WNV (Bentler *et al.*, 2007). The mechanism by which the virus perpetuates in endemic foci is not fully understood. This emphasis

represents an expansion of our ongoing effort to identify the factors that play a role in the transformation of epidemics of WNV to enzootic foci with continued incidence of outbreak of the disease (DeCarlo *et al.*, 2010). The focus of this study is in on how possible hosts act as potential reservoirs for WNV. We chose dairy cattle (domestic animals) in an agricultural nidus, and raccoons (wildlife) as components of the ecological niche on Long Island.

Material and Methods

Study design—We carried out a cross-sectional study stratified by animal species to address the stated objectives. Cattle and raccoon blood samples were collected from animals in areas in NYS where the disease is known to be endemic. The samples were examined for evidence of exposure to WNV using a serological test. This research conforms to the requirement of Cornell University's Institutional Animal Care and Use Committee (Protocol no. 2008-0153).

Dairy cattle in NYCW—Blood samples from the dairy cattle were obtained from tail veins using a 20 gauge vacutainer® needle and a 5 mL vacutainer® blood collection tube (BD, Becton Dickinson and Company, Franklin Lakes, New Jersey, USA). The samples were kept at 4°C and transported to our laboratory at Cornell University for testing. The cattle were selected from dairy operations located in the NYCW.

Raccoons on Long Island—Raccoon capture and blood-sample collection was performed as previously described (Boulanger *et al.*, 2008). The raccoons sampled in this study represent a population captured in a rabies vaccine study. Tomahawk box traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin, USA) baited with Fur King (Blackie's Blend, Glenmont, Ohio, USA), a commercial raccoon sweet paste, were

used to trap raccoons during September–October 2003–2005. Trap locations were selected to maximize capture rate and to avoid disturbances from people, pets, and other wildlife. If no raccoons were captured at a trap site within three nights, the two traps were moved to another location. The captured raccoons were sedated and blood samples (10 mL.) were collected from femoral blood samples in vacutainer® tubes (BD, Becton Dickinson and Company, Franklin Lakes, New Jersey, USA). Sex, relative age, and weight of each raccoon were recorded. Raccoons were released at the site of capture after a recovery period. This research conforms to the requirements of Cornell University's Institutional Animal Care and Use Committee (Protocol No. 95-79-01).

Blood samples were centrifuged at 800 X G for 10 minutes and 500 μL of serum was removed for the WNV plaque reduction neutralization test.

Plaque Reduction Neutralization Testing for WNV—The Animal Health Diagnostic Laboratory at Cornell University's College of Veterinary Medicine conducted the plaque reduction neutralization testing (PRNT) of serum samples using previously established procedures (Beaty *et al.*, 1989, Ostlund *et al.*, 2001). Titer > 20 was confirmatory for the animal to have WNV neutralizing antibodies. Titer ≤ 20 was seronegative.

Confidence interval for zero events—The 95% confidence interval for zero events was calculated using the formula $p \approx 3 / N$ (Hanley & Lippman-Hand, 1983).

Risk factors—Data collected for each animal (cattle and raccoon) enrolled in the study included date of blood draw, and host factors (age and sex). The prevalence of

seroconversion to WNV was computed as the proportion of animals that tested positive out of all tested samples. In situations where the numerator of the proportion was zero we used the approximation formula for the 95% confidence interval (CI) for no event (the upper limit for the CI equal to 3/N). The significance of association between each factor and the likelihood of seroconversion was evaluated using univariable logistic regression analysis. Factors that were significant in the univariable logistic regression were considered in the multivariable analysis to evaluate the significance of each factor while simultaneously controlling for the effect of the other factors. All the analyses were performed using Statistix 9.0 software (Analytical Software, Tallahassee, Florida, USA).

Results

Dairy cattle in NYCW—A total of 63 samples were collected from cattle in the NYCW and none of the dairy cattle tested positive for WNV (N = 63). The 95% confidence interval for zero events for dairy cattle in the NYCW (Delaware County) was 0.047, which was interpreted to mean that if the sampling were repeated in the population a hundred times the seroconversion rate would fall between 0 and 4.7% ninety-five times.

Raccoons on Long Island—A total of 32 raccoons were captured in Long Island (Nassau and Suffolk County) during the target period of high activities of mosquitoes. There was a relatively high exposure rate to WNV because 50% of the serum samples from these raccoons were seropositive for WNV.

Risk factors—The distribution on the risk factors hypothesized to associate with the likelihood of exposure to WNV is shown in Table 4.1. There was a greater risk in

summer than in fall [odds ratio (OR) = 1.8 and 95% CI (0.39, 8.29)]. Adults were at more risk than juveniles [odds ratio (OR) = 1.7 and 95% CI (0.40, 6.68)]. Males were at an increased risk compared with females [odds ratio (OR) = 1.3 and 95% CI (0.31, 5.33)].

Table 4.1. Risk factors of West Nile virus for raccoons on Long Island, New York

Factor	Number Positive	Number Negative	Odds Ratio (95% Confidence Interval)
Season			
Summer	6	4	
Fall	10	12	1.8 (0.39–8.21)
Age			
Adult	9	7	
Juvenile	7	9	1.7 (0.40–6.68)
Sex			
Male	7	6	
Female	9	10	1.3(0.31–5.33)

Discussion

This study represents a part of our long-term objective of shedding light on the factors that help in the evolution of endemic foci with enzootic cycles of WNW. The focus was on the possible hosts that are likely to play a role in the perpetuation of the infection in these niches.

Dairy cattle in NYCW—The confidence interval for zero events represents the maximum risk of infection. The maximum risk of WNV infection for dairy cattle in the NYCW was 4.7%. The low seroconversion rate or risk for dairy cattle in the

NYCW is in contrast to data collected in Europe and Asia. In Russia, 72% of cattle tested were antibody positive for WNV while 90% of bovids tested in Pakistan were antibody positive (Vasil'ev *et al.*, 2005, Reisen & Boreham, 1979). This large discrepancy in the exposure rate between our study and the other studies could be attributed to several factors including the difference between the demography of the sampled populations, the time of sampling, and the test used to detect seroconversion. Our test is a two-step testing strategy with high specificity (Ostlund *et al.*, 2001). While the majority of scientists are under the impression that WNV does not infect cattle, a recent study in Mexico reported a seroconversion rate of 11% among sampled animals (Ulloa *et al.*, 2009). The authors in that study concur with the lack of a biological rationale for their findings. We believe that our study has confirmed the common belief that cattle do not play a role in the perpetuation of the infection of WNV in endemic foci. A recent study on the surveillance for WNV in Italy supported this perception (Busani *et al.*, 2010).

Raccoons on Long Island—The role of mesopredators as possible hosts of WNV has not been determined in Long Island. In other states the seroprevalence of WNV in raccoons combined with their peri-domestic tendencies indicated that this species could be a useful sentinel for monitoring WNV activity within suburban communities. In Wisconsin researchers found that the prevalence of WNV antibody in raccoons was 19% (Docherty *et al.*, 2006). After an outbreak of WNV infections in Slidell, Louisiana, in 2002, neutralizing antibodies to WNV were detected in 60% of raccoons tested (N = 5) (Dietrich *et al.*, 2005). Sera from raccoons were collected during 2003 and 2004 in California, Arizona, Texas, Louisiana, Ohio, and Wyoming, and similar prevalence rates were observed in these mesopredators (45.6%) (Bentler *et al.*, 2007). High prevalence rates for WNV antibody were noted among raccoons (100%, with a

very small sample size, N = 2), during late summer and fall 2003 from Colorado, Louisiana, New York, Ohio, and Pennsylvania (Root *et al.*, 2005). Additionally, raccoons have been found to be a favorite blood meal of mosquitoes (Hamer *et al.*, 2008).

Summer being a greater risk factor corresponds to the peak incidence of human cases and of virus isolations made from mosquitoes, with both happening in early September (Andreadis *et al.*, 2004). However, a study conducted in Wisconsin with mesopredators (2003–2006) did not find any kind of prevalence trend by month (Docherty *et al.*, 2009). This study included additional species of mesopredators [Virginia opossums (*Didelphis virginiana*) and coyotes (*Canis latrans*)], and this risk factor was not looked at for each species separately. Additionally, the authors found no difference in sex or age, but this could mean that there are risk factors within the species due to the geography. We found a greater risk for WNV seroprevalence in adults than in juveniles. A study done in the eastern United States with 11 wild mammalian species found a significant increase of risk for adults (Gomez *et al.*, 2008). It does make biological sense that adults have a greater likelihood of being exposed or infected over time since they have had more seasons of WNV exposure.

The role of raccoons in the perpetuation of WNV cannot be concretely judged since there are no laboratory studies that quantitate their peak viremia levels. The viremia level needed for transmission is 10^5 pfu mL^{-1} (Turell *et al.*, 2000, Sardelis *et al.*, 2001, Blitvich, 2008).

In this study we investigated the role of cattle and raccoons in the perpetuation of the enzootic cycle for WNV in endemic foci in NYS. The results confirmed the general

perception that cattle do not play a significant role, whereas raccoons are likely to contribute to the transmission of the virus. Further virology studies are needed to support or shed more light on the role of raccoons in the dynamic of infection of WNV.

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CONCLUSION

What We Know About West Nile Virus, and Where Research Should be Focused in the Future

The existence of West Nile virus has been known for over seven decades. Although the body of research on the disease has grown propitiously, much still needs to be explored, including factors in the development of the neuroinvasive form of the disease, how WNV affects the brain and kidneys, the two lineages of the disease, the effect of the environment on WNV, and how to prevent and treat WNV.

First, a strong association has been found between age and the development of WNV encephalitis. Older people are more likely than younger people to develop neuroinvasive disease, with their risk doubling by age 70 (Lindsey *et al.*, 2010). WNV infection can be fatal in this at-risk population. Fortunately, the incidence of neuroinvasive disease resulting from WNV infection decreased from 1.02 to 0.23 between 2002 and 2008 (Lindsey *et al.*, 2010). Still, the highest risk factor for WNV is advancing age, with an incidence of 1.35 per 100,000 population among persons 70 years or older. Overall, 9% of neuroinvasive disease cases were fatal, but fatalities occurred in 17% of neuroinvasive disease cases in the older group (Lindsey *et al.*, 2010).

Second, the virus can persist for years after initial infection in the kidneys of those who have been infected (Murray *et al.*, 2010). This can potentially lead to kidney failure. Viral RNA could be detected in the urine of patients for at least six years following infection. In addition, hamsters experimentally infected with WNV developed chronic renal infection, shedding the virus in the urine for up to eight months (Tonry *et al.*, 2005). In a longitudinal study of 112 hospitalized WNV-infected

patients, five patient deaths were attributed to chronic renal failure (Murray *et al.*, 2010). This long-term effect of WNV is an area of study that will hopefully be continuing.

Third, two lineages of WNV have been identified (Lanciotti *et al.*, 2002). The original African strain is lineage 2 and the strain of WNV seen in Europe, Australia, and North America is lineage 1. WNV lineage 2 was previously seen only in Africa but is now showing up in infected horses in Europe (Bakonyi *et al.*, 2006). A cause of concern about this is that WNV vaccines currently on the market for equines were not tested against lineage 2 strains; therefore, the vaccines might or might not cross-protect against lineage 2. Consequently, research is needed to assess whether current equine vaccines can result in cross-protection. Four WNV vaccines are now licensed in the United States (US) for equine veterinary use. All four have been demonstrated to be protective for lineage 1. Equine owners should be encouraged, as they have been up to this point, to provide yearly WNV vaccinations for their animals.

Fourth, WNV transmission involves a mosquito vector and an avian reservoir host (Blitvich, 2008). For one thing, vector competence is increased in warmer temperatures (Brault, 2009), but a variety of environmental factors might be related to horses being infected with WNV (Rios *et al.*, 2009). Increased risk factors for WNV transmission to horses are related to the availability of mosquito larval habitats, animal housing conditions, and animal management practices. Having a natural source of water on the farm property is protective. Factors increasing the risk of WNV are running electric fans and housing horses in stables constructed of solid wood or cement. Quarter horses are the most commonly affected breed of equine. They are also the largest breed registry in the US.

Fifth, using only environmental-variable or animal-sentinel data was less predictive of WNV than a model that considered all variables (Liu *et al.*, 2009). Population density, growing degree-days, temperature, and the presence of WNV-positive mosquitoes, dead birds, and WNV-positive birds were significant risk predictors of human infection. Currently, no vaccine has been approved for humans. While such a vaccine would be beneficial, especially in endemic areas, the cost-benefit factor of return on investment may not make a marketable product feasible. Nevertheless, the 2000 census showed that 12% of the population is now 65 or older (USCensusBureau), and this population is a prime target for the most severe form of the disease. There may be a possible growing market share for any future vaccine manufacturers. In fact, the US Census Bureau has predicted that in 20 years, 20% of the population in the United States will be in this age group.

Sixth, therapeutics is a crucial area of continued WNV research on combating the disease once it has been contracted. Interferon stimulation may prove to be a viable way to help the innate immune system recognize and limit WNV replication and infection (Rios *et al.*, 2010, Zhang *et al.*, 2010, Jiang *et al.*, 2010). A new therapeutic made from tobacco plants has been shown to arrest WNV infection in mice (Lai *et al.*, 2010). Hu-E16 IgG1, a humanized anti-WNV monoclonal antibody (mAb), binds to a highly conserved epitope on the envelope protein and blocks viral fusion. A single dose of plant-derived Hu-E16A protected mice against WNV mortality four days after infection, and plant-derived Hu-E16 mAb can be rapidly scaled-up for commercial production and produced inexpensively. However, this post-exposure therapeutic has not yet been licensed for human use.

After WNV has been introduced in the skin by a mosquito bite, the virus infects and replicates in Langerhans cells (Johnston *et al.*, 2000). Langerhans cells in the skin are possibly the first cells to produce type I interferon and form the first line of defense against WNV. During secondary viremia, cells of monocyte lineage are infected (Hayes *et al.*, 2005). Host factors are important in determining the outcome of WNV infection. Deficiencies in the host have been linked to more severe outcomes for the patient. Virus replication and increasing susceptibility for developing neurological disease have been linked to a genetic defect in the 2'-5'-linked oligoadenylate synthetase (OAS) in both humans and horses (Lim *et al.*, 2009, Rios *et al.*, 2010). A single nucleotide polymorphism may prove to be the difference between being able to fight the infection and succumbing to a virulent form of WNV.

Finally, a group of proteins naturally found in cells can protect the cell against the invasion of many viruses including WNV (Brass *et al.*, 2009). Interferon-inducible transmembrane (IFITM) proteins boost the natural defense of the body against viral infection. Thus, IFITM3 protects cells against WNV. Switching off these genes using RNA interference makes cells vulnerable to contracting the disease when exposed to the virus and allows the virus to replicate 5 to 10 times faster. Overproduction of IFITM3 makes the cells resistant to WNV. This work is being conducted in culture at the cellular level, so its therapeutic value for humans is still to be determined.

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