Graduate Theses and Dissertations   Graduate School

4-1-2004

Comparative Analysis Of Serologic Assays For The Detection Of Antibodies To Eastern Equine Encephalomyelitis Virus In Sentinel Chickens

Christy L. Voakes
University of South Florida

Follow this and additional works at: https://scholarcommons.usf.edu/etd

Part of the American Studies Commons

Comparative Analysis Of Serologic Assays For The Detection Of Antibodies To Eastern

Equine Encephalomyelitis Virus In Sentinel Chickens

by

Christy L. Voakes

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Public Health
Department of Global Health
College of Public Health
University of South Florida

Major Professor: Lillian M. Stark, Ph.D.
Azliyati Azizan, Ph.D.
Roger Sanderson, M.A.

Date of Approval:
April 1, 2004

Keywords: arboviruses, alphaviruses, hai, igm elisa, prnt, surveillance

Dedication

To Eric

Who asked me,

"What about public health?"

Acknowledgments

I have been extremely fortunate to work with some of the most talented and knowledgeable colleagues, faculty, and students in the public health field. I am especially grateful to Lillian Stark, Ph.D., M.P.H. for her encouragement, expertise in virology, and assistance with experimental design. I would also like to thank Azliyati Azizan, Ph.D. for her valuable advice and for keeping me on track with this project. I would also like to express my sincere gratitude to Roger Sanderson, R.N., M.A. for stepping in and providing insight from an epidemiologist's perspective.

I would like to thank everyone in the Arboserology Department at the Florida Department of Health- Tampa Branch Laboratory for their assistance and contributions to this research. I would especially like to thank Maribel Castaneda and Rita Schofield for their years of dedication in ensuring high quality results in the hemagglutination inhibition assay and Efe Akhigbe for her assistance with the IgM antibody capture enzyme-linked immunosorbent assay. I am especially grateful to Ana Quevedo for her assistance in the BSL-3 laboratory and to Jennifer Kehoe for her help with the Vero cells.

I am very grateful to Angela Butler for all of her time and statistical expertise. I also extend my thanks to everyone in my thesis support group- Kristina Carter, Claudia Marcelo, Logan Wolpin, Matt Smith, and Brenda Brennan.

Special thanks to my families, the Voakes', the Welshans', and the Ottendorfer's, who have encouraged me to pursue my dreams.

Table of Contents

List of Tables

List of Figures

List of Symbols and Abbreviations

Symbol and Abbreviations	Description
%	Percent
°C	Degrees Centigrade
Ab	Antibody
Ag	Antigen
CO_2	Carbon Dioxide Gas
CDC	Centers for Disease Control and Prevention
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
EEE	Eastern Equine Encephalomyelitis serocomplex
EEEV	Eastern Equine Encephalomyelitis virus
EMEM	Essential Minimal Eagle Media
FCS	Fetal Calf Serum
FBE	Florida Bureau of Epidemiology
FDOH	Florida Department of Health
FMV	Fort Morgan virus
g	gravity
HAI	Hemagglutination Inhibition Assay
HJV	Highlands J virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MAC-ELISA	IgM Antibody Capture Enzyme-Linked Immunosorbent Assay
MHC	Major Histocompatibility Complex
µL	microliter
mL	milliter
min	minute
NPV	Negative Predicted Value
PPV	Positive Predicted Value
PRNT	Serum Neutralization Plaque Reduction Test
SINV	Sindbis virus
SLEV	St. Louis Encephalitis virus
SVD	Serum Virus Diluent
VEE	Venezuelan Equine Encephalitis serocomplex
VEEV	Venezuelan Equine Encephalitis virus
WEE	Western Equine Encephalitis serocomplex
WEEV	Western Equine Encephalitis virus
WNV	West Nile virus

Comparative Analysis of Serologic Assays for the Detection of Antibodies to Eastern

Equine Encephalomyelitis Virus in Sentinel Chickens

Christy L. Voakes

ABSTRACT

Florida's mild climate supports year round enzootic transmission of arthropod-borne (arbo) viruses, such as St. Louis Encephalitis virus (SLEV), West Nile virus (WNV), and Eastern Equine Encephalomyelitis virus (EEEV). First isolated in 1960 from two Florida blue jays, Highlands J virus (HJV) is endemic to the state and vectored by the same mosquitoes as EEEV (Henderson et al, 1962). EEEV and HJV are both *alphaviruses*, but HJV is not pathogenic to humans, occasionally causes encephalitis in horses, and is a recognized pathogen in some bird species (turkeys, emus, etc) (Cilnis et al, 1996).

The Florida Sentinel Chicken Arboviral Surveillance Program, established in 1978, utilizes sentinel chickens to detect arboviral activity throughout the state. Current serologic antibody detection methods include the hemagglutination inhibition (HAI), IgM antibody capture enzyme-linked immunosorbent (MAC-ELISA), and serum neutralization plaque reduction (PRNT) assays (Blackmore et al, 2003).

In 2003, sentinel chickens detected significantly greater alphavirus activity than seen in the previous 15 years (Stark & Kazanis, 2003). This increase raised concerns that bridging into the human population had become a serious threat as well as an important

issue for veterinary health. The objective of this study was to determine if cross-reactions with Highlands J virus were impacting the serologic diagnostic tests routinely performed for identification of EEEV.

For 2003, the HAI test detected 476 *alphavirus* positive sentinels. We tested 316 of these chickens in the PRNT, which identified 176 EEEV positive sentinels and 75 HJV positive sentinels. Our results indicate that Highlands J virus is extensively cross-reactive in the HAI test and that the MAC-ELISA is more specific for the detection of antibodies solely to EEEV. We demonstrated that EEEV antibody titers in the HAI test were positively correlated to antibody titers in the PRNT assay. Analysis of *alphaviral* activity by county indicates widespread transmission of HJV across the northern and panhandle regions of the state; however EEEV activity was greater than HJV activity in all but four counties.

Consequently, distinguishing between the two agents can reduce the expenditure of resources on unnecessary vector control and medical alerts to protect the public health from Highlands J virus.

Arthropod-borne viruses (arboviruses)

An **ar**thropod-**bo**rne virus (arbovirus) is a virus that requires a hematophagous (blood-sucking) arthropod vector for transmission into vertebrate hosts to maintain its life cycle (Gubler, 2001). Most arboviruses are zoonoses with vertebrate hosts other than humans as their primary reservoir. Currently, 534 viruses are registered in the International Catalogue of Arboviruses. Only 134 of these registered viruses have caused documented disease in humans (Karabatsos, 1985). Arboviruses are taxonomically diverse and belong to eight viral families and fourteen genera. The arboviruses that are medically important belong to three virus families: the *Bunyaviridae*, *Flaviviridae*, and *Togaviridae* (Gubler & Roehrig, 1998).

Vertebrate infection can occur after an infected arthropod takes a blood meal, and arthropod infections occur after feeding on viremic (presence of virus in the bloodstream) hosts. Usually the virus remains undetected as it cycles between the primary arthropod vector and the primary vertebrate host until an ecologic change occurs that allows the virus to escape this focus (Centers for Disease Control & Prevention [CDC], 2001). For arboviruses, the most common amplifying hosts are birds and rodents and the most important arthropod vectors are mosquitoes and ticks for diseases of public health consequence. One exception to this rule is dengue virus, which has adapted completely

1

to humans and is maintained in a mosquito-human-mosquito transmission cycle in urban centers of the tropics and sub-tropics (Gubler, 2002). Important ecological parameters that govern these cycles include temperature, rainfall, and humidity which influence geographic distribution of the vectors and hosts (Gubler, 2002).

Arboviruses are globally distributed, but are primarily found in tropical areas where the climate can support year-round transmission by cold-blooded arthropods (Gubler, 2002). The past 20 years has witnessed changing epidemiological trends resulting in dramatically increased global epidemic arboviral activity. Population growth, new irrigation systems, deforestation, and uncontrolled urbanization in tropical developing countries have especially contributed to the emergence/resurgence of arboviral diseases (Gubler, 2001).

During this time, viruses once thought to be controlled or not of major public health significance caused epidemic disease in many regions of the world. For example, dengue virus expanded globally resulting in larger and more frequent epidemics. West Nile virus (WNV) was introduced into North America in 1999 with epidemics and epizootics of severe neurologic disease in humans, horses, and birds- an apparent shift from relatively less pathogenic strains found previously in the Middle East, Israel, India, France, and South Africa. This recent emergence of a new, more virulent strain of WNV with greater epidemic potential has raised important questions about the capacity of the public health infrastructure to implement surveillance, prevention, and control programs not only for WNV, but also in the event other pathogens reemerge in a more pathogenic state (Gubler, 2002).

2

Alphaviruses

Classification

Initially classified as serologic Group A arboviruses (Casals & Brown, 1954), the Alphavirus genus (Family *Togaviridae*) includes 25 viruses, all transmitted by arthropods, restricted geographically in distribution, and sharing a common replication strategy. The pathogenic *alphaviruses* can be divided into two groups, those causing human diseases characterized by encephalitis, usually found in the New World (e.g. Eastern Equine Encephalomyelitis virus), and viruses that cause arthritis and rash, found primarily in the Old World (e.g. Sindbis virus) (Griffin, 2001).

Alphaviruses are positive strand RNA viruses with icosahedral symmetry and a lipid envelope. E1 and E2 are viral encoded coat glycoproteins that have important immunologic as well as diagnostic properties. The *alphavirus* serogroup is currently divided into seven broad antigenic complexes: Barmah Forest (BF), Eastern Equine Encephalomyelitis (EEE), Middelburg (MID), Ndumu (NDU), Semliki Forest (SF), Venezuelan Equine Encephalitis (VEE), and Western Equine Encephalitis (WEE). The EEE, BF, NDU, and MID complexes each only have a single virus (Calisher & Karabatsos, 1988), although Eastern equine encephalomyelitis virus (EEEV) has two antigenic varieties that are geographically restricted to either North America or South America (Brault et al, 1999). However, the SF, WEE, and VEE complexes each contain several viruses (Calisher & Karabatsos, 1988). For example, the WEE antigenic serocomplex includes the New World viruses Highlands J (HJV), Fort Morgan (FMV), Buggy Creek, and Aura, as well as the Old World viruses Sindbis (SINV), Whataroa, and

3

Kyzlagach (Calisher et al, 1988). The major alphavirus serocomplexes found in the New World are EEE, VEE, and WEE (Weaver et al, 1999).

Phylogeny and Evolution

The ability of *alphaviruses* to replicate in arthropods, avians, and mammals is thought to influence geographic distribution patterns and potentially impact their genetic diversity and evolution (Cilnis et al, 1996). *Alphaviruses* can be transmitted by a wide range of mosquito species, but each virus typically has a preferred mosquito vector for the enzootic cycle that uses either birds or mammals as primary amplifying hosts (Scott & Weaver, 1989). Viruses which use avian amplifying hosts, such as EEE and WEE complex viruses in North America, may be more efficiently dispersed over wide geographic regions, enhancing gene flow, and thus far have remained highly conserved. On the other hand, viruses which utilize mammalian enzootic hosts, such as Venezuelan equine encephalitis virus (VEEV) and strains of EEEV which amplify in rodent hosts in the tropics, have a smaller range of dispersal due to limited mobility of the host, which has resulted in genotypic evolution within multiple geographic foci in South America (Cilnis et al, 1996; Weaver et al, 1997).

Recombination between *alphaviruses* has been difficult to achieve *in vitro*, but is estimated to have occurred naturally thousands of years ago when a Sindbis-like virus and EEEV recombined to form WEEV, HJV, and FMV. *Alphaviruses* likely originated in the Americas and later spread to the rest of the world, based upon evidence that one WEE complex virus (Aura) in the New World does not have a recombinant genome (unlike WEEV, HJV and FMV), nor does Old World members of the WEE complex

4

(SINV, Whataroa). In addition, there is greater sequence divergence in New World *alphaviruses* than in Old World viruses (Weaver et al, 1997).

Important Biologic Characteristics

Hemagglutination can be described as the clumping together of erythrocytes (RBCs) to form interlocking matrices due to the binding of agglutinin (protein) molecules on the surface of each cell. *Alphaviruses* have the ability to hemagglutinate avian RBCs, especially goose and chicken erythrocytes, which continues to be a valuable method for virus quantification, and for measuring antiviral antibody by the inhibition of hemagglutination (HAI) test (Clarke & Casals, 1958). Hemagglutination is dependent primarily on the E1 glycoprotein since it binds to lipids in the RBC membrane (Griffin, 2001).

The first *alphavirus* cellular receptor to be identified was the major histocompatibility complex (MHC) class 1 molecule for SF virus on human and mouse cells, but it is not strictly required for infection since cells lacking MHC molecules can also be infected with SFV. Other identified cellular molecules include the high-affinity laminin receptor as well as heparan sulfate, which is an important initial binding molecule for some viruses. *Alphaviruses* may bind to several receptors, or possibly use receptor-co receptor combinations in order to infect such a wide range of hosts (Griffin, 2001). The E2 viral glycoprotein interacts with these receptors, and antibodies directed against the E2 protein can neutralize virus infectivity. The E2 glycoprotein also has hemagglutination properties (Schlesinger & Schlesinger, 2001).

Alphaviruses replicate quickly in most vertebrate cell lines, releasing progeny virus between 4 to 6 hours after infection. Infection leads to extensive cytopathic effects

5

(cpe) characterized by cell rounding, shrinking, and cytoplasmic blebbing. Many alphaviruses cause cell death by inducing apoptosis (and through non-apoptotic mechanisms), often with death of the infected cell by 24 to 48 hours (Griffin, 2001).

Pathogenesis and Pathologic Changes

Alphaviruses are commonly injected into the host through the bite of an infected mosquito, where they initially replicate in skeletal muscle at the puncture site (e.g. EEEV, WEEV) or may be taken up and infect Langerhans cells in the skin (e.g. VEEV). Langerhans cells could then transport the virus and infect lymph nodes draining the inoculation site. A substantial plasma viremia (existence of virus in the bloodstream) is induced in amplifying and disease-susceptible hosts by the continued production of virus at the primary site of replication, delivery into the vascular system, and slow clearance of the virus from the blood (virulent strains are cleared more slowly than avirulent strains). Consequently, *alphaviruses* are able to spread through the bloodstream to infect more distant skeletal muscles from site of inoculation and into lymphatic tissues (Griffin, 2001).

The exact mechanism by which encephalitic *alphaviruses* enter the central nervous system (CNS) is unclear, but infection of choroid plexus epithelial cells has been proposed for EEEV. Once the virus gains entry to the CNS, it can be spread cell to cell or through cerebrospinal fluid (CSF). Most encephalitic *alphaviruses* target the neuron leading to severe and irreversible damage to the cell (Griffin, 2001).

Pathologic changes in the CNS of humans with fatal disease include perivascular extravasation of erythrocytes, inflammation, and demyelination. Equine pathology associated with VEEV includes depletion of bone marrow, lymph node tissue, and spleen

cells, as well as pancreatic necrosis (Griffin, 2001). Avian pathology associated with EEEV includes ascites, hepatomegaly, splenomegaly, and visceral gout (Hansen & Docherty, 1999).

Antigenic Characteristics

All alphaviruses share common antigenic sites as they are closely related to each other, as proven by extensive cross-reactivity in hemagglutination inhibition (HAI) and complement-fixation assays with polyclonal immune sera (Calisher et al, 1980). E1 proteins are most important for hemagglutination, but antibodies to the E1 glycoprotein are more likely to cross-react with other alphaviruses (due to high sequence conservation). These close antigenic relationships among alphaviruses can impact the hemagglutination test, such that confirmatory tests are necessary to identify the true infective agent. However, neutralizing antibodies to the E2 protein are not as cross-reactive, and are usually virus specific (Griffin, 2001).

Immune Response

Innate immune responses to alphaviral infections include induction of type 1 interferon (IFN), increase in tumor necrosis factor α (TNF), and interleukins, which actually may do more physiological harm than good. Fever and production of inflammatory cytokines may contribute to mortality (Griffin, 2001).

Adaptive cellular and humoral immune responses induced by alphaviral infection are important in recovery from infection. Virus-specific immunoglobulin M (IgM) antibodies are produced very early in the course of disease, usually detectable in serum within 3 to 4 days, and are the targeted molecules for rapid diagnosis of infection. IgM antibodies are generally not long lasting, often diminishing within 14 to 30 days, and

7

their presence denotes a recent infection (Olson et al, 1991; Martin, 2000). Conversely, immunoglobulin G (IgG) antibodies are first detectable in serum 7 to 14 days after infection (Calisher et al, 1986c; Olson et al, 1991), but can remain at high levels for years, especially after secondary exposure to the same infectious agent (Figure 1). Appearance of antibody correlates with declining viremia as well as neutralization of virus infectivity. Experimental passive transfer of antibodies prior to infection has been shown to protect against fatal disease, yet the more practical applications for prevention are the inactivated vaccines available for EEEV, VEEV, and WEEV (Griffin, 2001). The humoral antibody response not only provides protection from alphaviral disease, but also allows for rapid serologic antibody testing to diagnose the infection.

Figure 1 Theoretical Primary and Secondary Antibody Response after Exposure to an Infectious Agent. Within 3-4 days, IgM antibodies are quickly produced but decline as IgG antibodies rise, which can remain elevated for months to years. Secondary exposure to the same antigen rapidly results in high levels of IgG.
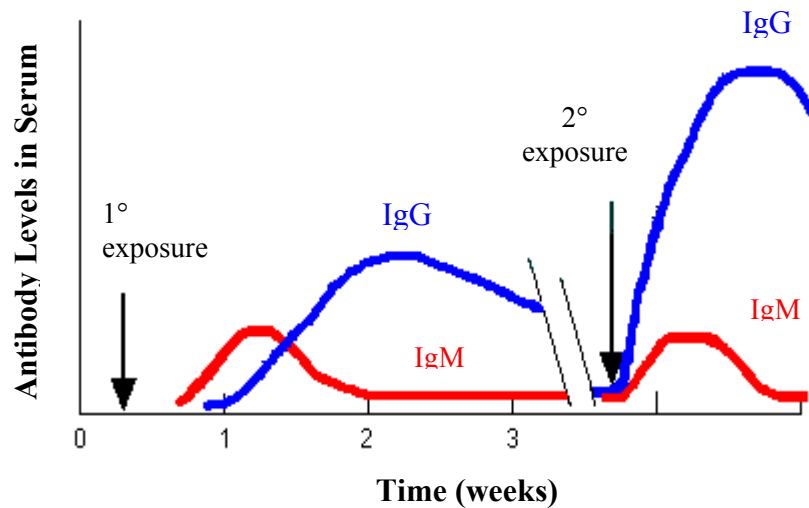
*Diagram modeled after Immune Response by Dr. Thomas Terry. Available from URL http://sp.uconn.edu/~bi102vc/102f01/terry/immunity.html

8

Eastern Equine Encephalomyelitis Virus

History and Discovery

Historians and scientists theorize that EEEV has appeared along the eastern seaboard of North America for centuries. The origin of EEEV disease is unknown, but was first believed to be associated with epizootics recorded in 1831, 1845, and 1933. The first clear record of epidemic encephalitis comes from Massachusetts in the summer of 1831 when 75 horses died (Hanson, 1957). Published reports in the New England Farmer at the time describe symptoms characteristic of EEEV disease in equines (lethargy, listing to one side, spastic paralysis, etc), as well as inflammation of the brain at necropsy, and higher attack rates in horses on pasture versus those kept stabled (Peck, 1831; Phinney, 1831; Thompson, 1831). Although epizootics were also recorded in New York, North Carolina, Florida, and Maryland between 1845 and 1912, the virus was first isolated in 1933 from the brains of diseased horses in New Jersey and Virginia. In 1938, EEEV was first isolated from the CNS of humans (Griffin, 2001). Florida's first documented human case was in 1952 (Bigler WJ et al, 1976). In Florida, EEEV naturally occurs in swampy areas throughout the year and the virus is maintained in native bird species and mosquitoes (CDC, 2001).

Virus Maintenance and Amplification Cycles

Vector

EEEV was first isolated from other non-mosquito arthropod vectors, such as chicken mites and lice, which transmit the virus inefficiently. However, EEEV is vectored most effectively by mosquitoes, and has been found in *Culiseta* sp., *Mansonia* sp*., Coquillettidia* sp., *Aedes* sp., and *Culex* sp. (Griffin, 2001). In Florida, EEEV has

9

been isolated from five mosquito species: *Culiseta* sp., *Aedes* sp., *Coquillettidia* sp., *Culex* sp., and *Anopheles* sp. (Bigler WJ et al, 1976; Wellings et al, 1972; Henderson et al, 1962).

The primary enzootic vector for EEEV in North America is the ornithophilic (affinity for birds) mosquito *Culiseta melanura*. The overwintering mechanism (persistence of an infectious agent in the vector during winter months when no reinfection of mosquitoes occurs) in *Culiseta melanura* is unknown. *C. melanura* overwinters in the larval stage, from which EEEV has been isolated once (Monath, 1979). Overwintering through transovarial transmission has largely been rejected as the mechanism due to an inability to consistently isolate EEEV from pools of male mosquitoes and larvae, as well as the failure of experimental studies to reproduce the phenomenon artificially (Brault et al, 1999).

The enzootic cycle, where the virus silently circulates between birds and mosquitoes, is commonly maintained in coastal areas and fresh water swamps. Outbreaks can occur when the virus spreads into mosquito species that feed on a variety of hosts, not just birds. These bridge vectors (e.g. *Aedes* sp., *Coquilletidia* sp., and *Culex* sp.) are necessary to spread EEEV outside of the swamp regions and into different hosts, which may result in disease (CDC, 2001) (Figure 2).

Amplifying Host

Hosts for the virus to amplify in are also critical to maintenance of the enzootic cycle. Primary reservoir hosts for EEEV in North America are migratory passerine songbirds, starlings, and wading birds. Wild birds that can function as amplifying hosts outside of the fresh-water swamps include the American robin, cardinal, blue jay, and

common grackle, which may help disseminate EEEV to other habitats (CDC, 1993). Though infected with EEEV, these birds remain asymptomatic with prolonged viremia. Amplifying hosts that are silently infected with EEEV usually are not harmed by the virus (Griffin, 2001).

Incidental Host

Humans, horses, and some birds (pheasants, quail, and ostriches) are not preferred reservoir hosts and do not contribute to EEEV amplification; only low levels of virus circulate in the bloodstream. They are incidental hosts, a dead-end for the transmission cycle since mosquitoes will not be infected upon taking a blood meal from these hosts (CDC, 2001). Emus are an exception to the rule; they are highly susceptible to EEEV infection and develop high-titered viremias that could contribute to virus amplification in the peridomestic environment, potentially placing humans at increased risk (CDC, 1998). Although EEEV produces subclinical infections in a wide range of wild birds from raptors to songbirds, EEEV can cause mortality in the glossy ibis and several species exotic to the United States, including emus (Day & Stark, 1996a), whooping cranes, pheasants, pigeons, house sparrows, chukar partridges, and white Peking ducklings (Hansen & Docherty, 1999). Interestingly, birds do not develop encephalitis (with the exception of pheasants) even those that have fatal outcomes (Griffin, 2001). Significant morbidity and mortality occurs in these incidental hosts.

Equines are significantly affected by EEEV disease. Hundreds of equine cases occur each year, far exceeding human cases, despite the availability of an inactivated vaccine for horses. Horses are at higher risk for infection with arboviruses because of higher exposure levels; they remain outdoors and attract hordes of biting mosquitoes

11

(CDC, 2001). Encephalitis does develop in horses and symptoms of infection begin with depression, progressive incoordination, seizures, and prostration. Case fatality rates range from 80 to 90% (Weaver et al, 1999). Equine arboviral encephalitis cases are valuable surveillance tools, since morbidity in horses typically precedes bridging of the virus into human populations (CDC, 2001; Blackmore et al, 2003).

Figure 2 Enzootic and Epizootic Eastern Equine Encephalomyelitis Virus Amplification Cycles. This diagram depicts the interaction of vectors, amplifying and incidental hosts. The enzootic cycle is shown in blue. The epizootic transmission cycle is shown in red and includes incidental hosts that are affected when bridging between the two cycles occurs.

Cranes

Enzootic mosquito vector *Culiseta species*

Horses

Epizootic mosquito vector *Aedes*

Epizootic mosquito vector *Aedes species*

Songbirds & Swamp birds

Humans

Pheasants
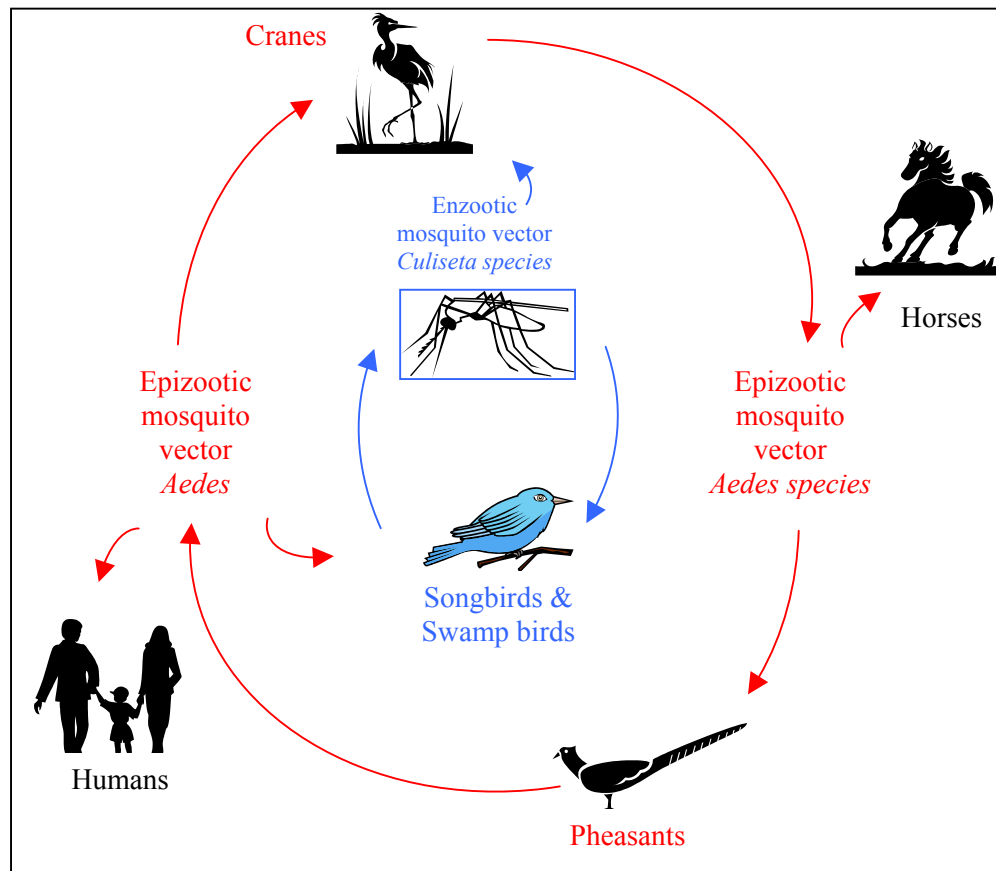
*Diagram modeled after Transmission of eastern equine encephalomyelitis *in* Field Manual of Wildlife Diseases, General Field Procedures and Diseases of Birds (Hansen & Docherty, 1999).

Epidemiology

In North America, peak activity for EEEV transmission occurs in the late summer to early fall months, although the virus is frequently transmitted year-round in Florida. The virus maintains localized enzootic foci from New Hampshire along the Atlantic seaboard and Gulf Coast over to Texas, with inland foci in the Great Lakes region and South Dakota. Most clinical cases are associated with exposure to wooded areas near fresh-water swamps (Bigler WJ et al, 1976). In South America, EEEV is also enzootic, but human infections in this area are mild or subclinical (Griffin, 2001).

Although EEEV is the rarest mosquito-borne arboviral encephalitide for humans in the United States (CDC, 1992), it is also the most severe with a human case-fatality rate of 33-50% (Villari et al, 1995). In addition, 35 % that survive the disease sustain neurological lesions that permanently impair function (CDC, 2003a) and often require life-long institutionalized care (Villari et al, 1995). On average, 4 human cases are diagnosed each year in the United States. Cases occur sporadically and in limited epidemics: 182 cases have been reported to the CDC between 1964 and 2000 (CDC, 2002), although reported case numbers to the CDC peaked at 36 in 1959 (Monath, 1979). Florida led all states with a total of 53 confirmed cases between 1964 and 2000 (CDC, 2002). Epidemics are cyclic often occurring every 9 years (CDC, 1993). Demographics indicate that gender does not play a role in EEEV infection, children 10 years of age and under are most susceptible, with higher case fatality rates found in children and the elderly (Griffin, 2001).

13

Clinical Disease and Pathology

After a bite from an infected mosquito, the incubation period is 4 to 10 days in humans (CDC, 2001). Clinical cases are not always life-threatening; some infections resemble mild flu-like illnesses with one to two weeks of malaise, fever, chills, and myalgias, followed by recovery (CDC, 2003a; Griffin, 2001). Cases of encephalitis usually begin with sudden onset of fever, muscle pains, and headache of increasing severity (CDC, 2001). Associated symptoms with onset of encephalitis are continued fever, worsening headache, vomiting, seizures, and coma. Death usually occurs within 2 to 10 days afterwards. Serious sequelae are common and include mental retardation, paralysis, and seizures (Griffin, 2001). No specific treatment is available for EEEV infections, only supportive care is available (CDC, 2001).

Economic Burden

Even though human infection with EEEV is sporadic and infrequent, its severe sequelae and potential need for life-long institutionalized care for survivors may make expensive preventive programs much more cost-effective over time for a community. An early 1990s study, conducted in Massachusetts, on the economic burden imposed by EEEV found that for transiently affected patients (with full recovery) the average cost per case was $21,000, which mostly was for direct medical services. However, for a person with residual sequelae the average lifetime cost of EEEV infection approached $3 million, including loss of potential income earned. These figures have likely increased over the last decade as medical costs have soared so that treatment for EEEV disease is much more expensive now. In contrast, massive aerial applications of ultra-low volume (ULV) malathion for insecticidal interventions that are very effective at preventing

14

outbreaks of EEEV infection, cost between a quarter ($0.7 million) to one half ($1.4 million) less than the expense of a single residually affected child (Villari et al, 1995). Vector control technologies have also improved in the last decade allowing for targeted pesticide applications based on surveillance data which is much more cost-effective.

Public Health Implications

Eastern Equine Encephalomyelitis virus is a significant threat to public health in North America. Endemic in many eastern states, the consequences of infection are severe and are associated with high case fatality rates, permanent neurological damage in survivors, and incredible economic costs to communities. Early identification of arboviral activity is crucial for timely implementation of risk-reduction strategies, such as vector-control practices, medical alerts, and educational campaigns to promote use of repellents and avoid insect bites (CDC, 1994).

Highlands Jay (J) Virus

History and Discovery

Initially described as a variant strain of Western Equine Encephalitis virus (WEEV), Highlands J virus (HJV) was first isolated in the eastern United States in 1960 from two blue jays in Highlands County, Florida (Henderson et al, 1962; Karabatsos et al, 1963). During this survey for arboviruses in south-central Florida, this misnamed WEEV-HJ strain was also isolated from one *Aedes* sp. salt marsh mosquito pool and a sentinel mouse (Henderson et al, 1962). Four years later, the WEEV-HJ strain was again isolated from 6 mosquito pools and from a fatal equine case in Hillsborough County, Florida. This was the first equine fatality associated with WEEV, i.e. Highlands J virus,

in the eastern part of the United States (Jennings et al, 1966). Reevaluation of alphaviral taxonomy and the establishment of HJV as a distinct virus, no longer a subtype of WEEV (Calisher et al, 1980, 1988), led to the retraction of WEEV as the cause of death of the Florida equine case. It was re-classified as Highlands J virus associated encephalitis and mortality (Karabatsos et al, 1988).

Epidemiology

The virus has only been isolated in the United States, where HJV is enzootic to the eastern seaboard. Highlands J virus is transmitted among passerine birds by *Culiseta melanura* mosquitoes in freshwater swamps in an amplification cycle similar, if not identical, to that of EEEV (Hayes and Wallis, 1977).

Highlands J virus is not considered to be an important pathogen to public or veterinary health due to its low virulence for humans and equines, with the exception of the 1964 equine case (Karabatsos et al, 1988). However, HJV is recognized as an important poultry pathogen and was associated with widespread infection of domestic turkeys in North Carolina (Ficken et al, 1993). Like EEEV, Highlands J virus also has been known to cause disease in other domestic avian species including pheasants, ducks, emus, whooping cranes, and chukar partridges (Weaver et al, 1997).

Prevalence of the virus in nature is not well described due to sporadic identification of HJV in cases of encephalitis and its initial misclassification as WEEV. Nonetheless, high rates of infection in mosquito vectors are found annually in the eastern states (Monath, 1979). In addition, a 1960 study in Florida showed (when the virus was initially described) that the seroconversion (development of antibodies after exposure to an infectious agent) rate to HJV in wild birds was 23%, but in 1961 the prevalence of

16

HJV was much lower at 3.4% (Henderson et al, 1962). In 2003, HJV was isolated from 2 dead birds, a turkey and Eurasian collared-dove, in Florida (Collins & Blackmore, 2003).

Public Health Implications

Although HJV is not a significant cause of morbidity or mortality in humans and equines, it does have important implications for protection of public health against arboviral disease. Its close antigenic relationship to EEEV and nearly identical transmission cycle makes it difficult to readily distinguish HJV from its highly pathogenic relative, yet the viruses must be differentiated in order to prevent waste of costly resources such as vector control and educational campaigns on a less pathogenic agent. Consequently, in areas where HJV is known to coincide with transmission of EEEV, enhanced surveillance detection methods should be employed to garner a measure of true EEEV activity versus Highlands J virus transmission.

Surveillance for Arboviral Activity

A surveillance system can quantify viral activity at a specific time, predict the likely future course of the transmission cycle, as well as indicate when control should be implemented to prevent epizootic or epidemic viral transmission.   An effective surveillance program requires long-term commitment and proactive projects to gather data in epidemic and non-epidemic years. This allows for baseline (background) levels to be set and can provide the basis for setting thresholds and prompt decision making for vector control and medical alerts when increased activity is detected. Due to the complex life cycles of arboviruses, no single technique can collect all the data necessary for accurate risk assessment of vector-borne diseases. Thus, multiple detection methods are necessary and threshold levels and indicator parameters may vary by region and season.

17

Current year data should be compared with historical data for the same region as well (CDC, 1993).

Successful EEEV surveillance programs focus on components of both the enzootic cycle and the epizootic cycle. An ideal program should monitor meteorological data (rainfall and temperature patterns which promote development of large mosquito populations), vector data (field infection rates, density and age structure of both *Culiseta melanura* and epizootic species, *Aedes* and *Coquillitidia*), avian morbidity and mortality, and vertebrate host data (high EEEV antibody prevalence in wild passerine birds will prohibit further viral amplification). Active or passive surveillance of encephalitis in unvaccinated equine cases can also be useful predictors (CDC, 1993; Blackmore et al, 2003).

A few states including Florida, California, Delaware, and Utah use sentinel chicken flocks scattered throughout regions at greatest risk for EEEV, WNV, SLEV, or WEEV infection (CDC, 1993). Sentinel chickens may not be perfect indicators for all regions as they were not useful for detecting EEEV activity in New Jersey (Crans, 1986). Despite this, domestic chickens are still one of the most widely used sentinel animals for detection of arboviruses, and it is likely that one ideal captive avian sentinel truly does not exist (CDC, 1993). The primary advantage of captive bird sentinels is that time and place of exposure are known. Conversely, sentinels only detect focal transmission and multiple flocks must be placed to accurately represent geographic areas (CDC, 2003b).

Florida Sentinel Chicken Program

Periodic outbreaks of St. Louis Encephalitis virus (SLEV) over the last forty years (e.g. 1959 to 1962- 315 human cases/55 deaths; 1977- 110 cases/8 deaths; 1990- 223

18

cases/11 deaths) led to the formation and continuation of an arboviral surveillance program in Florida (Bigler B, 1999). At the heart of this program is the Florida Sentinel Chicken Arboviral Surveillance Network, which was established in 1978 (Nelson et al, 1983). Chickens are chosen as sentinels because they are susceptible to infection and will develop antibodies, the infection is not life-threatening, and significant viremia does not develop, thus they are non-infectious to handlers, mosquitoes, and other chickens (Langevin et al, 2001).

In 2003, the rate of sentinel chicken seroconversions to EEEV in Florida was significantly greater than the fifteen year average historical rate (1988-2003), where activity was traditionally highest in the northern and panhandle regions, and peaking in July. Fortunately, this increased activity did not result in an epidemic of human cases, with only 3 confirmed EEEV positive (Stark & Kazanis, 2003).

Chicken Serosurveillance Guidelines

Six flocks of six chickens each are recommended for each county, where they are placed at potential enzootic transmission sites. Sentinel sites are permanently positioned in areas free from public access and vandalism, within 2-3 miles of active mosquito breeding areas, in both swamps and near residential areas. Chickens are protected from the elements and predators, and an additional flock of chickens is recommended to be kept in a mosquito-proof building for replacements due to seroconversion or mortality. All chickens are properly identified by numbered wing or leg bands. Leghorn, Rhode Island Red, or Barred Rock chickens that are 10-12 weeks old at onset are recommended. Chickens are pre-bled and baseline antibody levels were analyzed prior to placement in

19

the field. It is recommended that all chickens in a flock are sampled every week (FBE and FDOH, 2000).

Seroconversion (development of antibodies after exposure to an infectious agent) rates in sentinel chickens, along with data collected by mosquito control agencies, are utilized by public health officials as indicators of the intensity of enzootic transmission activity as a predictor of epizootic arboviral transmission in an area and to determine control measures needed to prevent clinical disease. The recent emergence of West Nile virus (WNV) in Florida mobilized public health agencies statewide and reaffirmed the importance of the sentinel chicken program as an early warning system not only for endemic diseases, like SLEV and EEEV, but also allows for the detection of new or re-emerging diseases (Blackmore et al, 2003).

Detection Methods

It is rare to isolate these viruses from blood (or cerebrospinal fluid (CSF) from humans) taken during the acute phase of infection because the viremic stage is often completed prior to onset of illness. Consequently, diagnostic methods primarily are based upon detection of antibodies. Serologic detection methods are complex due to close antigenic relationships within virus families, including the *alphavirus* and *flavivirus* (i.e. WNV and SLEV) families. After a sample is determined to be a certain virus group positive by the screening test, specialized diagnostic tests are required to differentiate between specific viruses, including cross-reactions between EEEV and HJV (FBE & FDOH, 2000).

20

Hemagglutination Inhibition (HAI) Test

Hemagglutinating antigens from SLEV, WNV, WEEV and dengue virus were first demonstrated by Sabin and his associates. These studies indicated that hemagglutination was characteristic of most arboviruses, and that hemagglutinins had specific requirements for type of erythrocyte and pH (Sabin & Buescher, 1950; Sabin, 1951; Chanock & Sabin, 1953, 1954a, 1954b; Sweet & Sabin, 1954). Later studies were able to use this property to extend the list of arboviruses with HA activity (after acetone- and ether-extracted antigen preparation) and show that most of the arboviruses fall into three immunologically distinct groups: A, B, and C (Casals & Brown, 1954; Casals, 1957). Antiviral antibodies in sera can specifically inhibit the hemagglutination reaction such that this test can be utilized for diagnostic purposes (Clarke & Casals, 1958).

Previously one of the most common laboratory techniques used to determine arboviral infections, the HAI test now is only used by a few states for this purpose. The HAI test is used for a variety of etiologic agents; it is inexpensive and easy to perform, and is able to test large numbers of specimens at one time. However, when testing a large number of specimens, this test is not rapidly performed. Laboratories (mostly state diagnostic facilities) that use this method are typically located far from the surveillance site. The HAI test as used by these laboratories is cost-effective, reproducible, and is useful for analyzing hundreds or over a thousand specimens (only Florida has this capability) at once. However, results may not be reported for a week and may effect local agencies response time to initiate or intensify control measures (Olson et al, 1991).

The best source of HA antigens is sucrose-acetone extracted suckling mouse brain. Suckling mice are inoculated with virus, and the brain tissue is harvested, which

contains high titers of virus. Acetone extraction removes non-specific lipoprotein inhibitors, and treatment of the test sera with protamine sulfate also removes inhibitors and broadens the pH range for HA activity. Goose erythrocytes are routinely used to remove naturally occurring agglutinins in the sera. The sera are then serially diluted and appropriate antigens containing 4-8 units of hemagglutinin are added. The serum-virus mixture is incubated overnight to allow for antigen-antibody binding. Standardized goose RBCs are added to the test sera-antigen mixtures. Inhibition of agglutination is indicated by a button of red cells (Clarke & Casals, 1958).

Cross-reactivity within a virus group is common and can complicate interpretation of HAI test results. As such, HAI tests are valuable screening assays (with a high sensitivity), but a positive test result requires additional confirmatory tests. Both the IgM and IgG antibody fractions are involved in the HAI reaction so that a fourfold rise in titer between acute and convalescent sera may be diagnostic of recent infection (FBE & FDOH, 2000).

IgM Antibody Capture Enzyme-Linked Immunosorbent Assay (MAC-ELISA)

The first immunoglobulin produced following immunization, IgM antibodies are efficient agglutinating molecules, excellent complement-activators, and largest in size. Although they cannot pass through the placenta and are inefficient in the neutralization of viruses, IgM antibodies are very important as they are typically the first class of immunoglobulins produced following infection. Elevated levels of IgM usually indicate recent exposure to antigen or a recent infection (Benjamini et al, 2000).

The immunoglobulin M antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) has proven to be an excellent technique for measuring IgM antibodies in

22

response to viral infection (Duermeyer et al, 1979; Hofmann et al, 1979; Schmitz et al, 1980; Roggendorf et al, 1981; Burke & Nisalak, 1982; Jamnback et al, 1982; Monath et al, 1984; Calisher et al, 1986a; Calisher et al, 1986b; Calisher et al, 1986c; Olson et al, 1991; Martin et al, 2000; Johnson et al, 2003).   This diagnostic method has been standardized and allows for a consistent rapid approach for monitoring arboviral disease, but is restricted by species type (Martin et al, 2000). The MAC-ELISA is more specific than the hemagglutination inhibition test, which can only indicate viral group infection (i.e. Togavirus or Flavivirus) due to extensive cross reactions between family members. The presence of virus-specific IgM antibodies in a single serum specimen indicates that it is a presumptive positive. This eliminates the need for a convalescent-phase serum to be drawn (Martin et al, 2000), which often is difficult to achieve due to improper timing and loss to follow-up for humans. Chickens can be lost to follow-up as well; they may escape, lost to predation, or are just never re-bled (personal observation, sentinel submission sheets).

The test is performed as follows: anti-species IgM capture antibody is coated on 96-well microplates, and the wells are blocked with milk to decrease background. Serum from the animal species is added followed by non-infectious viral antigen. The presence of antigen is detected using an enzyme-conjugated anti-viral antibody that interacts with a chromogenic substrate to generate a colorimetric result (Martin et al, 2000).

Serum Neutralization Plaque Reduction Test (PRNT)

The serum neutralization plaque reduction test (PRNT) is the gold standard for differentiating between closely related etiologic agents. Time consuming, expensive, and laborious, the assay utilizes live infectious agents to challenge suspect sera for the

23

presence of specific neutralizing antibodies. This assay may also be used to determine the identity of viral isolates. The test is highly sensitive and specific and, for arboviruses, is usually performed in African green monkey kidney (Vero) cell cultures. The principle of the assay is that specific neutralizing antibodies in serum will block viral infectivity so that the virus cannot attach to cells. Any virus that is not neutralized will initiate as a plaque, which develops when the infected cell monolayers are maintained under media solidified by agarose. To easily visualize the plaques, the media is supplemented with neutral red, a vital dye. Plaques characteristically form as colorless, round areas, where the cells have been killed by the virus. These appear against a red background of viable cells, and can be quantified. If antibodies are present, a reduction in the number of plaques occurs if the virus is neutralized (Beaty et al, 1989).

Serum neutralizing antibody is primarily IgG antibody (FBE & FDOH, 2000). Unlike IgM molecules, IgG antibodies are effective at neutralizing viruses and predominate in the blood, lymph, and CSF. The IgG class also has the longest half-life of all the immunoglobulin isotypes, as well as agglutinating properties (Benjamini et al, 2000). Serum neutralizing antibody, especially IgG molecules, may persist for life after some viral infections, as seen for SLE and dengue viruses (FBE & FDOH, 2000).

The PRNT is a quantitative assay that requires precision in pipetting and must be performed under stringent biosafety requirements (Beaty et al, 1989). A protocol developed nearly 30 years ago at the CDC is still in use today, with minor modifications (Lindsey et al, 1976). Briefly, a virus stock is titrated so that the challenge virus contains approximately 200 PFU/0.1 mL (plaque forming unit). Test sera are inactivated (56°C for 30 min) to destroy endogenous complement and are serially diluted two-fold in

24

Minimal Essential Media, Earle's Salts (EMEM) (Sigma, Cat. No. M0275), 1% Bovine Serum Albumin (BSA), 1% hepes and then combined with an equal volume of challenge virus diluted in the same media plus labile serum factor (Beaty et al, 1989; Chappell et al, 1971). The serum-virus mixtures are incubated overnight at 4°C before 100 µl of this mixture (containing 100 PFU) is inoculated onto Vero cells in six-well plates. After a one hour adsorption period, cells are overlaid with media solidified with agarose. Timing of the second overlay with the vital dye is virus specific and depends on the incubation period for each virus. Plaques are counted and serum antibody titers are determined based on specified plaque reduction levels, commonly 80, 90, or 95% (Beaty et al, 1989).

Surveillance Case Definition for Arboviral Encephalitis

Clinical Description

A spectrum of illness in humans may result from arboviral infections including asymptomatic infections to CNS disease of variable severity. Arboviral encephalitis is characterized by such symptoms as fever, headache, and altered mental status ranging from confusion to coma. Cases are classified as either probable or confirmed based upon laboratory diagnosis (CDC, 1993).

Laboratory Criteria for Diagnosis

For humans, a fourfold or greater rise in virus-specific serum antibody titer; virus isolation or detection of genomic sequences in blood, tissue, or CSF; virus-specific IgM antibodies detected by antibody capture enzyme immunoassay; or detection of virus-specific serum IgG antibodies in the same or convalescent specimen by HAI or PRNT are utilized to confirm an arboviral infection (CDC, 1993). For chickens, demonstration of

25

virus-specific IgM antibodies by MAC-ELISA or detection of virus-specific serum IgG antibodies by PRNT is confirmation of arboviral infection (FBE & FDOH, 2000).

Surveillance: A Team Approach

Surveillance systems are most effective when data is shared and feedback strategies are built into the program, so that results can trigger a change in action. For example, the Florida Arbovirus Response Plan is designed for control measures that must be activated when surveillance detects arboviral activity in an area. At Response Level 2, where wide-spread detection in sentinel flocks, wild birds, or mosquitoes occurs, a Department of Health (DOH) declared medical alert will be considered for affected counties. Additional sentinel chickens may be placed to increase surveillance activities and the Department of Agriculture and Consumer Services (DACS) can issue a mosquito declaration, with increased vector control measures, such as aerial adulticiding. Sentinel chicken surveillance data are summarized and reported weekly by the Florida Bureau of Laboratories (FBOL). The state Bureau of Community Environmental Health summarizes the data on a weekly basis and provides the information to interagency partners, DOH County Health Departments, and the CDC (FDOH, 2002). Consequently, coordination between multiple state agencies, counties and mosquito control districts, is crucial for implementation and success of the arboviral surveillance program.

Objectives

The use of sentinel chickens for surveillance of arbovirus transmission is well-established. A sentinel chicken surveillance program is flexible; it may be expanded or decreased depending on the season and risk for mosquito-borne diseases, and fiscal concerns. Most importantly, this program may be able to detect emerging or reemerging arboviral diseases, as seen with the emergence of West Nile virus in North America. The adaptability of this type of surveillance makes a sentinel chicken program a powerful tool for detection of arboviral diseases, and enable prevention and control strategy implementation before bridging into the human population occurs. The dramatic resurgence of arboviral diseases at the end of the 20th century necessitates more emphasis to be placed on flexible surveillance methods that can accurately detect viral activity and provide early warning.

The HAI assay and the MAC-ELISA are serological detection methods routinely used to diagnose arboviral infections. As a screening assay, the HAI test is very sensitive but broadly reactive with closely related viruses, often resulting in detection of group (e.g. *alphavirus*) antibodies. The MAC-ELISA is utilized as a confirmatory test due to its higher specificity, such that it primarily detects virus-specific (e.g. EEEV) antibodies. For the year 2003, a higher annual seroconversion rate (7.2%) to EEEV was detected in avians in the state of Florida as compared to the average historical rate data. In addition, significantly increased sentinel chicken seroconversion rates to EEEV were detected in

27

the panhandle and northern regions of the state for most months of the year (Stark & Kazanis, 2003). Based upon these findings, an additional confirmatory test, the serum neutralization plaque reduction test (PRNT), may also be warranted to improve existing laboratory methods for the evaluation of EEEV activity in areas where HJV is also enzootic, especially when the MAC-ELISA is not definitive. Inclusion of the PRNT for EEEV/HJV should confirm true EEEV positive sentinels and impact public health response through appropriate medical alerts, vector control, and educational campaigns.

My hypothesis is that the hemagglutination inhibition (HAI) test and IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) are not equivalent in specificity for the detection of antibodies to EEEV when measured against the serum neutralization plaque reduction test (PRNT) due to cross-reactions with Highlands J virus.

This study has four specific aims:

1) To compare the HAI and MAC-ELISA assays with the PRNT to calculate sensitivity, specificity, and predictive values.

2) To evaluate HAI titer as a potential indicator of infecting alphavirus, either EEEV or HJV.

3) To assess the impact of flock management and specimen handling procedures by comparing results obtained by county.

4) To determine counties with HJV activity, and its impact on the Tampa Branch Laboratory (TBL) testing algorithm, including additional cost analysis.

Materials and Methods

Sentinel Chicken Sera Submission and Testing

4,361 adult sentinel chickens were maintained at 285 potential enzootic transmission sites in 35 Florida counties. The chickens were bled and serum samples analyzed for baseline antibody level before placement in the field. Once placed in the field, 1.5 mL to 2.0 mL of blood was collected from each chicken up to 4 times per month, with weekly sampling during peak transmission months (July through December) as in previous years (Blackmore et al, 2003). Clotted blood was centrifuged at 1200 xg for 15 minutes to separate serum and sent for laboratory analysis.

Sample Selection

County Mosquito Control Districts or County Health Departments throughout the state submitted 44,364 sentinel chicken sera specimens to the Florida Department of Health – Bureau of Laboratories, Tampa Branch (TBL) for arboviral serology testing. Samples submitted between 01/01/2003 to 12/31/2003 were screened for presence of antibody to *alphavirus* (EEEV) and *flavivirus* group (SLEV) antigens with the hemagglutination inhibition (HAI) antibody test. Sentinel chickens that were positive for the first time were confirmed EEEV positive with the IgM-capture enzyme-linked immunosorbent assay (MAC-ELISA), quantity of sera permitting. Specimens positive in HAI (titer $\geq$ 1:10) and MAC-ELISA (P/N > 1.6) for *alphavirus* antibody were stored at -70°C for future PRNT assays (Figure 3).

29

Based upon a population size of 4,361 individual birds, a power of 80% and a

95% confidence interval, a total sample size of 353 for PRNT was determined using the

NCS Pearson Sample Size Calculator (www.ncs.com).

Figure 3 Florida Department of Health- Tampa Branch Laboratory Diagnostic Testing
Algorithm for Detection of EEEV in Sentinel Chicken Sera.

Sera Received/Assigned Lab #/Centrifuged

HAI Test

EEEV +   EEEV –

IgM Capture ELISA   Discard

EEEV +   EEEV –

Confirmed   Confirm in PRNT
EEEV & HJV
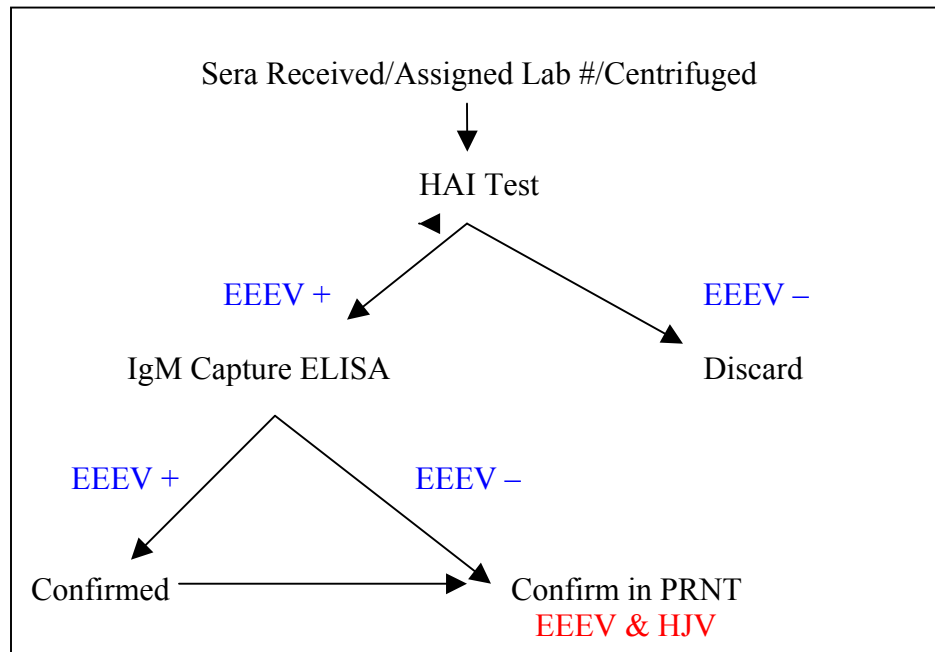
Sera Analysis

Hemagglutination Inhibition (HAI) Test

Serum samples received each week by noon on Wednesday were assayed in the same test. All sera were centrifuged at 2000 xg for 10 min, 4°C (Beckman Coulter, Allegra 6R) for further clarification. For the HAI antibody test, serum samples were treated with protamine sulfate (Holden et al, 1966), acetone extracted and assayed by the method of Clarke and Casals (1958) in microtiter plates. Hemagglutinating antigens were prepared from suckling mouse brains by the sucrose-acetone-extraction (Schmidt, 1979) and betapropriolactone-inactivation (Sever et al, 1964) method.

Antigens were titrated and screened for optimal pH activity as determined by the highest dilution that yielded complete agglutination. For EEEV antigen, the optimal pH was usually 6.2-6.4. Working dilutions of antigens were pre-titered before use in the HAI assay, using the predetermined pH value, to assure a working antigen dilution containing 8 HA units per 25 µl. Twofold serial dilutions of chicken sera (25 µl) starting at a 1:10 dilution through 1:40 were tested, including a serum control for each sample, as well as known positive and negative controls. 25 µl of antigen was added to all sample wells, except the sera control well, and incubated overnight at 4°C. The next morning a 1:40 dilution of standardized goose RBCs (50 µl) was added to each well and a back titration of the antigen was performed. Plates were incubated at room temperature for approximately 90 minutes before being evaluated and scored for presence of agglutination (complete, partial, trace, or none). Antibody titers were recorded for positive (1:10, 1:20, or ≥ 40) and negative (<10) specimens.

31

The HAI assay detected the presence of group antibody to *flavivirus* (with SLEV P15 SMB sucrose-acetone-extracted antigen) and to *alphavirus* (with EEEV D64-837 SMB sucrose-acetone-extracted antigen). Sentinel chicken sera that were positive for the first time, "acute phase", for SLEV group or EEEV group antibody were designated serum 1 (S1) and were tested in the MAC-ELISA for specific antibody to WNV or EEEV, respectively. Eastern equine encephalomyelitis virus IgM-negative birds were re-bled within one week to confirm infection. These later "convalescent phase" serum samples were designated S2, S3, etc. Repeat positive specimens in the HAI assay were presumed EEEV confirmed (Blackmore et al, 2003), and S2 sera that were negative for hemagglutinating antibodies to EEEV were designated "non-confirms". Sera from positive sentinel chickens were saved, regardless of later negative HAI results for S2 or S3 samples. Samples were then confirmed in the MAC-ELISA and PRNT assays for EEEV infection, if sufficient volume of sera remained.

IgM Antibody Capture Enzyme-Linked Immunosorbent Assay (MAC-ELISA)

Specimens positive in the HAI assay were tested for virus-specific antibodies to EEEV in the MAC-ELISA test, performed as previously described (Martin et al, 2000). Viral and normal antigens were prepared by sucrose-acetone extraction of suckling mouse brains (Beaty et al, 1989) and were obtained from the Centers for Disease Control and Prevention (CDC), Fort Collins, Colorado. Alphavirus horseradish peroxidase conjugated monoclonal antibodies (2A2C-3) (WEE strain McMillan, Cat. No. VS2371) were also obtained from the CDC, Fort Collins.

Protocol

Briefly, the test was performed as follows: goat anti-chicken IgM, lyophilized (ICN Biomedicals, Inc., Cat. No. 64-395) capture antibody was diluted 1:1000 in carbonate-bicarbonate buffer (0.015M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6) and used as a coating buffer (75 µl/well) for Immulon-2 flat-bottom 96-well microtiter plates (Dynatech Industries, Inc., Cat. No. 011-010-3450). Plates were incubated in humidified chambers overnight at 4°C after the addition of capture antibody and antigen as well as for 1 hour at 37°C after addition of sera and conjugated monoclonal antibodies (Martin et al, 2000).

Coating antibody was removed from the plates, which were then blocked with phosphate-buffered saline (PBS) containing 5% nonfat dry milk and 0.5% Tween 20 solution (200 µl/well) for 30 minutes at room temperature. A standard 5 washes with 0.05% Tween 20 PBS solution (wash buffer) using an automatic microtiter plate washer (Skatron, SkanWasher 400) were used except before the addition of substrate where 10 washes were employed to reduce overall background (Martin et al, 2000). Chicken sera (test and controls) were diluted 1:400 in wash buffer (50 µl/well). Sera were assayed in duplicate, and known positive and negative control chicken sera for IgM antibodies to EEEV were included on each plate tested. Antigens were diluted 1:400 as determined by standardized assays in wash buffer, and 50 µl of EEEV suckling mouse brain (SMB) antigen or normal mouse brain (NMB) antigen was added to the appropriate wells (Martin et al, 2000).

Conjugated alphavirus horseradish peroxidase (2A2C-3-HRP) monoclonal antibodies were diluted 1:4000, and 50 µl was added to each well. 75 µl of 3,3'5,5'

33

tetramethylbenzidine substrate (TMB) (Sigma, Cat. No. T-8665) was added to each well.

The plates were immediately covered to block out light (TMB is light sensitive) and

incubated for 10 minutes at room temperature. A "stop" solution of 1 N H_2SO_4 (Fisher

Scientific, Cat. No. SA212-1) was added to each well (50 µl/well) to stop the reaction.

Plates were swirled to gently mix the solutions and allowed to stand for 1 minute. Plates

were read in the microtiter plate reader (Beckman Coulter, AD340) at a wavelength of

450 nm (Martin et al, 2000).

Figure 4 Simplified Flow Chart of Steps for the Sentinel Chicken MAC-ELISA, as
  Performed at the Florida Department of Health, Tampa Branch
  Laboratory. Tests were performed in 96-well microplates and steps were
  followed in the sequence outlined here.

IgM Capture antibody

↓ wash x 5

Blocking buffer

↓ wash x 5

Chicken sera, + & - controls

↓ wash x 5

SMB & NMB Antigens

↓ wash x 5

Alphavirus Conjugate

↓ wash x 10

TMB Substrate

↓

Stop Solution

↓

Read Plate @ 450 nm

34
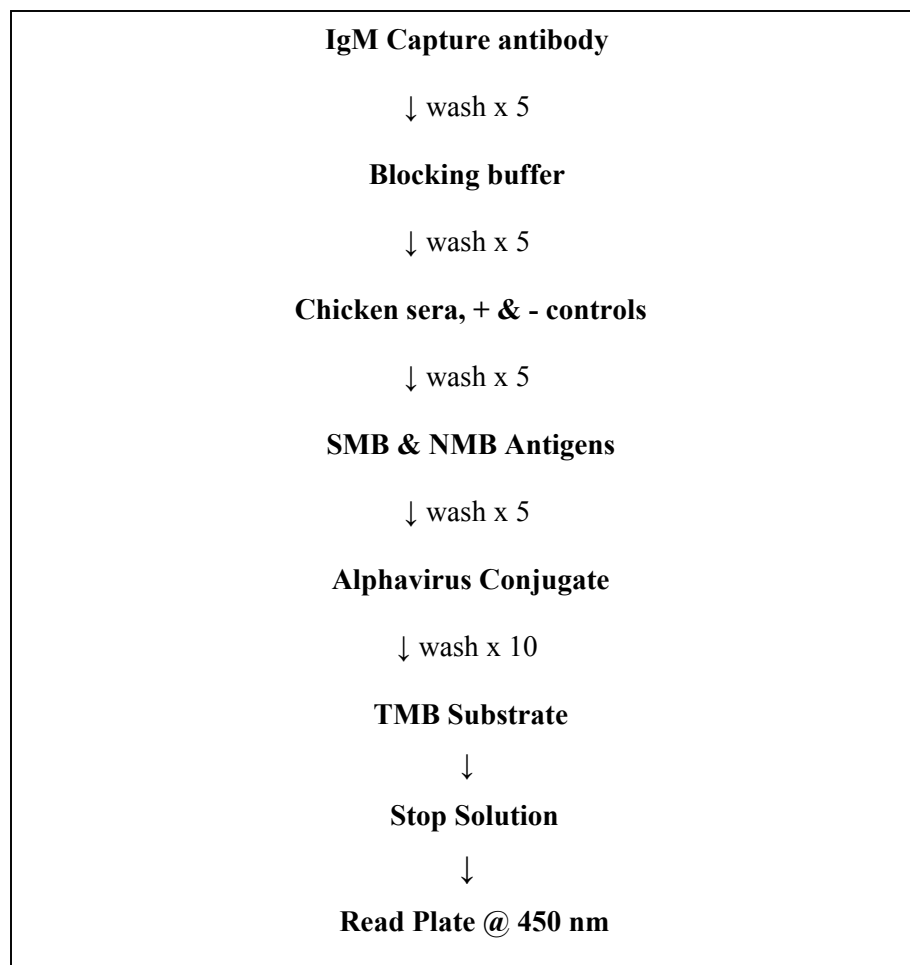
Interpretation of Results

Positive to negative (P/N) ratios were computed for the positive control serum equaling the mean optical density (OD) of the positive control serum with viral antigen divided by the mean OD of the negative control serum with viral antigen. The P/N value for the positive control serum must be ≥ 2.0 and the negative control serum P/N must be < 2.0 for a valid test. Additionally, the ratio of the OD of the positive control serum with viral (SMB) antigen to that of the positive control serum with control (NMB) antigen must be >2.0. P/N values do not indicate absolute antibody concentration, and as such are not quantitative. For test specimens, the P/N value was computed by dividing the mean OD of the test serum with viral antigen by the mean OD of the negative control serum with viral antigen. A specimen was considered positive for IgM antibodies if the P/N value was ≥ 2.0. A specimen was considered to have an equivocal result if the P/N value was in the range of 1.6 to 1.999. In addition, the mean OD of the test serum with viral antigen divided by the mean OD of the test serum with NMB was calculated for each sample (P/N ≥ 2.0) to ensure that non-specific background noise was not generated (Martin et al, 2000).

Samples positive/equivocal for IgM antibodies to EEEV were reported positive for infection. Eastern equine encephalomyelitis virus IgM-negative chickens were re-bled within one week to confirm alphavirus infection. Repeat HAI-positive (S2) sera without S1 IgM antibodies to EEEV were also classified as EEEV infected.

Serum Neutralization Plaque Reduction Test (PRNT)

Volume of samples permitting, all S1 sera with positive, equivocal, or negative results for IgM antibodies to EEEV in the MAC-ELISA, as well as S2 sera, were assayed

35

in a serum neutralization plaque reduction test (PRNT) challenged with Eastern Equine Encephalomyelitis virus strain D64-837 (SM8-BGM2 04/17/94) and Highlands J virus strain 64A-1519 (SM4-BGM 04/17/94). PRNT assays were performed with African green monkey kidney (Vero) cells, as previously described (Schmidt, 1979; Beaty et al, 1989). All PRNT assays were performed in a biosafety cabinet in a BioSafety Level 3 laboratory due to security concerns regarding the Select Agent status of EEEV, despite its classification as a BioSafety Level 2 agent. Endpoints were determined at a 90% plaque reduction level.

Vero Cell Cultures

Vero cells (ATCC, Cat. No. CCL-81 passage 143-149) were seeded into tissue culture six-well plates (Falcon, Cat. No. 35520), grown in 3 mL/well EMEM and 5% fetal calf serum (FCS). Plates were incubated at 37°C, 4% CO_2 until approximately 90% confluent (4 days).

Titration of Viral Stocks

EEEV and HJV stock ampoules were removed from a -70°C freezer and quick thawed in a water bath at 37°C. On ice, viruses were diluted in serum virus diluent (SVD), consisting of EMEM with antibiotics, 1% Bovine Serum albumin, and 1 % Hepes. For viral titrations only, 8% FCS, not heat-inactivated (Chappell et al, 1971), was added to the SVD. Each virus was titrated (from a dilution of 10^{-1} through $10^{-6.5}$ for EEEV and from 10^{-1} through $10^{-5.3}$ for HJV) and inoculated onto Vero cells to determine the challenge concentration containing 200 PFU/100 μl (Beaty et al, 1989). For EEEV, the challenge virus titer was determined to be $10^{-4.3}$/100 μl. The challenge virus titer was shown to be $10^{-2.7}$/100 μl for HJV. For each assay, back titration plates were prepared in

36

duplicate for each virus so that the virus titer obtained in that test could be computed and plaque reduction levels determined (Beaty et al, 1989).

Serum Dilution

Each sentinel chicken serum sample was aliquoted into a sterile tube and diluted 1:5 in SVD. Three known positive and three negative control chicken sera (based on MAC-ELISA results) were included with each assay run. Diluted sera were incubated at 56°C for 30 minutes to inactivate endogenous complement in the specimens. Serial twofold dilutions of the sera were performed in sterile 96-well microtiter plates (Falcon, Cat. No. 353072), starting at the 1:5 dilution. Each serum sample was assayed simultaneously for EEEV and HJV in two separate 96-well plates. Addition of an equal volume of challenge virus dilution to each well resulted in final sera concentrations of 1:10, 1:20, and 1:40. Sera/viral mixtures were incubated overnight at 4°C (Beaty et al, 1989). For sera specimens resulting in antibody titers >40 for both EEEV and HJV, additional dilutions (up to 20,480) were performed in another assay run to distinguish the infecting virus in the heterlogous reaction.

Preparation of Media/Agarose Overlays

Overlay media (2X EMEM) and Seakem agarose (Cambrex BioScience, Cat. No. 50004) for the first overlay were prepared prior to serum/virus inoculations into Vero cells. 2X EMEM, 4% FCS media was prepared fresh for use, volume dependent on the number of plates required in each test. Agarose and reagent grade water were mixed in a separate flask larger than two times the volume of the water, and autoclaved at 15 psi, 121° for 15 minutes, 30 minutes prior to end of the sera/virus adsorption period in Vero

37

cells. The agarose was allowed to cool until the flask was just warm to the touch before aseptically combining with the 2X EMEM media (Beaty et al, 1989).

Inoculation of Vero Cell Cultures

Media was aspirated from the Vero cells leaving approximately 50 µl per well. Plates were inoculated with 100 µl of each serum/virus dilution, one dilution per well, and with 100 µl back titration virus dilutions. Plates were incubated for 1 hour in a 37°C, 4% CO_2 incubator, and were rocked every 10 minutes. 3 mL of media agarose overlay was added to each well and plates were swirled gently to mix the liquid inoculum into the overlay. Plates were allowed to cool completely on a level surface before incubation in an inverted position at 37°C, 4% CO_2 (Beaty et al, 1989).

Timing for the second overlay containing the neutral red indicator dye was dependent on the growth characteristics of each virus. Observation indicated that for EEEV, the second overlay should be added one day post-inoculation (PI) and plates counted at 48 hours PI (similar to timing described by Main et al, 1988). However, the second overlay for HJV was added 2 days PI and plates were counted at 72 hours PI, instead of 6 days later (Beaty et al, 1989). Plates were inspected on a light box and plaques were counted for each on two consecutive days. Specimens with 90% plaque reduction levels as compared with the back titration controls were confirmed positive for viral infection with either EEEV or HJV.

38

Results

A retrospective study of previously tested sentinel chicken sera was conducted to determine potential cross-reactions between EEEV and HJV in the HAI antibody test and MAC-ELISA by assay in the PRNT. In 2003, 285 of 4,361 sentinel chickens were confirmed EEEV positive. Of those, 126 were confirmed by repeat positive in the HAI test and 159 were confirmed positive by the MAC-ELISA.

A total sample size of 353 birds was determined with the use of the NCS Pearson: Sample Size Calculator (www.ncs.com) as appropriate for this study. The actual number of birds tested in the PRNT was 316, including a mixture of the above confirmed positive chickens (214 were tested, 71 had insufficient sera for PRNT) and birds that did not confirm by either the HAI or MAC-ELISA assays. Some chickens had to be removed from the study due to apparent switching of the birds (n=6) where PRNT results indicated these birds were positive for antibodies to EEEV for their S1 sera, negative for EEEV but positive for HJV in the S2 sera, and positive for antibodies to EEEV for their S3 sera but negative for HJV. These results are doubtful based upon physiologic properties of IgG antibodies, which remain elevated over time, and indicate that these birds were either switched in the field or mislabeled when received at the lab. In addition, chickens with insufficient S2 sera for testing in the MAC-ELISA (n=3) had to be discarded, as these sentinels' first and second sera were not confirmable in the MAC-ELISA. Sample populations in the two tests were identical (i.e. the same birds were tested in all assays)

39

for comparative purposes. The sample size would have exceeded the ideal number except volume of sera remaining did not permit testing in the PRNT for many chickens, likely decreasing the power of the study. A total of 428 sera samples were tested, including 94 sentinels with paired sera (188 total sera), which are only included for comparison of S1 versus S2 outcomes in the PRNT.

The HAI and MAC-ELISA tests were compared to the PRNT assay, the gold standard, for arboviral antibody detection, and to calculate sensitivity, specificity, positive predicted value (PPV), and negative predicted value (NPV) using two-by-two tables (Table 1). Univariate comparison of dichotomous outcomes in the HAI and MAC-ELISA was performed using McNemar's Test (statistical analyses generated from SAS 8.02 © 1999-2001, SAS Institute Inc. Cary, North Carolina). Regression modeling for HAI titers and MAC-ELISA P/N ratios were correlated to PRNT titers (statistical analyses generated from Analyse-it® General 1.71 © 1997-2002, Analyse-it Software, LTD, a Microsoft Excel add-in). Statistical analyses were generated with the assistance of Angela E. Butler.

Detection of *Alphavirus* Antibodies by Serum Neutralization Plaque Reduction
Hemagglutination Inhibition (HAI) Test

Eastern Equine Encephalomyelitis virus

From a sample size of 316 chickens, the HAI test correctly identified 175 positive sentinel seroconversions after exposure to EEEV. The HAI test falsely detected 119 chickens as EEEV antibody positive: 68 of these sentinels were positive for HJV antibodies and 51 birds were negative for antibodies to both viruses. Conversely, a negative HAI titer (<10) resulted in 1 chicken as EEEV antibody positive, 7 birds as HJV

40

Table 1 General 2x2 Table for Calculations of Sensitivity, Specificity, Positive Predictive Value, and Negative Predictive Value.

Exposure (Serologic Assay)	Disease (Outcome in PRNT)			Calculations:
	+	-	Totals	Sensitivity = A/A+C
+	A	B	A + B	Specificity = D/B+D
-	C	D	C + D	PPV = A/A+B NPV = D/C+D
Totals	A + C	B + D	A + B+ C + D	**x 100 = %**

antibody positive, and 14 birds as true negatives (Table 2). A total of 140 sentinels were determined to be EEEV antibody negative, including a mixture of HJV positive birds and true *alphavirus* antibody negative.

In a 2x2 comparison of these results, sensitivity of the HAI assay is 99%, specificity 15%, positive predicted value (PPV) 60%, and negative predicted value (NPV) 95% (Table 3). The detection of antibodies to EEEV in sentinel chicken sera by the HAI and PRNT diagnostic methods was shown to be significantly different by McNemar's test (P = <.0001 and McNemar's statistic = 116.033).

Linear regression modeling of HAI total antibody and PRNT IgG antibody titers to EEEV (converted to logarithmic scale) showed a positive correlation (P <.0001, r^2 = 0.30) (Figure 5).

Highlands J virus

An evaluation of the 140 negative sentinels described above resulted in 75 HJV antibody positive birds and 65 sentinels negative for both viruses as compared with the PRNT. This comparison determined a sensitivity of 91%, specificity 21%, PPV 57%, and NPV 67% for the detection of antibodies to HJV in an EEEV antigen based HAI test (Table 4).

IgM Antibody Capture Enzyme-Linked Immunosorbent Assay (MAC-ELISA)

Eastern Equine Encephalomyelitis virus

A positive result (P/N $\geq$ 1.6) in the MAC-ELISA test correctly identified 132 positive sentinel seroconversions after exposure to EEEV. The MAC-ELISA falsely indicated 13 chickens as EEEV antibody positive (P/N values ranging from 1.63 to 8.62): 2 sentinels were positive for antibodies to HJV and 11 were negative for both viruses.

42

Table 2 Detection of Antibodies to EEEV and HJV in Sentinel Chickens by the HAI and MAC-ELISA Assays as Compared with the PRNT. A total of 316 sentinels were assayed for the presence of *alphavirus* neutralizing antibodies resulting in 251 *alphavirus* positive sentinels.

| PRNT	HAI EEEV Antigen		MAC-ELISA EEEV Antigen	
Result	+	-	+/equiv	-
EEEV +	175	1	132	44
HJV +	68	7	2	73
Negative	51	14	11	54
Total # of Birds	316		316	

43

Table 3 Detection of Antibodies to EEEV in the HAI Test vs. PRNT. Sensitivity, specificity, and predictive values were calculated based upon this 2x2 comparison for detection of antibodies to Eastern Equine Encephalomyelitis virus. The EEEV – column includes negative and HJV+ sentinels as detected by PRNT and is shaded yellow to indicate further 2x2 analysis of this subset of 140 sentinel chickens.

HAI	Serum Neutralization Plaque Reduction			
	EEEV +	EEEV -	Totals	
EEEV +	175	119	294	Sensitivity 99% Specificity 15% PPV 60% NPV 95%
EEEV -	1	21	22	
Totals	176	140	316	

Table 4 Detection of Antibodies to HJV Based Upon Group *Alphavirus* Reactions in the HAI Test with EEEV Antigen. A 2x2 comparison of the 140 EEEV - sentinels (Table 3- shaded yellow) by PRNT determined sensitivity, specificity, and predictive values.

HAI	Serum Neutralization Plaque Reduction			
	HJV +	Negative	Totals	
EEEV +	68	51	119	Sensitivity 91% Specificity 21% PPV 57% NPV 67%
EEEV -	7	14	21	
Totals	75	65	140	

Figure 5 Correlation Between HAI Antibody Titers and PRNT Antibody Titers for
EEEV Detection. Linear regression modeling detected a positive correlation
between the two tests, indicating that as EEEV antibody titers in the HAI
increased this correlated with rising antibody titers to EEEV in the PRNT.

46
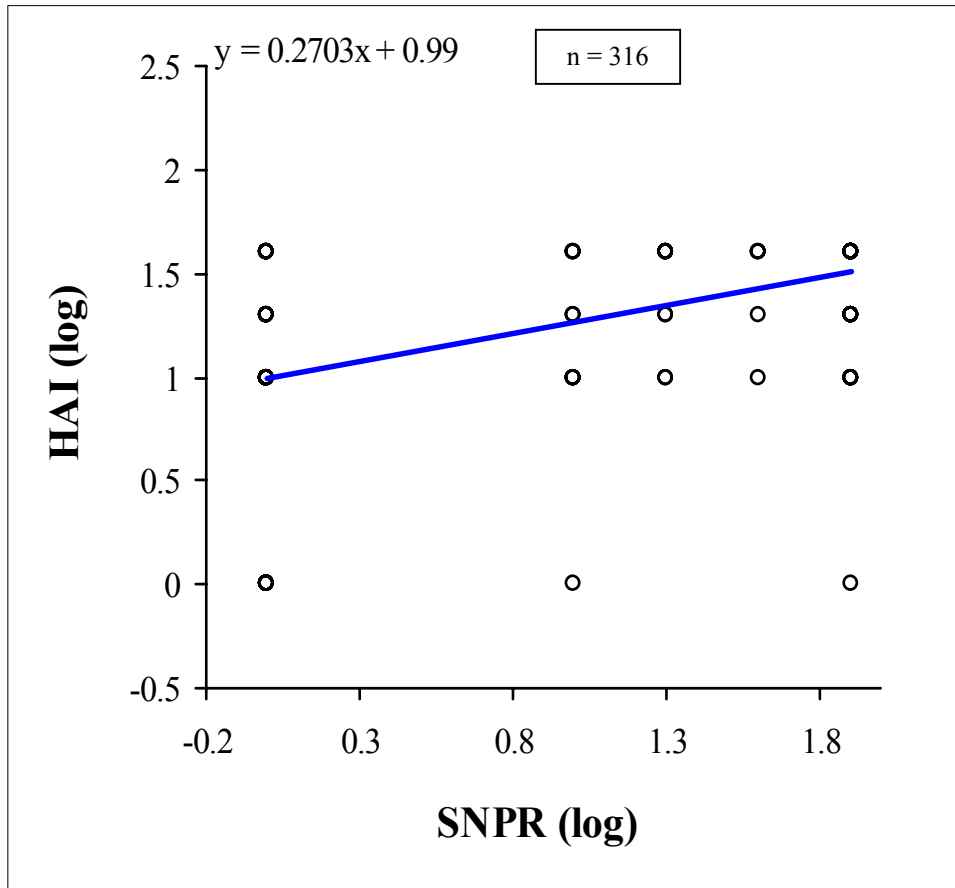
S2 sera were unavailable for confirmation. Of the 117 specimens that did not confirm in the MAC-ELISA (P/N < 1.6), 44 birds had seroconverted to EEEV and 73 seroconverted to HJV as detected in the PRNT. The MAC-ELISA confirmed 54 birds as true *alphavirus* antibody negatives (Table 2).

A 2x2 comparison of these results with those of the PRNT yielded a sensitivity of 75%, specificity 91%, positive predicted value (PPV) 91%, and negative predicted value (NPV) 74% (Table 5). The detection of antibodies to EEEV in sentinel chicken sera by the MAC-ELISA and PRNT diagnostic methods was also shown to be significantly different by McNemar's test (P = <.0001 and McNemar's statistic = 16.860).

Linear regression modeling of MAC-ELISA P/N values (IgM antibody) and PRNT IgG antibody titers to EEEV (converted to logarithmic scale) showed a slight positive correlation (data not shown).

Highlands J virus

An evaluation of the 140 negative sentinels in the MAC-ELISA (shown in Table 5) resulted in 75 HJV positive birds and 65 sentinels negative for both viruses as compared with the PRNT. This comparison indicated a sensitivity of 3%, specificity 83%, PPV 15%, and NPV 42% for the detection of antibodies to HJV in an EEEV antigen based MAC-ELISA (Table 6).

S1 and S2 Paired Sera Analysis

A total of 94 sentinels (out of 316 chickens) had at least two serum samples that were tested in the PRNT. These results indicated that 83% of the S1 (acute) and S2 (convalescent) sera were in complete agreement (n=78). The S1 and S2 sera were discordant in 17% of the sentinels (n=16), due to seroconversions and seroreversions.

47

Table 5 Detection of Antibodies to EEEV in the MAC-ELISA vs. PRNT. Sensitivity, specificity, and predictive values were calculated based upon this 2x2 comparison for detection of antibodies to Eastern Equine Encephalomyelitis virus. The EEEV – column includes negative and HJV+ sentinels as detected by PRNT and is shaded yellow to indicate further 2x2 analysis of this subset of 140 sentinel chickens.

MAC-ELISA	Serum Neutralization Plaque Reduction			
	EEEV +	EEEV -	Totals	
EEEV IgM+/equiv	132	13	145	Sensitivity 75% Specificity 91% PPV 91% NPV 74%
EEEV IgM -	44	127	171	
Totals	176	140	316	

Table 6 Detection of Antibodies to HJV Based Upon Group *Alphavirus* Reactions in the MAC-ELISA with EEEV Antigen. A 2x2 comparison of the 140 EEEV - sentinels (Table 5- shaded yellow) by PRNT determined sensitivity, specificity, and predictive values.

MAC-ELISA	Serum Neutralization Plaque Reduction			
	HJV +	Negative	Totals	
EEEV IgM+/equiv	2	11	13	Sensitivity 3% Specificity 83% PPV 15% NPV 42%
EEEV IgM -	73	54	127	
Totals	75	65	140	

Two sentinels seroconverted from IgG negative to IgG positive after the collection of the S1 sera (PRNT<10) and before the collection time of the second sera drawn (PRNT>40). One sentinel seroconverted within 7 days and the other within 14 days for PRNT IgG antibodies. Conversely, 8 EEEV positive sentinels (7 EEEV + PRNT>40 & 1 EEEV + PRNT=1:10) reverted to negative status (PRNT<10) in their S2 sera, within a range of 7 to 14 days. Six HJV positive sentinels (PRNT>40) reverted to *alphavirus* negative (PRNT<10) in the S2 sera, within a range of 7 to12 days (data not shown).

Analysis of EEEV and HJV Activity for the State of Florida

Out of 30 counties with sentinels tested in the *alphavirus* PRNT, 19 had EEEV activity and14 also had HJV activity. Citrus, Flagler, Jackson, Osceola, and Pinellas counties had EEEV activity, but did not have sentinel seroconversions to HJV (Figure 6). Orange County and Reedy Creek Mosquito Control Districts (Figures 6 & 9), as well as North and South Walton Counties are represented separately due to different sentinel chicken programs within the same county (Figure 6). Data from the two programs within each county were combined, respectively, for a total number of sentinel seroconversions in these two counties.

Alphavirus seroconversion rates were calculated for select panhandle and northern counties based upon confirmation in the PRNT (Figures 7-10). Eleven counties were negative for both viruses when confirmed in the PRNT (Figure 11).

EEEV and HJV activity for the calendar year 2003 was calculated based upon number of sentinel chicken seroconversions per month as determined by PRNT for the sample size tested in this study (n=316) (Figure 12). Onset of EEEV activity occurred in March, peaked in June, and declined over the remainder of the year. HJV activity first

50

appeared in the month of May, peaked in July, and rapidly declined through the month of

November (Figure 12).

Figure 6 Number of *Alphavirus* Antibody Positive and Negative Sentinel Chickens by
County. Out of 30 counties tested with sentinel chickens, 19 had EEEV activity,
14 also had HJV activity. Five of the 19 counties with EEEV activity did not
have concurrent HJV activity. Eleven counties without *alphavirus* positive
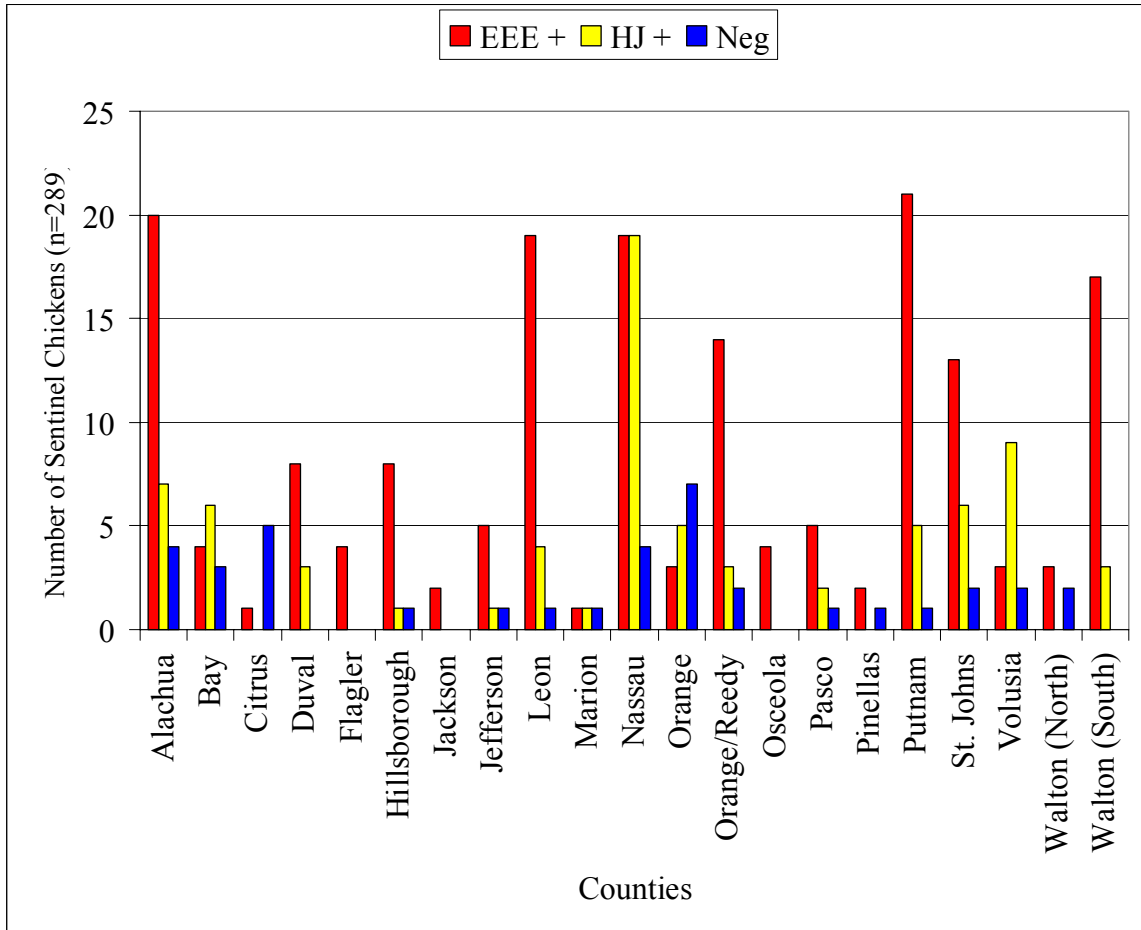sentinels are not shown.

Figure 7 Comparison of Four Florida Panhandle Counties for *Alphavirus* Seroconversion
Rates in Sentinel Chickens.

Bay County (n=13)

23 31
46
■ % EEE + □ % HJ + ■ % Negative

Jefferson County (n=7)

14
14
72
■ % EEE + □ % HJ + ■ % Negative

Leon County (n=24)

4
17
79
■ % EEE + □ % HJ + ■ % Negative

S. Walton County (n=20)

15 0
85
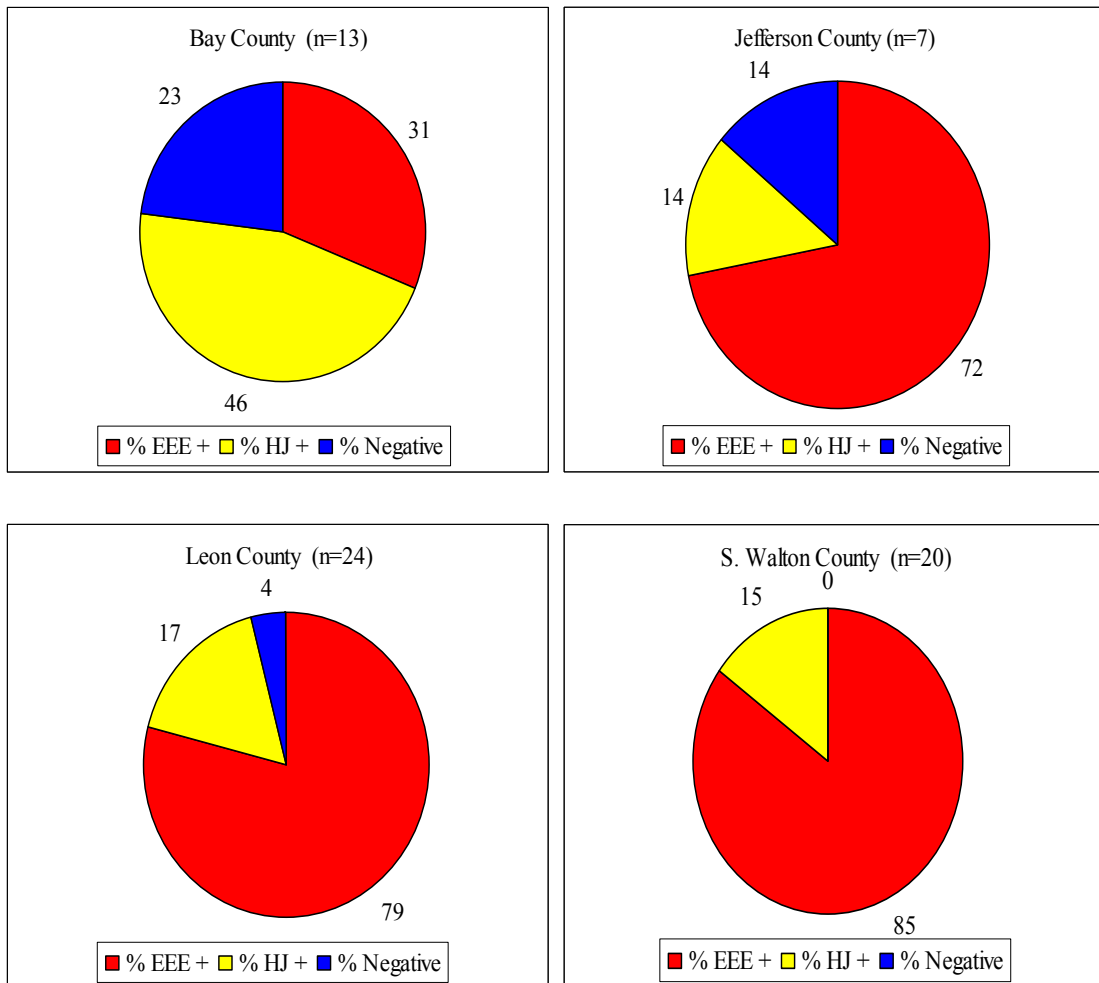■ % EEE + □ % HJ + ■ % Negative

53

Figure 8 Comparison of Four Northern Florida Counties for *Alphavirus* Seroconversion
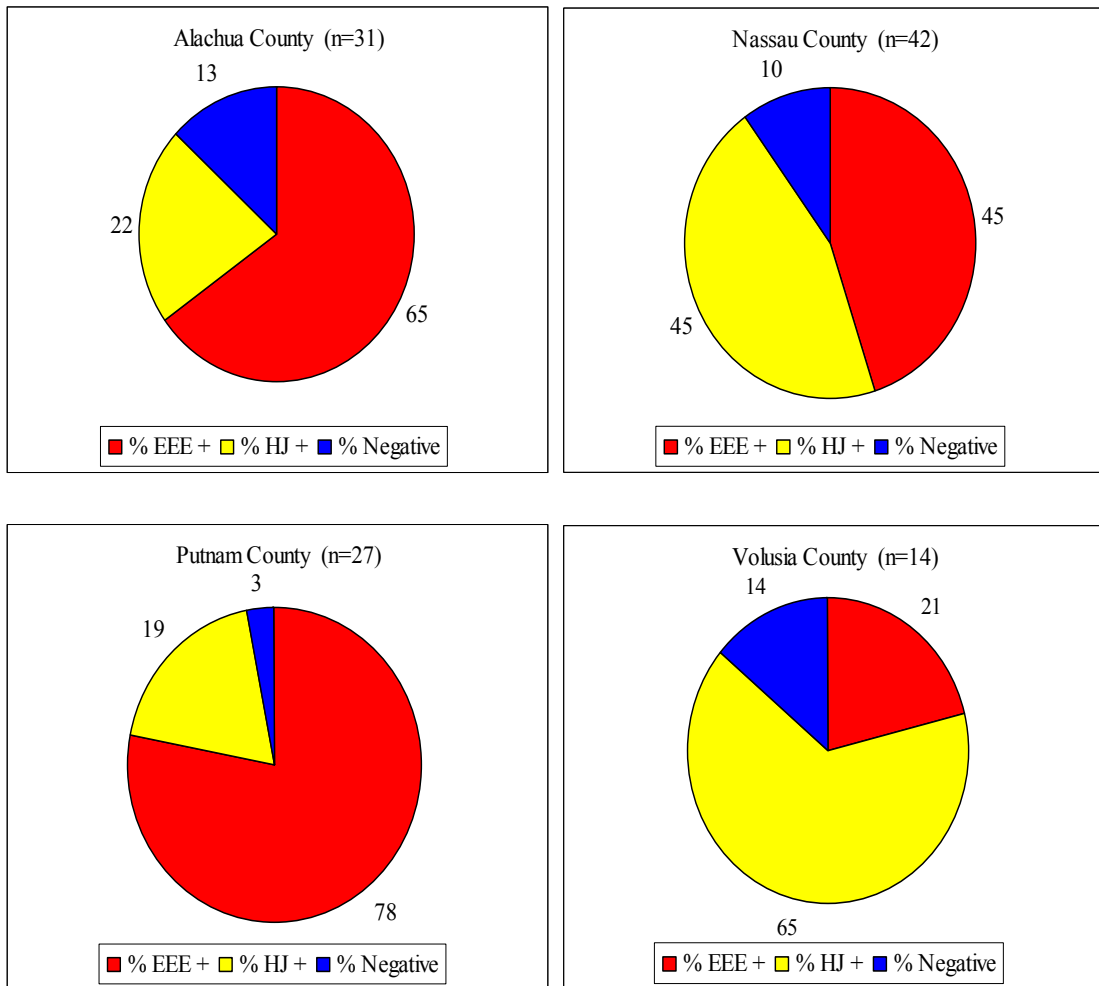   Rates in Sentinel Chickens.

Figure 9 Comparison of Florida's Orange County and Reedy Creek Mosquito Control Districts for *Alphavirus* Seroconversion Rates in Sentinel Chickens.
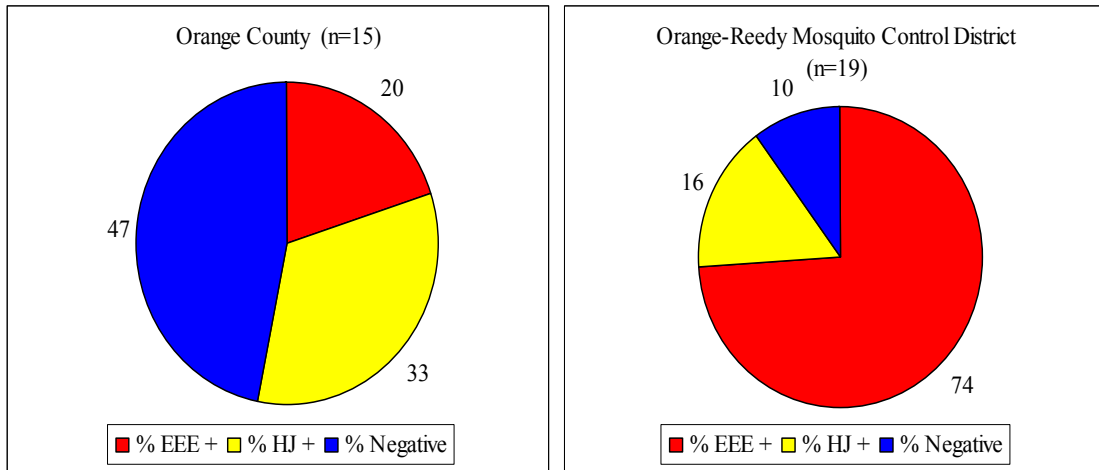
Figure 10 Comparison of Two Northern Florida Counties, St. Johns and Citrus, for *Alphavirus* Seroconversion Rates in Sentinel Chickens.
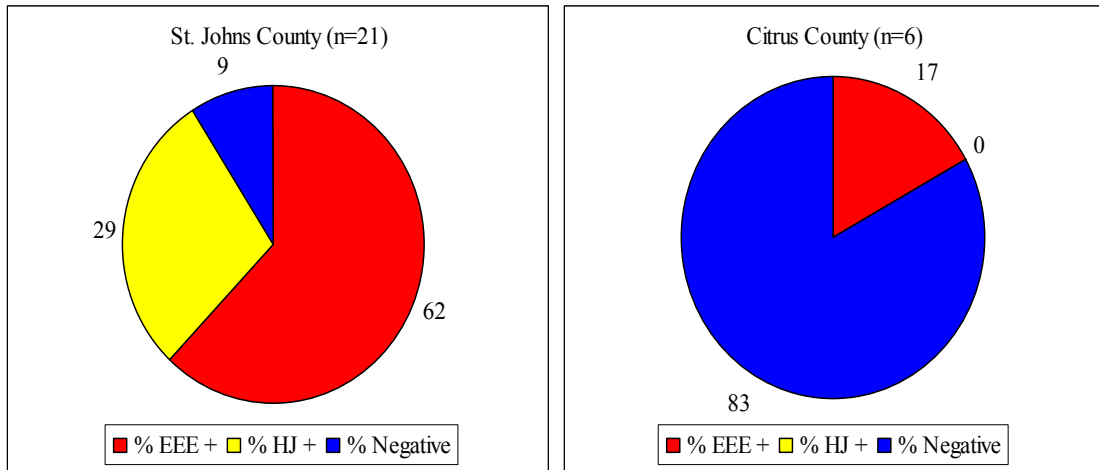
Figure 11 Map of the State of Florida Identifying Counties with Eastern Equine
Encephalomyelitis Virus and Highlands J Virus Activity as Confirmed by
PRNT of Sentinel Chicken Sera Submitted. Five counties (Citrus, Flagler,
Jackson, Osceola, and Pinellas) did not have sentinel seroconversions to
Highlands J virus when EEEV activity was also detected. In counties colored
blue, sera samples were tested but were negative for antibodies to EEEV and
HJV. One county (Citrus) had one sentinel positive for antibodies to EEEV,
but five sentinels negative for both viruses, as shown on the map with a
mixture of red and blue colors. Counties not shaded either did not participate
in the Sentinel Chicken Program or were not tested in the PRNT for detection
of antibodies to EEEV or HJV due to insufficient sera remaining for analysis.

**Florida Counties with Eastern
Equine Encephalomyelitis virus &
Highlands J virus Activity**

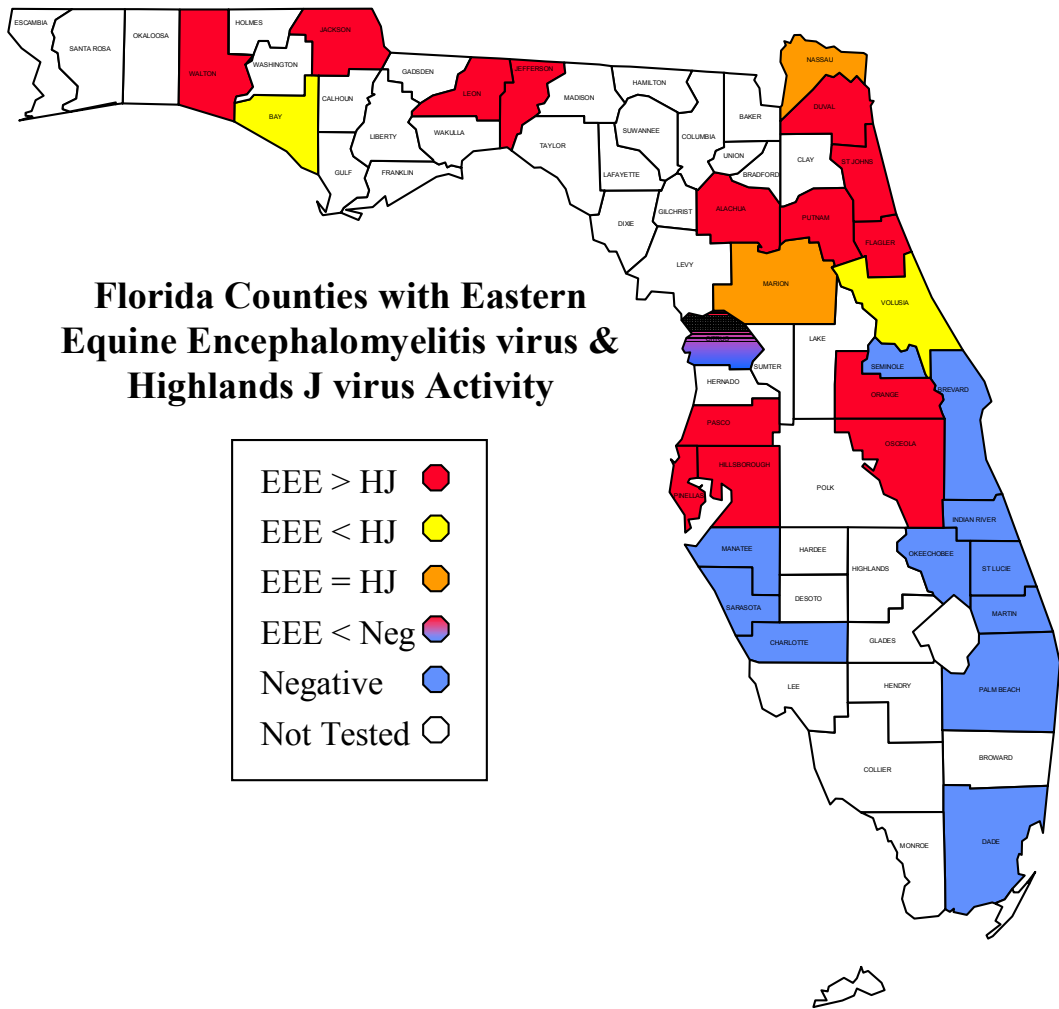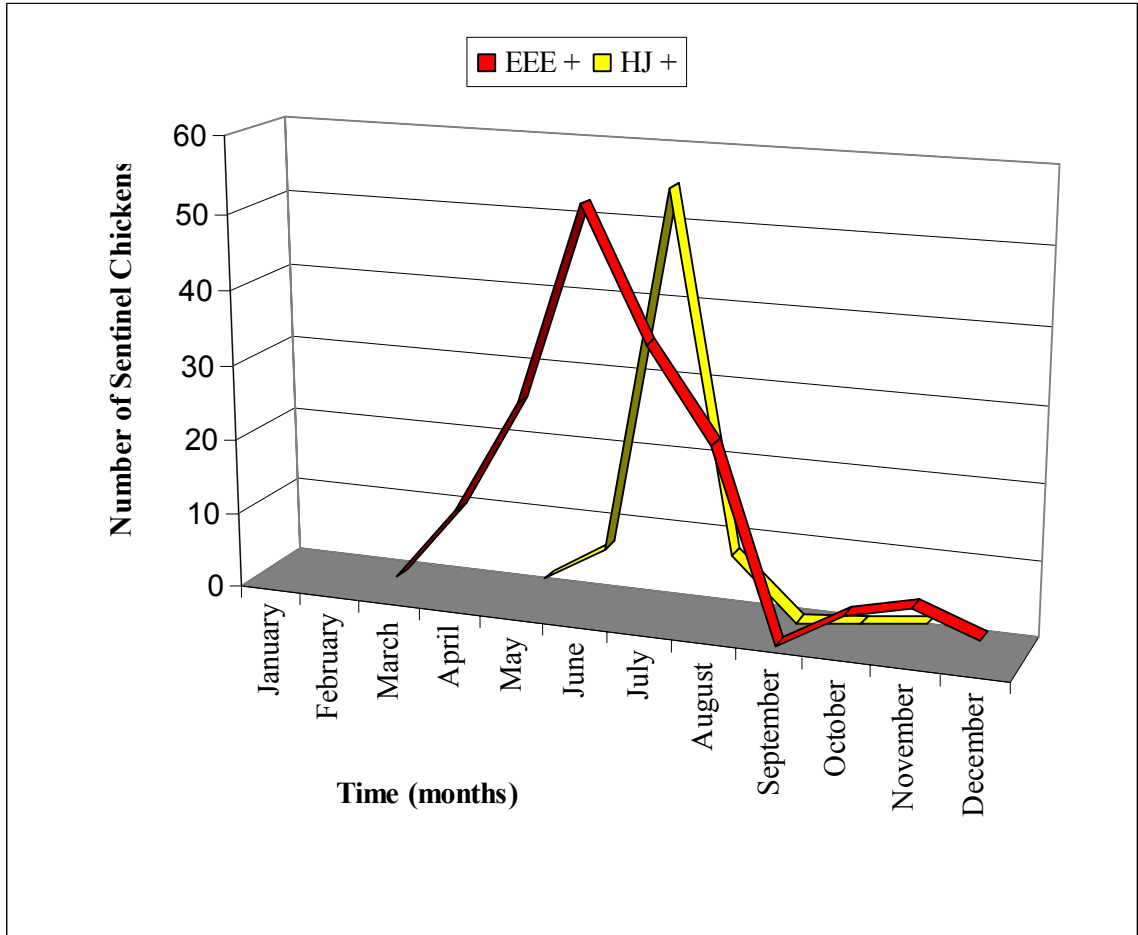|	
EEE > HJ	🔴
EEE < HJ	🟡
EEE = HJ	🟠
EEE < Neg	🔵
Negative	🔵
Not Tested	⚪

Figure 12 *Alphavirus* (EEEV and HJV) Activity Over Time (in months) for 2003. Onset
of EEEV activity occurred in March, peaked in June, and declined over the
remainder of the year. HJV activity first appeared in the month of May,
peaked in July, and rapidly declined through the month of November.

Discussion

Eastern equine encephalomyelitis virus is an important public health pathogen that maintains year-round enzootic transmission foci in Florida. Surveillance for this highly virulent agent and other endemic arboviruses, such as SLE and WN viruses, is necessary to prevent epidemic transmission activity and protect the health of the human and veterinary population. The Sentinel Chicken Program is an integral component of arboviral surveillance for the state of Florida and has proven to be a successful monitoring tool for early detection of arboviral activity (Blackmore et al, 2003).

Sentinel chickens detected significantly greater EEEV activity in 2003 than seen in the previous fifteen years (Stark & Kazanis, 2003). This increase raised concerns that bridging into the human population was a serious threat as well as an important issue for veterinary health. Fortunately, only 3 human cases were detected in 2003, although EEEV was still a significant contributor to equine morbidity and mortality with 207 infected horses (Collins & Blackmore, 2003).

This study was initiated to determine if cross-reactions with Highlands J virus, a closely related *alphavirus*, were impacting the serologic diagnostic tests routinely performed for identification of EEEV. In addition, the results of this study may help, in part, our understanding of why sentinel chickens have historically been less effective prognosticators of EEEV than SLEV transmission in Florida (Day & Stark, 1996b; Bigler WJ et al, 1976).

59

Based upon comparison with the serum neutralization plaque reduction test (PRNT), results indicate that the HAI test is highly sensitive but poorly specific for the detection of antibodies only to EEEV whereas the MAC-ELISA is both sensitive and highly specific (Figure 13). The predictive values (PPV and NPV) for the HAI test and the MAC-ELISA are very different (Figure 14), indicating that the MAC-ELISA is the better test for confirmation of true EEEV positive sentinel chicken seroconversions, whereas the HAI test is effective at detecting antibody negative sera.

Our results have shown that Highlands J virus is extensively cross-reactive in the hemagglutination inhibition (HAI) test as exemplified by its sensitivity of 91% for the detection of antibodies to HJV (Figure 15). By virtue of this fact, the HAI assay has proven to be very sensitive for the detection of *alphavirus* positive sentinels. Conversely, an EEEV antigen based MAC-ELISA is effective at limiting the false detection of cross-reactive viruses with 3% sensitivity and 83% specificity for the identification of antibodies to HJV (Figure 15). The predictive values for the HAI test and MAC-ELISA reflect the influence of cross-reactions with Highlands J virus on these serologic assays (Figure 16).

All of these results indicate that the HAI assay is an excellent screening test for the presence of *alphavirus* antibodies in serum and that the MAC-ELISA is highly effective for the confirmation of EEEV positive sentinels. This is in agreement with our statistical analyses comparing each assay with the PRNT by McNemar's test. Both assays were highly sensitive for the detection of antibodies to EEEV (P = <.0001). However, McNemar's statistical value equaled 16.86 for the MAC-ELISA indicating that it is more comparable to the PRNT than the HAI test, which had a greater statistical value of

60

116.03. Therefore, we conclude that the HAI and MAC-ELISA tests are not equivalent in specificity for the detection of antibodies to EEEV when compared with the serum neutralization plaque reduction test (PRNT).

Linear regression modeling of HAI antibody titers vs. PRNT antibody titers show a positive correlation (P = <.0001, r^2=0.30) indicating that as HAI antibody titers increase so do PRNT antibody titers for EEEV. Consequently, cautious interpretation of sentinel chickens with repeat low HAI titers (< 40), especially if their S1 sera are EEEV IgM negative, is recommended until confirmation of the infectious agent by PRNT as probable EEEV or HJV.

The PRNT identified 176 EEEV antibody positive sentinel chickens and 75 HJV antibody positive sentinels from a total of 316 tested. Sixty-five sentinels were negative for antibodies to both viruses as shown in the PRNT. Out of these neutralizing antibody negative sera, the MAC-ELISA found 11 sentinels positive or equivocal for EEEV IgM antibodies. It is likely that these sera were drawn early in the stage of infection, so that IgM antibodies had developed but neutralizing IgG antibodies were not yet present at a detectable level (Figure 1) resulting in no antibody quantifiable by the PRNT. This hypothesis could not be tested since second sera samples were unavailable for verification, however, these specimens may have contributed to a decreased sensitivity and lower predictive positive value for the MAC-ELISA as compared with the PRNT, if they were in fact acute EEEV viral infections.

Out of 94 birds with paired sera, 2 sentinel seroconversions to EEEV were noted. One sentinel was negative in the MAC-ELISA (and PRNT) for its S1 serum, but positive in the MAC-ELISA for the S2 serum sample with a PRNT antibody titer > 40. This

61

sentinel was included in the statistical tests by its S2 sample. The other sentinel was negative in the MAC-ELISA for both the S1 and S2 sera, but the S2 serum resulted in a PRNT antibody titer > 40 for EEEV. As with the other sentinel seroconversion, this chicken was analyzed in the statistical tests by its S2 sample.

Further analysis of MAC-ELISA negative S2 sera resulted in 5 EEEV + chickens, 10 HJV + sentinels, and 11 true negative sentinels based upon the PRNT. Negative results in the MAC-ELISA were even more compelling for S1 sera where the PRNT detected 39 EEEV + sentinels, 58 HJV + chickens, and 42 true negatives. These findings indicate that confirmation of HAI positive and IgM negative sentinel chickens needs to be performed by PRNT since 25% of the EEEV antibody positive sentinels found in this study were MAC-ELISA negative (n=44). IgM levels in chickens are transitory in nature and can diminish seven to fourteen days following infection (Olson et al, 1991). This likely impacted these MAC-ELISA results, especially if the blood samples were drawn during this period when IgM levels are low or non-detectable and IgG levels are rising, so that a sentinel can be negative in the MAC-ELISA but positive by PRNT for antibodies to EEEV.

Reversions (seropositive to seronegative) were detected in fourteen of the chickens with paired sera, from either EEEV + or HJV + to negative. They were mostly found in low HAI titered S1 sera (9 had titers less than 40), and three were bacterially contaminated. In 1988, a study by Main et al noted that the antibody response following natural exposure to EEEV or HJV may be variable in different avian species and suggested that hemagglutination-inhibiting and neutralizing antibodies may not be detectable for life. Reversions occurred at different intervals for several wild bird

62

species, such that some birds (e.g. black-capped chickadees and veeries) that were seropositive for EEEV or HJV later reverted to seronegative status, within one to two weeks or even years later. These reversions were attributed to natural variation in the sensitivity of PRNT, such that the antibodies may have been present but were not detectable (Main et al, 1988). This phenomenon was observed in our study and may also be due to PRNT variability from changing viral titers and individual bird variation in antibody production.

The first detection of Highlands J virus activity occurred in May. This was nearly two months after the initial indication of Eastern equine encephalomyelitis virus activity in March. From our sample size of 316 birds, sentinel seroconversions to EEEV peaked in June at 54 birds and seroconversions to HJV peaked in July with 55 birds. EEEV activity slowly tapered off through December, whereas HJV activity quickly declined to just 8 positive sentinel chickens in August to no activity in December. Neither virus was detected by sentinel chickens during the month of September.

EEEV was identified from a total of 20 dead birds in 14 counties, with the first dead bird found in March. HJV was associated with 2 bird fatalities, both occurring in June with one in Jefferson County and one in Hamilton County. Only one mosquito pool tested positive for EEEV collected by Escambia County in June (Collins & Blackmore, 2003).

Analysis of *alphavirus* activity at the county level indicates widespread transmission of Highlands J virus across the northern and panhandle regions of the state (Figure 11) where sentinel chicken programs were in operation (Figure 17). However, Eastern equine encephalomyelitis virus transmission activity was higher than the sentinel

seroconversion rates to HJV in all but four counties. Bay County had a higher rate of confirmed sentinel seroconversions to HJV (46%) than EEEV (31%) (Figure 7). Volusia County also had an increased rate of HJV activity at 65% versus 21% noted for EEEV (Figure 8) as confirmed by PRNT. Nassau and Marion Counties have equal sentinel seroconversion rates for both viruses at 45% and 33%, respectively (Figure 8). This may not be an accurate representation of *alphavirus* transmission activity in Marion County due to the low number of sera tested (n=3), where one sentinel seroconverted to HJV, one to EEEV, and one sentinel was negative for both viruses (data not shown). In addition, HJV was not detected in five counties (Citrus, Flagler, Jackson, Osceola, and Pinellas) where EEEV activity was found. These findings may also be due to small sample sizes, with 6 or fewer sentinels tested for each of these counties.

Differences in *alphavirus* seroconversion rates were noted even within counties. Walton County has two separate sentinel chicken programs as designated by north and south. We were able to confirm four times as many sentinels in the PRNT for South Walton (n=20) than for North Walton (n=5). In South Walton, the PRNT confirmed sentinel seroconversion rates for EEEV and HJV were 85% and 15%, respectively. All sentinels available for testing were *alphavirus* positive by PRNT.

Comparatively, North Walton had a 60% confirmed sentinel seroconversion rate to EEEV (n=3) and 40% of the sentinels tested were negative (n=2) by PRNT. Highlands J virus activity was not detected. An additional 15 sentinels could not be confirmed for North Walton in the PRNT due to insufficient sera remaining, compared to only 5 sentinels with insufficient sera for South Walton. Consequently, our ability to test only a subset of the actual number of sentinels exposed in North Walton likely affects the

64

sentinel seroconversion rates calculated not only for this county but for the other counties as well.

Orange County also had two separate sentinel chicken programs in operation. Orange County and Reedy Creek Mosquito Control Districts (MCD) had significantly different sentinel chicken *alphavirus* confirmed seroconversion rates. Sentinels from Reedy Creek MCD had a significantly higher seroconversion rate to EEEV (74%) than Orange County MCD sentinels (20%) as confirmed by PRNT. Reedy Creek MCD had a lower level of HJV activity at 16% as compared to Orange County MCD, where HJV transmission was 33%. A striking difference was that 47% of the sentinels in Orange County MCD were negative for both viruses as compared to only 10% in Reedy Creek MCD. Of all sentinels screened *alphavirus* positive in the HAI test for these two counties, Orange County actually had fewer sentinels that were unavailable for confirmation (n=2) as compared to Orange-Reedy MCD (n=26). These results may be a reflection of flock sites and management strategies. Particular caution should also be given to interpretation of results for Dade, Flagler, Osceola, and St. Lucie counties as more than half of the sentinels screened positive in the HAI assay were not available for confirmation in the PRNT. In addition, none of the sentinels screened EEEV positive in the HAI for Hendry County (n=4) were available for testing in the PRNT.

This study had several limitations including a non-randomized testing design that selected sentinel chickens which had screened positive for the presence of *alphavirus* antibodies in the HAI test at some point during the year. In addition, flock management and bleeding practices varied by county, such that some counties with more resources were better able to maintain more chicken flocks within their regions than in other areas.

65

Collecting blood from chickens is difficult under the best circumstances but is much more challenging during a hot, humid Florida summer. Identifier leg bands are small making the bird numbers difficult to read, which contributed to mislabeling of submitted samples. Amount and quality of sera collected and shipped for testing were also highly variable between counties; with insufficient sera for confirmatory tests impacting this study the most.

Since *alphaviruses* are focally transmitted, placement of the sentinel chicken flocks is also critical to the success of the program. Consequently, this study was limited in that it could only detect viruses in areas where the chickens were placed, perhaps underestimating the true risk of transmission activity. Proper timing of sentinel blood sampling is also very important for the confirmation of viral infection by MAC-ELISA, especially since IgM antibodies are transient in nature.

Nonetheless, this surveillance project has strong points. A large susceptible population size (n=4361) was followed over the course of the year and monitored for the development of antibodies to arboviruses. A representative sample (n=316) was chosen for further testing to determine if cross-reactions between closely related *alphaviruses* were influencing results obtained from the HAI test and the MAC-ELISA for detection of antibodies to EEEV. In addition, weekly or biweekly sampling of the sentinel chickens provided accurate estimates of arboviral activity across the state on a timely basis.

The primary advantage with this surveillance system is that time and place of arboviral exposure is known. Confirmation of *alphavirus* sentinel seroconversions in the PRNT has enhanced our diagnostic capabilities and provides a clearer picture of EEEV and HJV activity in the state of Florida. The addition of this technique for the improved

detection of antibodies to EEEV should aid vector control strategies in areas experiencing Eastern equine encephalomyelitis virus activity and Highlands J virus transmission.

A study in the early 1990s in Massachusetts estimated that prophylactic aerial ultra-low volume application of malathion for the prevention of EEEV outbreaks could cost anywhere from $0.7 million to $1.4 million (Villari et al, 1995). These estimates have likely increased over the last ten years. The Tampa Branch Laboratory cost to test one serum sample in the serum neutralization plaque reduction test (PRNT) against two viruses is $50, as compared to a $6 per sample charge for testing in the HAI assay or the MAC-ELISA for two antigens. For this study, the additional cost of testing 428 sera in the PRNT was $21,400. These costs are negligible when compared to the expense of costly vector control measures (from $0.7 to $1.4 million) that may be implemented to prevent outbreaks of arboviral disease. Not only would these resources be wasted on mosquitoes carrying a non-human pathogen like Highlands J virus, but additional costs related to pesticide use may be incurred, especially if environmental damage and adverse health events occur (CDC, 2003c).

The cost to society of one child infected with Eastern equine encephalomyelitis virus has been estimated at 3 million dollars (Villari et al, 1995), which is insignificant when compared to the incalculable burden when a life is lost due to the high fatality rate associated with EEEV. Communities can also be burdened when medical alerts and advisories are issued through increased demands on medical sector services, as well as public complaints and illness related to increased insecticidal control measures (CDC, 2003c). An increase in EEEV transmission is important to human health but is also costly to veterinary health. EEEV is a significant contributor to equine morbidity and

67

mortality every year (despite an available equine vaccine). Epizootics of EEEV have been associated with up to 90% of infected horses developing acute and lethal disease (Weaver et al, 1999). Unlike the average of 4 human cases each year (CDC, 2003a), EEEV infects hundreds of equines annually. This creates a burden on large animal veterinary health providers as well as a tremendous cost to individual horse owners.

Consequently, this study recommends implementation of additional confirmatory tests by the serum neutralization plaque reduction test (PRNT) for the detection of antibodies to Eastern equine encephalomyelitis virus and Highlands J virus in sentinel chickens for the state of Florida. This testing can greatly assist county health departments and mosquito control agencies in the prevention and control of EEEV at a minimal extra cost. In addition, efforts to improve the quality of sera and blood drawing techniques are suggested to assist the Tampa Branch Laboratory (TBL) in its continued pursuit of timely and accurate test results. With higher quality and larger volumes of sera, the accuracy of these serologic assays will improve and can allow for further confirmatory testing as necessary. The success of the sentinel chicken program truly depends upon the performance of the collaborating mosquito control districts and county health departments to produce quality sera specimens and on the TBL to provide accurate test results.

A measure of the usefulness of a surveillance system can be estimated by evaluating sensitivity, positive predictive value, flexibility, acceptability, simplicity, timeliness, and representativeness (CDC, 1988). The Sentinel Chicken Program for the surveillance of arboviruses in the state of Florida exemplifies many of these attributes. Although the sentinel chicken surveillance system is complex, it is accepted, timely,

68

representative, and flexible. Diagnostic testing methods for sentinel chicken sera are highly sensitive with high predictive positive values. Weekly reports of sentinel seroconversions to arboviruses immediately influence public health response and prevention strategies by a coordinated effort between multiple state agencies, counties, and mosquito control districts. Consequently, accurate diagnoses of arboviral infections are critical to the success of the program, especially in light of the many cross-reactions that can occur within these viral families.

In conclusion, the sentinel chicken program is an important component of arboviral surveillance for the state of Florida. The results from 2003 indicate that Eastern equine encephalomyelitis virus still poses a considerable threat to public and veterinary health. This study has shown that extensive cross-reactions with Highlands J virus have occurred in the HAI test and a lowered sensitivity in the MAC-ELISA necessitates confirmatory testing of MAC-ELISA negative sera by PRNT. Accurate diagnostic methods for the detection of antibodies to EEEV in sentinel chickens are of critical importance for various state agencies to implement risk assessment measures. Ultimately, it is important to maintain an arboviral sentinel surveillance program that can provide early warning when activity is detected in an area, so that medical alerts/advisories, vector control, and educational campaigns can be declared to provide protection for the population.

Figure 13 Sensitivity and Specificity of the HAI Assay and the MAC-ELISA for the Detection of Antibodies to EEEV.
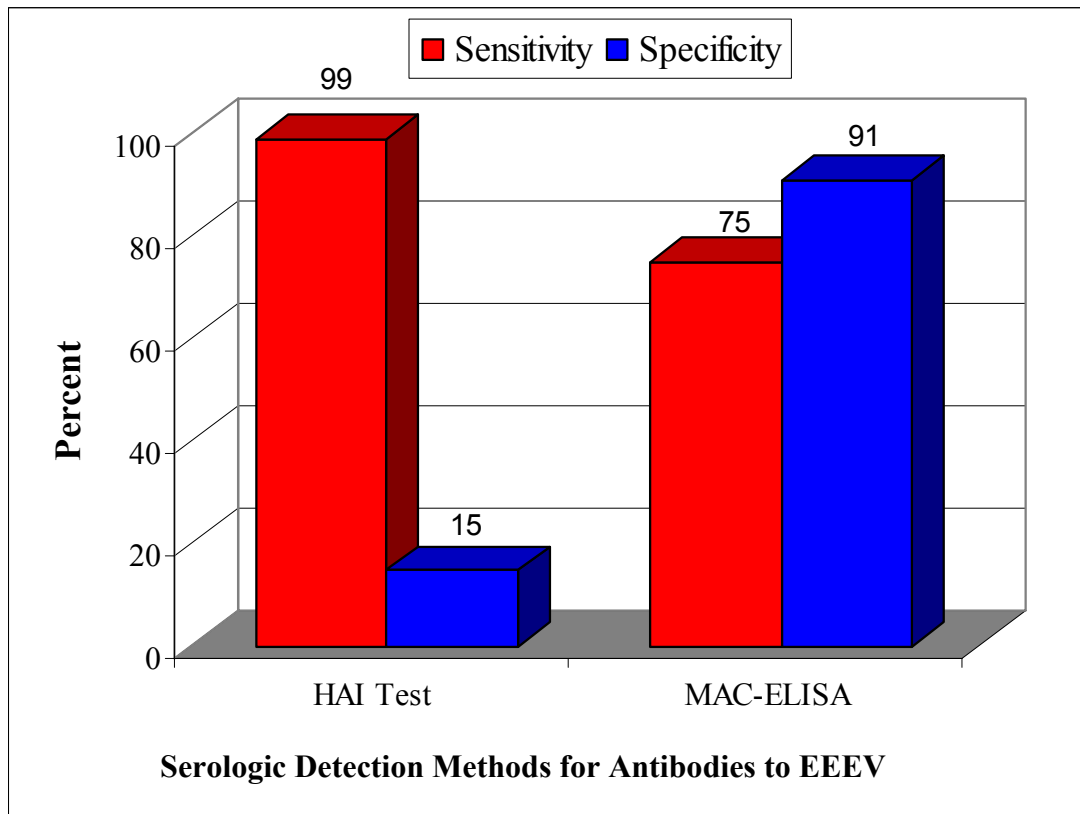
Figure 14 Positive and Negative Predicted Values for the Detection of Antibodies to
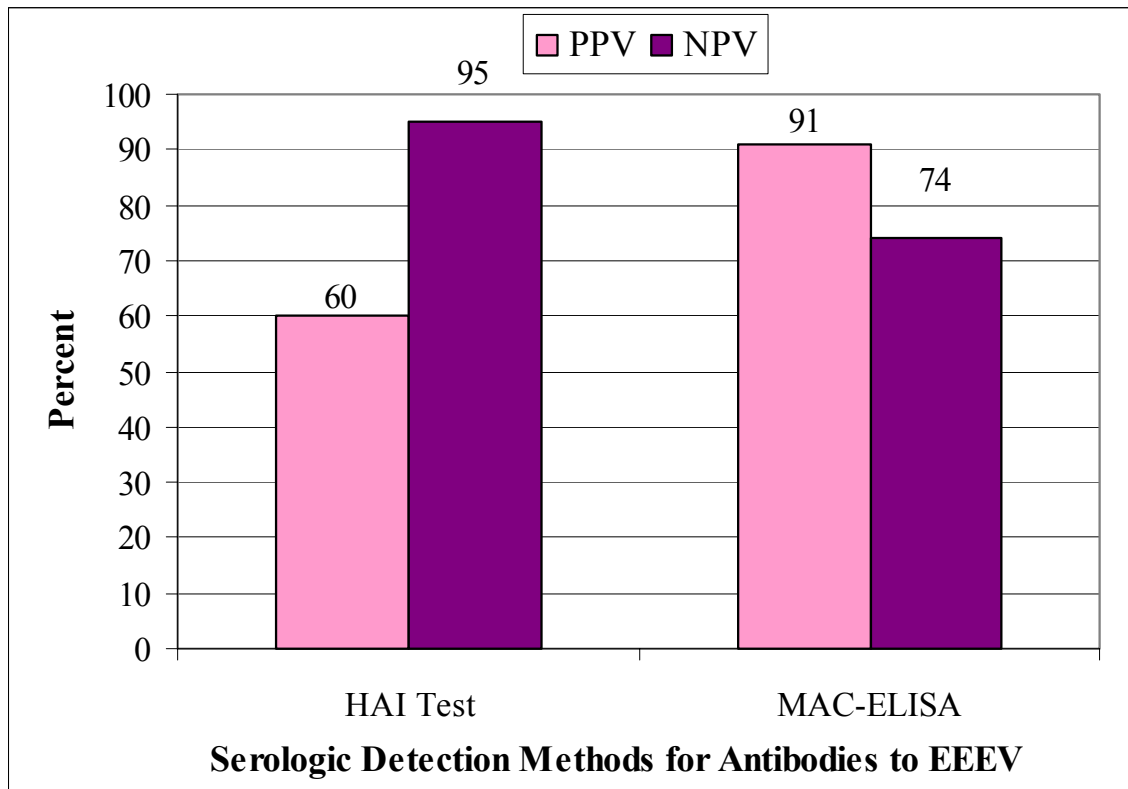EEEV by the HAI Assay and the MAC-ELISA.

Figure 15   Sensitivity and Specificity of the HAI Assay and the MAC-ELISA for the
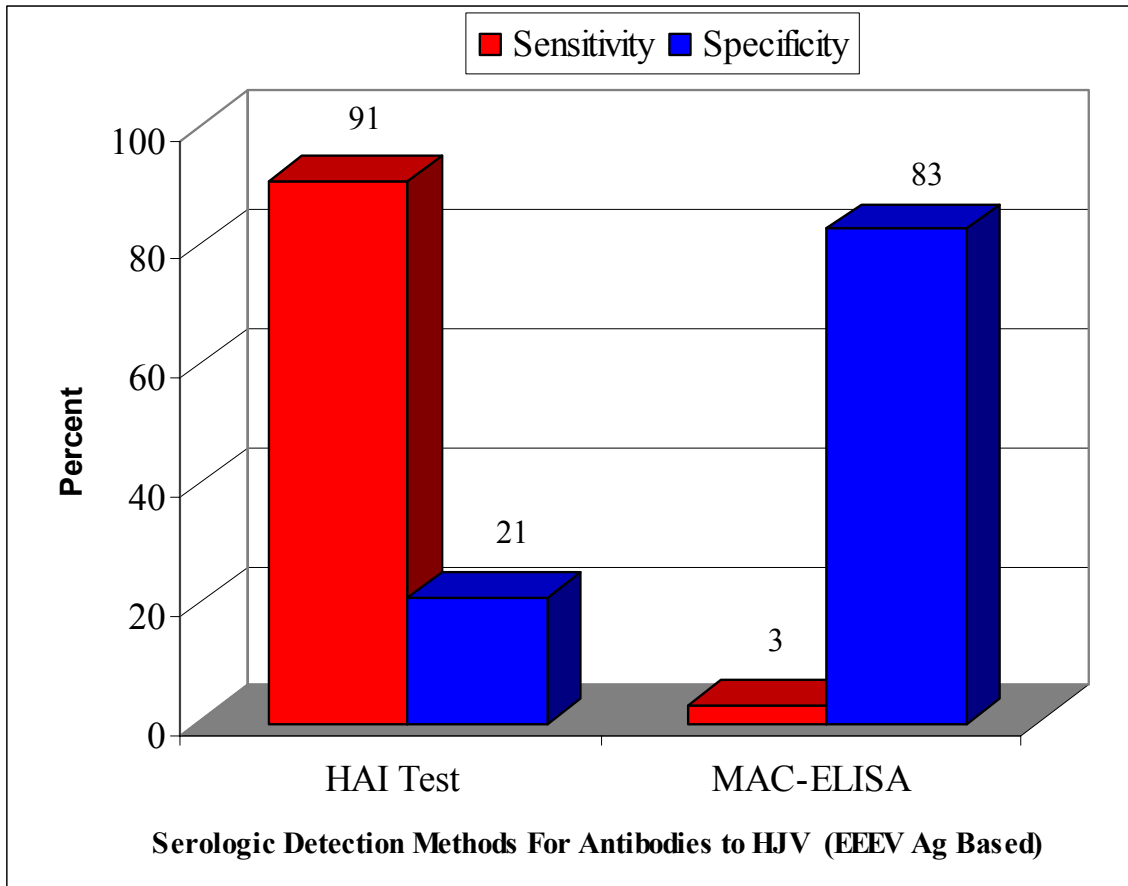Detection of Antibodies to Highlands J virus by Testing with EEEV antigen.

Figure 16 Positive and Negative Predicted Values for the Detection of Antibodies to
Highlands J virus by the HAI Assay and the MAC-ELISA by Testing with
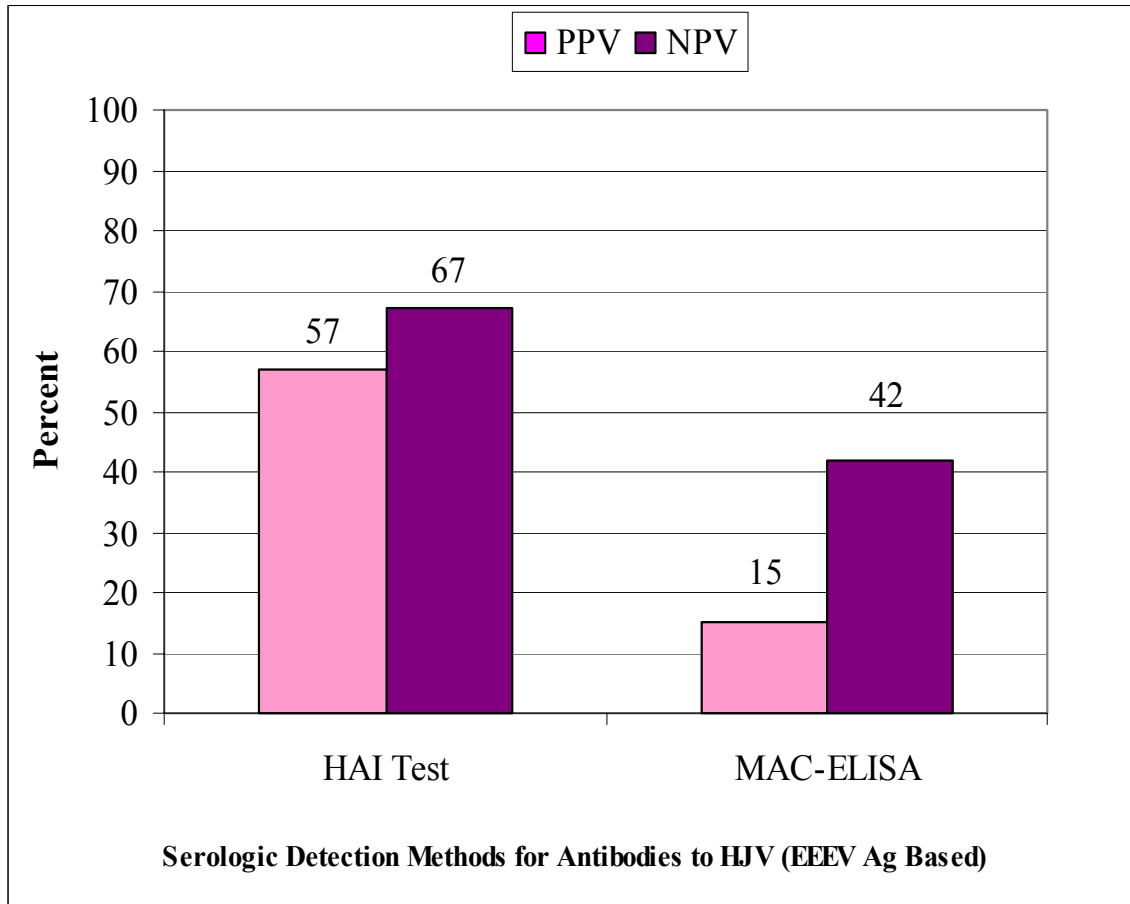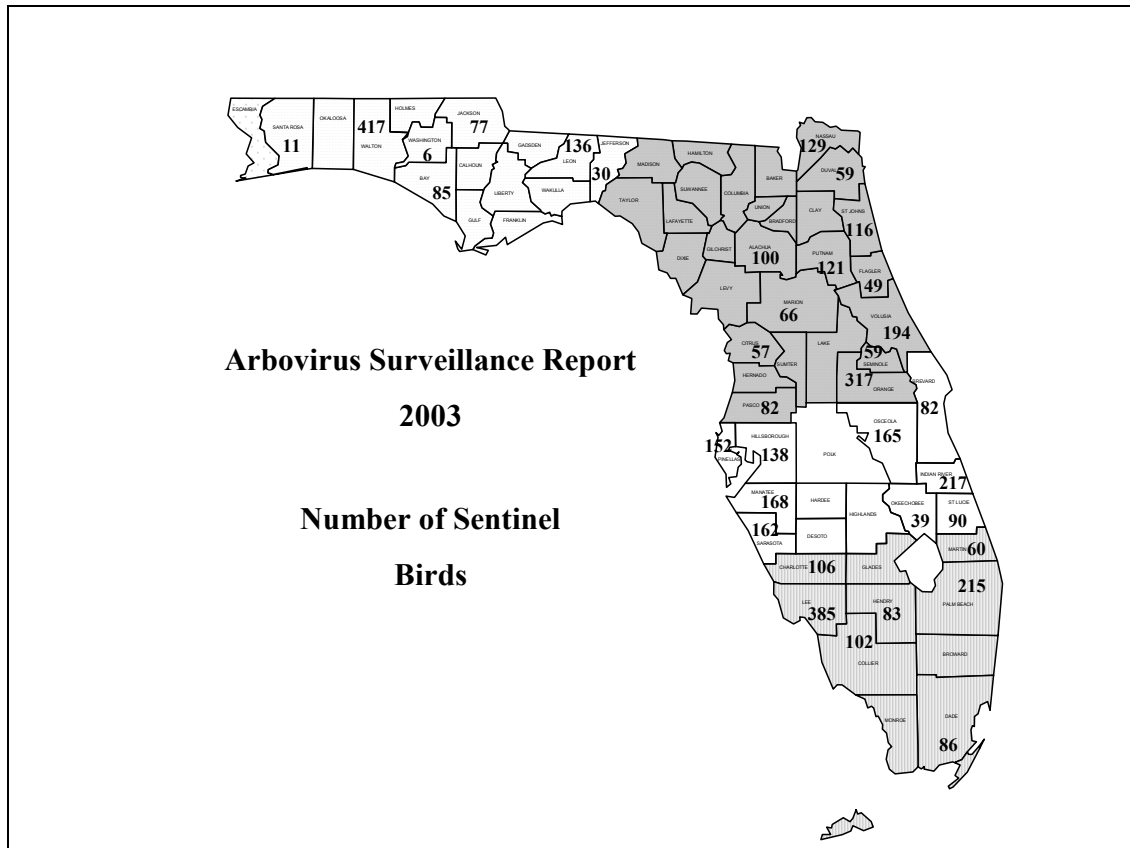EEEV antigen.

Figure 17 Number of Sentinel Birds by County for the State of Florida, in 2003.

74

References Cited

Beaty BJ, Calisher CH, and Shope RE (1989). *In* Diagnostic procedures for viral, rickettsial and chlamydial infections. Schmidt, NJ and Emmons, RW, eds. *Arboviruses, chapter 24.* American Public Health Association, Washington, DC.

Benjamini E, Coico R, and Sunshine G (2000). Immunology: A Short Course, 4th ed. John Wiley & Sons, New York, USA.

Bigler B (1999). St. Louis Encephalitis. Available from URL http://www.doh.state.fl.us/disease_ctrl/epi/htopics/reports/slepres2.pdf

Bigler WJ, Lassing EB, Buff EE, et al (1976). Endemic Eastern Equine Encephalomyelitis in Florida: a Twenty-year Analysis, 1955-1974. *Am J Trop Med Hyg* 25(6):884-890.

Blackmore CG, Stark LM, Jeter WC, et al (2003). Surveillance results from the first West Nile virus transmission season in Florida, 2001. *Am J Trop Med Hyg* 69(2):141-50.

Brault AC, Powers AM, Chavez CLV, et al (1999). Genetic and Antigenic Diversity Among Eastern Equine Encephalitis Viruses From North, Central, and South America. *Am J Trop Med Hyg* 61(4):579-586.

Burke DS, Nisalak A (1982). Detection of Japanese Encephalitis Virus Immunoglobulin M Antibodies in Serum by Antibody Capture Radioimmunoassay. *J Clin Micro* 14:353-361.

Calisher CH, Monath TP, Muth DJ, et al (1980). Characterization of Fort Morgan virus, an alphavirus of the western equine encephalitis virus complex in an unusual ecosystem. *Am J Trop Med Hyg* 29:1428-1440.

Calisher CH, El-Kafrawi AO, Mahmud MIA, et al (1986a). Complex-Specific Immunoglobulin M Antibody Patterns in Humans Infected with Alphaviruses. *J Clin Micro* 23(1):155-159.

75

Calisher CH, Berardi VP, Muth DJ, Buff EE (1986b). Specificity of Immunoglobulin M and G Antibody Responses in Humans Infected with Eastern and Western Equine Encephalitis Viruses: Application to Rapid Serodiagnosis. *J Clin Micro* 23(2):369-372.

Calisher CH, Fremount HN, Vesley WL, et al (1986c). Relevance of Detection of Immunoglobulin M Antibody Response in Birds Used for Arbovirus Surveillance. *J Clin Micro* 24(5):770-774.

Calisher CH, and Karabatsos N (1988). *In* The Arboviruses: Epidemiology and Ecology, Vol 1. Monath, TP, ed. *Arbovirus serogroups: definition and geographic distribution*. CRC Press, Boca Raton, Florida, 19-57.

Calisher CH, Karabatsos N, Lazuick JS, et al (1988). Reevaluation of the Western Equine Encephalitis Antigenic Complex of Alphaviruses (family Togaviridae) as Determined by Neutralization Tests. *Am J Trop Med Hyg* 38(2):447-452.

Casals J (1957). The arthropod-borne group of animal viruses. *Tr NY Acad Sc* 19:219-235.

Casals J, and Brown LV (1954). Hemagglutination with arthropod-borne viruses. *J Exp Med* 99:429-449.

Centers for Disease Control and Prevention (1988). Guidelines for Evaluating Surveillance Systems. *MMWR Morb Mortal Wkly Rep* 37(S-5):1-18.

Centers for Disease Control and Prevention (1992). Eastern Equine Encephalitis Virus Associated with Aedes albopictus—Florida, 1991. *MMWR Morb Mortal Wkly Rep* 41(7):115-121.

Centers for Disease Control and Prevention (1993). Guidelines for Arbovirus Surveillance Programs in the United States. Available from URL http://www.cdc.gov/ncidod/dvbid/arbor/arboguid.pdf

Centers for Disease Control and Prevention (1994). Arboviral Disease—United States, 1994. *MMWR Morb Mortal Wkly Rep* 44(35):641-644.

Centers for Disease Control and Prevention (1998). Arboviral Infections of the Central Nervous System--United States, 1996-1997. *MMWR Morb Mortal Wkly Rep* 47(25):517-522.

Centers for Disease Control and Prevention (2001). Information on Arboviral Encephalitides. Available from URL http://www.cdc.gov/ncidod/dvbid/arbor/arbdet.htm

76

Centers for Disease Control and Prevention (2002). Chart: Confirmed and Probable Eastern Equine Encephalitis Cases, Human, United States, 1964-2000, By State. Available from URL http://www.cdc.gov/ncidod/dvbid/arbor/cases-eee-1964-2000.htm

Centers for Disease Control and Prevention (2003a). Eastern Equine Encephalitis Fact Sheet. Available from URL http://www.cdc.gov/ncidod/dvbid/arbor/eeefact.htm

Centers for Disease Control and Prevention (2003b). Epidemic/Epizootic West Nile Virus in the United States: Revised Guidelines for Surveillance, Prevention, and Control. Retrieved November 19, 2003 from URL http://www.cdc.gov/ncidod/dvbid/westnile/resources/wnv-guidelines-aug-2003.pdf

Centers for Disease Control and Prevention (2003c). Surveillance for Acute Insecticide-Related Illness Associated with Mosquito Control Efforts—Nine States, 1999-2002. *MMWR Morb Mortal Wkly Rep* 52(27):629-634.

Channock RM, and Sabin AB (1953). The hemagglutinin of St. Louis encephalitis virus. *J Immunol* 70:271-316.

Channock RM, and Sabin AB (1954a). The hemagglutinin of Western Equine encephalitis virus: Recovery, properties, and use for diagnosis. *J Immunol* 73:337-351.

Channock RM, and Sabin AB (1954b). The hemagglutinin of West Nile virus: Recovery, properties and antigenic relationships. *J Immunol* 73:352-362.

Chappell WA, Sasso DR, Toole RF, Monath TP (1971). Labile Serum Factor and Its Effect on Arbovirus Neutralization. *Applied Microbiology* 21(1):79-83.

Cilnis MJ, Kang W, Weaver SC (1996). Genetic conservation of Highlands J Viruses. *Virology* 218(2):343-351.

Clarke DH, and Casals J (1958). Techniques for hemagglutination and hemaglutination-inhibition with arthropod-borne viruses. *Am J Trop Med & Hyg* 7:561-573.

Collins C, and Blackmore C (2003). Mosquito-Borne Disease Summary Through the Week Ending December 29, 2003. Florida Department of Health. Available from URL http://www.doh.state.fl.us/Environment/hsee/arbo/data/2003/03-12-29.pdf

Crans WJ (1986). Failure of chickens to act as sentinels during an epizootic of eastern equine encephalitis in southern New Jersey, USA. *J Med Entomol* 23(6):626-629.

Crans WJ, Caccamise DF, and McNelly JR (1994). Eastern equine encephalomyelitis virus in relation to the avian community of a coastal cedar swamp. *J Med Entomol* 31(5):711-728.

Day JF, and Stark LM (1996a). Eastern Equine Encephalitis Transmission to Emus (Dromaius novaehollandiae) in Volusia County, Florida: 1992 through 1994. *J Am Mosq Control Assoc* 12(3):429-436.

Day JF, and Stark LM (1996b). Transmission patterns of St. Louis encephalitis and eastern equine encephalitis viruses in Florida: 1978-1993. *J Med Entomol* 33(1):132-139.

Duermeyer W, Wielaard F, Vanderveen J (1979). A New Principle for the Detection of Specific IgM Antibodies Applied in an ELISA for Hepatitis. *Am J Med Virol* 4:25-32.

Ficken MD, Wages DP, Guy JS, et al (1993). High mortality of domestic turkeys associated with Highlands J virus and eastern equine encephalomyelitis virus infections. *Avian Dis* 37:585-590.

Florida Bureau of Epidemiology and Florida Department of Health (2000). Surveillance of Selected Arthropod-borne Diseases in Florida, 2000. Available from URL http://www.doh.state.fl.us/disease_ctrl/epi/vetvector/arbo99.pdf

Florida Department of Health (2002). Florida ArboVirus Response Plan, 2002. Arbrovirus Interagency Working Group. Available from URL http://www.co.leon.fl.us/mosquito/mceduc/diseases/factsht/arboplan02.pdf

Griffin DE (2001). *In* Fields Virology, Vol 1, 4th edition. Knipe, DM and Howley, PM, eds. *Alphaviruses*. Lippincott Williams & Wilkins, 917-962.

Gubler DJ, and Roehrig JT (1998). *In* Topley and Wilson's Microbiology and Microbial Infections, Vol 1, Virology. Mahy, BWJ, Collier, L, eds. *Arboviruses (Togaviridae and Flaviviridae)*. Oxford University Press, New York, 29:579-600.

Gubler DJ (2001). Human Arbovirus Infections Worldwide. *Ann NY Acad Sc* 951:13-24.

Gubler DJ (2002). The global emergence/resurgence of arboviral diseases as public health problems. *Arch Med Res* 33(4):330-342.

Hansen W, and Docherty DE (1999). *In* Field Manual of Wildlife Diseases, General Field Procedures and Diseases of Birds. Friend, M and Franson, JC, eds. *Chapter 20: Eastern Equine Encephalomyelitis.* US Geological Survey, Biological Resources Division, Reston, Virginia, 171-174.

Hanson RP (1957). An Epizootic of Equine Encephalomyelitis That Occurred in Massachusetts in 1831. *Am J Trop Med Hyg* 6:858-862.

Hayes CG, and Wallis RC (1977). Ecology of Western equine encephalomyelitis virus in the eastern United States. *Adv Virus Res* 21:37-83.

Henderson JR, Karabatsos N, Bourke ATC, et al (1962). A Survey For Arthropod-Borne Viruses in South-Central Florida. *Am J Trop Med Hyg* 11:800-810.

Hofman H, Frisch-Niggemeyer W, Heinz F (1979). Rapid Diagnosis of TBE by Means of Enzyme-Linked Immunosorbent Assay. *J Gen Virol* 42:505-511.

Holden P, Muth D, Shriner RB (1966). Arbovirus Hemagglutination-inhibiting in Avian Sera: Inactivation With Protamine Sulfate. *Am J Epidemiol* 84:67-73.

Jamnback TL, Beaty BJ, Hildreth SW, et al (1982). Capture Immonoglobulin M Systems for Rapid Diagnosis of La Crosse (California Encephalitis) Virus Infections. *J Clin Micro* 16:577-580.

Jennings WL, Allen RH, and Lewis AL (1966). Western equine encephalomyelitis in a Florida horse. *Am J Trop Med Hyg* 15:96-97.

Johnson AJ, Langevin S, Wolff KL, Komar N (2003). Detection of Anti-West Nile Virus Immunoglobulin M in Chicken Serum by an Enzyme-Linked Immunosorbent Assay. *J Clin Micro* 41(5):2002-2007.

Karabatsos N, Bourke ATC, Henderson JR (1963). Antigenic Variation Among Strains of Western Equine Encephalomyelitis Virus. *Am J Trop Med Hyg* 12:408-412.

Karabatsos N (1985). International Catalogue of Arboviruses, including certain other viruses of vertebrates. American Society of Tropical Medicine and Hygiene. San Antonio, Texas. 2001 update.

Karabatsos N, Lewis AL, Calisher CH, et al (1988). Identification of Highlands J Virus From a Florida Horse. *Am J Trop Med Hyg* 39(6):603-606.

Langevin SA, Bunning M, Davis B, et al (2001). Experimental Infection of Chickens as Candidate Sentinels for West Nile Virus. *Emerg Infect Dis* 7(4):726-729.

Lindsey HS, Calisher CH, Mathews JH (1976). Serum Dilution Neutralization Test for California Group Virus Identification and Serology. *J Clin Micro* 4(6):503-510.

Main AJ, Anderson KS, Maxfield HK, et al (1988). Duration of Alphavirus neutralizing antibody in naturally infected birds. *Am J Trop Med Hyg* 38(1):208-217.

Martin DA, Muth DA, Brown T, Johnson AJ, et al (2000). Standardization of Immunoglobulin M Capture Enzyme-Linked Immunosorbent Assays for Routine Diagnosis of Arboviral Infections. *J Clin Micro* 38(5):1823-1826.

Monath TP (1979). Arthropod-borne encephalitides in the Americas. *Bull World Health Organ* 57:513-533.

Monath TP, Nystrom RR, Bailey RE, et al (1984). Immunoglobulin M Antibody Capture Enzyme-Linked Immunosorbent Assay for Diagnosis of St. Louis Encephalitis. *J Clin Micro* 20(4):784-790.

Nelson DB, Kappus KD, Janowski HT, et al (1983). St. Louis encephalitis—Florida 1977. Patterns of a widespread outbreak. *Am J Trop Med Hyg* 32:412-416.

Olson JG, Scott TW, Lorenz LH, Hubbard JL (1991). Enzyme Immunoassay for Detection of Antibodies Against Eastern Equine Encephalomyelitis Virus in Sentinel Chickens. *J Clin Micro* 29(7):1457-1461.

Peck GM (1831). Disease in Horses. *New England Farmer* 10:217-218.

Phinney E (1831). An Alarming Disease of Horses. *New England Farmer* 10:70.

Roggendorf M, Heinz F, Deinhardt F, Kunz C (1981). Serologic Diagnosis of Acute Tick-Borne Encephalaits by Demonstration of Antibodies of the IgM Class. *J Med Virol* 7:41-50.

Sabin AB (1951). Hemagglutination by viruses affecting the human nervous system. *Fed Proc* 10:573-578.

Sabin AB, and Buescher, EL (1950). Unique physico-chemical properties of Japanese B encephalitis virus hemagglutinin. *Proc Soc Exp Biol Med* 74:222-230.

Schmidt NJ (1979). *In* Diagnostic procedures for viral, rickettsial and chlamydial infections, 5th ed. Lennette, EH and Schmidt, NJ, eds. *Cell culture techniques for diagnostic virology.* American Public Health Association, Washington, DC.

Schmitz H, von Deimling V, Fleming B (1980). Detection of IgM Antibodies to CMV Using an Enzyme-Labelled Antigen. *J Gen Virol* 50:59-68.

Schlesinger I, and Schlesinger MJ (2001). *In* Fields Virology, Vol 1, 4th edition. Knipe, DM and Howley, PM, eds. *Togaviridae: The Viruses and Their Replication.* Lippincott Williams & Wilkins, 895-916.

Scott TW, and Weaver SC (1989). Eastern equine encephalomyelitis virus: epidemiology and evolution of mosquito transmission. *Adv Virus Res* 37:277-328.

Sever JL, Castellano GA, Pelon W, et al (1964). Inactivation of the infectivity of viral hemagglutinating antigens with the use of betaprone. *J Lab Clin Med* 64:983-988.

Stark LM, and Kazanis D (2003). Florida Department of Health. Arbovirus Surveillance: Annual Summary Report 2003.

Sweet BH, and Sabin AB (1954). Properties and antigenic relationships of hemagglutinins associated with the dengue viruses. *J Immunol* 73:363-373.

Terry, Thomas (2001). *In* Biology 102 Lecture Notes: The Immune System. Primary and Secondary Immune Responses. Available from URL http://sp.uconn.edu/~bi102vc/102f01/terry/immunity.html

Thompson A (1831). Epidemic in Horses. *New England Farmer* 10:108.

Villari P, Spielman A, Komar N, et al (1995). The Economic Burden Imposed by a Residual Case of Eastern Encephalitis. *Am J Trop Med Hyg* 52(1):8-13.

Weaver SC, Kang W, Shirako Y, et al (1997). Recombinational History and Molecular Evolution of Western Equine Encephalomyelitis Complex Alphaviruses. *J Virol* 71(1):613-623.

Weaver SC, Powers AM, Brault AC, Barrett AD (1999). Molecular Epidemiological Studies of Veterinary Arboviral Encephalitides. *The Veterinary Journal* 157(2):123-138.

Wellings FM, Lewis AL, Pierce LV (1972). Agents Encountered During Arboviral Ecological Studies: Tampa Bay Area, Florida, 1963 to 1970* *Am J Trop Med Hyg* 21(2):201-213.

Bibliography

Bigler, B (1999). Eastern Equine Encephalitis. Available from URL
  http://www.doh.state.fl.us/disease_ctrl/epi/htopics/reports/eeeprs.pdf

German RR (2000). Sensitivity and Predictive Value Positive Measurements for Public
  Health Surveillance Systems. *Epidemiology* 11:720-727.

Halperin W, Baker EL, and Monson RR (1992). Public Health Surveillance. John Wiley
  & Sons, New York, USA.

Nandalur, M. and Urban, AW (2002). Eastern Equine Encephalitis. Available from URL
  http://www.emedicine.com/med/topic3155.htm

http://www.cdc.gov/ncidod/eid/index.htm

http://www.cdc.gov/ncidod/dvbid/index.htm

http://www.doh.state.fl.us/Environment/hsee/arbo/index.html

http://www.doh.state.fl.us/disease_ctrl/epi/Epi_Updates/Epi_Weekly/Epi_Update.htm

http://ipm.ifas.ufl.edu/about/index.htm

http://www.promedmail.org/pls/askus/f?p=2400:1000

http://www.tulane.edu/~dmsander/Big_Virology/BVHomePage.html

http://virology-online.com/viruses/Arboviruses.htm