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Luminex microsphere immunoassay offers an improved method in testing for antibodies to Eastern Equine Encephalitis virus in sentinel chicken sera

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Luminex Microsphere Immunoassay Offers an Improved Method in Testing for Antibodies to Eastern Equine Encephalitis Virus in Sentinel Chicken Sera

by

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Dedication

To my family who provided the encouragement, argument, love and attitude to make my goals a reality, and to my friends who leant their shoulders in support of my mentality.

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List of Symbols and Abbreviations

Symbol and Abbreviations	Description
%	Percent
°C	Degrees Centigrade
Ab	Antibody
Ag	Antigen
BABS	Bovine Albumin-Borate Saline
CDC	Centers for Disease Control and Prevention
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
EEEV	Eastern Equine Encephalomyelitis Virus
EIA	ELISA Immunoassay
FBE	Florida Bureau of Epidemiology
FDOH	Florida Department of Health
g	gravity
HAI	Hemagglutination Inhibition Assay
HJV	Highlands J Virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JE	Japanese Encephalitis Virus
MFI	Mean Fluorescent Intensity
MIA	Microsphere-based Immunoassay
MAC-ELISA	IgM Antibody Capture Enzyme-Linked
mRNA	Messenger Ribonucleic Acid
µg	microgram
µl	microliter
ml	milliliter
min	minute
NPV	Negative Predicted Value
NSMB	Normal Suckling Mouse Brain
PPV	Positive Predicted Value
PRNT	Serum Neutralization Plaque Reduction Test
SLEV	St. Louis Encephalitis Virus
VEEV	Venezuelan Equine Encephalitis virus
WEEV	Western Equine Encephalitis virus
WNV	West Nile Virus

Luminex Microsphere Immunoassay Offers an Improved Method in Testing for Antibodies to Eastern Equine Encephalitis Virus in Sentinel Chickens

Kelly Ann Fitzpatrick

ABSTRACT

Eastern Equine Encephalitis virus has a human mortality rate of 30% of those cases diagnosed, while 30% of those surviving infection remain with neurological sequelae for life (CDC.gov, 2007).

The use of sentinel chickens for surveillance of arboviruses that are known to use birds as a reservoir host, such as St. Louis Encephalitis (SLE), West Nile (WN) virus, Eastern Equine Encephalitis (EEE) and Highlands J (HJ) virus, in Florida began with the Sentinel Chicken Arboviral Surveillance Network in 1978 (Day and Stark, 1996). This network enables the activation of an early warning system for citizens, as well as, county epidemiologists and those in mosquito control, allowing for a coordinated effort of disease prevention.

Methods currently used at the Florida Department of Health, Tampa Branch Laboratory include screening of submitted sera for antibodies to these arboviruses of epidemiologic importance by way of the hemagglutination inhibition test (HAI), and confirmation by the IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) and Plaque Reduction Neutralization test if the MAC-ELISA proves to be

negative. While these tests combined are providing the results needed, the time to result can be a week or greater depending on the initial screening result in the HAI tests.

The Microsphere Assay Technology provides an accurate, more rapid (a day or two instead of a week or more) detection method including both a screening and confirmation protocol specifically designed to test for antibody to EEE in sentinel chicken sera. Two sera out of the thousands tested that were tested by HAI shown to be negative in standard testing, resulted as positive by the MIA method and therefore indicated a missed positive. The sensitivity and specificity, positive and negative predictive values of this new protocol as compared with MAC-ELISA as a reference standard indicated that both tests were remarkably similar; Providing sensitivity near 80%, specificity and PPV at 99%, and negative predictive values at 90% for MAC-ELISA and 94% for the MIA. Finally it was determined that Highlands J virus will not have any impact on the testing protocol and results of this test.

Introduction

Arthropod-Borne Viruses

Requirement of an arthropod transmission vector is a unique characteristic of arthropod-borne viruses (arboviruses). The endemicity of these viruses is dependent on three main components, including the virus, vector and the vertebrate host. These three components dictate the spread, severity and impact upon humans by each virus. Environmental factors are also an important impacting and controlling aspect of vector borne diseases; they have distinct effects upon the lifecycle of the known vectors thereby directly affecting the transmissibility of the virus to new hosts. These factors include temperature and rainfall, thereby directly affecting the transmissibility of the virus to new hosts. Vectors within the Phylum Arthropoda include mosquitoes, biting flies and midges, sand and black flies, mites and ticks, all capable of pathogen transmission to a suitable vertebrate host. In general, vectors have the ability to transmit both mechanically as well as biologically, the latter being vital to the propagative transmission of arboviruses; thus, arboviruses depend upon their vector hosts for multiplication as well as transportation between hosts (Chamberlain 1961).

Currently, the focus of public health in the United States is concentrated only on a short list of arboviruses including members of the Flaviviridae, Bunyaviridae and Togaviridae families; these include West Nile virus (WNV), Lacrosse virus (LAC), St.

Louis encephalitis (SLE), Western Equine Encephalomyelitis (WEE), and Eastern Equine Encephalomyelitis (EEE) viruses. Species of hematophagous mosquitoes are the known primary vectors of these viruses, infecting fowl with a cyclic, mostly enzootic pattern. Mammals including humans and horses are also involved in many cases as dead end hosts, which are defined as those hosts unable to provide sufficient viremia to further infect subsequent potential vectors or hosts. Through the work of Cupp et al. it is believed that ectothermic species, such as snakes and other amphibians could potentially be an overwintering reservoir host in the southeastern United States for Eastern Equine Encephalitis (Cupp et al, 2004).

The use of sentinel chickens for surveillance of arboviruses that are known to use birds as a reservoir host, such as St. Louis Encephalitis (SLE), West Nile (WN) virus, Eastern Equine Encephalitis (EEE) and Highlands J (HJ) virus, in Florida began with the Sentinel Chicken Arboviral Surveillance Network in 1978 (Day and Stark, 1996). Sentinel chickens are young immunologically naïve chickens that have not previously been exposed to the viruses being assessed and are therefore sero-negative, or do not have antibody specific to viral proteins in their sera. This network enables the activation of an early warning system for citizens as well as county epidemiologists and those in mosquito control, allowing for a coordinated effort of disease prevention. Prevention includes control of the vectors, as well as warning for medical personnel of possible encephalitic disease in the area, and the potential need for treatment of those individuals. Warnings for the public include press releases and media advisories, which are critical steps taken to inform the public to take defensive action against being bit by mosquito vectors.

There are 550 arboviruses currently identified, at least 100 of which are known to be pathogenic to humans (Gould 2006, Cann 2001). These are grouped into four viral families, which are the Togaviridae, Flaviviridae, Bunyaviridae and Arenaviridae (Table 1). All arboviruses are enveloped, measure 17-150 nm or more and are often spherical or occasionally rod shaped. Their genomes are coded as either positive or negative sense, single stranded RNA genomes. These RNA genomes are known to experience frequent mutation, thereby increasing the potential genetic variation within species and their phenotypic and genotypic differences (Calisher, 1994). These viruses infect humans by way of passage through the blood brain barrier from the peripheral circulation, where initial inoculation took place.

Arboviruses have evolved pathogen-vector relationships enabling not only their multiplication but also transmission from one host or species to another, thereby ensuring their ability to survive. In fact, scientific speculation suggests that ticks may have actually been the vectors first involved in the evolution of certain viral lineages, followed subsequently by the transmission by mosquitoes (Kuno and Chang, 2005). It also was once believed that viral multiplication was done with little to no damage caused to the arthropod host; recent studies however have found certain pathologies and decreases in function may occur to the vector during some infection with some viruses (Kuno and Chang, 2005).

In order for infection by the virus to take place, the vector must first be susceptible to infection and second, ingest a minimum threshold level of virus from the primary source (Chamberlain and Sudia, 1961). It is believed that the ability to be a

Table 1. Arthropod-borne viral families of significance: major Arboviruses and their significant non-arbovirus relatives (*) and the locations where they can be found. (ICTVdB Management, 2006)

Family Togaviridae **Genus Alphavirus Group A**		**Major locations**
Chikungunya virus		East Africa
Eastern equine encephalitis virus		North America, United States, South America, some Caribbean Islands
Everglades virus		North America, United States
Highlands J virus		North America, United States
Sindbis virus		Egypt
Venezuelan equine encephalitis		Caracas, Venezuela, United States
Western equine encephalitis		North America, United States
Family Flaviviridae **Group B**		
Genus Flavivirus		
Dengue virus		Africa, Tropical areas
St. Louis encephalitis virus		North America, United States
West Nile virus		Tropical and temperate regions worldwide, United states, Africa, Europe
Yellow fever virus		Africa, South America
Genus Pestivirus		
Bovine viral diarrhea virus *		Border disease virus
Genus Hepacivirus		
Hepatitis C virus *		Worldwide
Family Bunyaviridae		
Genus Ortho-Bunyavirus	California encephalitis virus	North America
Hantavirus	Sin Nombre virus *	North America, United States
Nairovirus	Crimean-Congo hemorrhagic fever virus	Eastern Europe, Asia, India, Middle east
Phlebovirus	Rift Valley fever virus	Africa, Saudi Arabia, Yemen
Tospovirus	Sandfly fever Sicilian virus	Middle East
Family Arenaviridae **Genus Arenavirus**		
Lassa virus *		West Africa

suitable vector is dependent upon the presence of certain binding receptors in the gut of the vector (Kuno and Chang, 2005). While evidence for this appears to be strong, primary studies have only been conducted using data extrapolated from the use of cell culture not studied in vivo, and therefore conclusions must be taken carefully if mosquito derived cell cultures were used (Kuno and Chang, 2005). The minimum infectious dose of the arboviruses varies by virus and species of vector; however, a correlation has been drawn between the threshold levels for arthropod infection and viremias in selected vertebrate hosts (Chamberlain and Sudia, 1961). Once infected, the vector, after an extrinsic incubation period, remains infected for the remainder of its life, for mosquitoes this is measured in terms of days or weeks, ticks on the other hand, due to their ability to become infected in their immature stages, may be infectious most of their two year life span (Kuno and Chang, 2005).

Primarily arboviruses are known to be zoonotic diseases, passing from one animal host to another via the vector intermediary. Susceptibility to infection is a primary determinant for the successful transmission and dissemination of arboviruses, both in the vector as well as the vertebrate host. Therefore, development of herd immunity or a decrease in population of the reservoir host has a significant impact on the continuance of infection transmission (Kuno and Chang, 2005). Arboviruses have developed three main strategies to ensure survival when these two limiting factors are in place. They include exploiting the inherent mobility of their vectors to new host populations, selecting for host vertebrate species with a high fecundity rate so that new naïve hosts are readily available, and lastly incorporating the means to evade the immune response in their hosts (Kuno and Chang, 2005).

Western and Eastern Equine Encephalitis viruses are both categorized as alphaviruses or Group A viruses, by terminology once used by the Center for Disease Control and Prevention (Kissling 1960); as of today there are known to be 27 alphavirus members identified (Calisher, 1994, Baron, 1996). Western equine encephalitis (WEE) is principally vectored by *Culex tarsalis* between passerine birds and intermittently a mammalian such as horses or humans (Kissling, 1960, Passler and Pfeffer, 2003)). It can be found at its peak between the months of June and September in western North America, in South America and Cuba however its transmission cycles are only narrowly understood (Kissling, 1960, Weaver et al, 1997). The extrinsic incubation time or time of multiplication within the mosquito has been determined to be approximately 8 days but may become infectious in as little as four days (Kissling, 1960). 639 confirmed human cases of this virus have been known to occur in the United States since 1964 (CDC.gov, 2007).

Also a member of the alphavirus genera is Eastern Equine encephalitis (EEE), while this virus will be discussed in more detail later; it is known that the primary enzootic vector in the infection of birds is *Culiseta melanura* (Kissling, 1960)(Service, 2004). The primary vectors to the mammalian hosts include members of *Aedes, Anopheles, Culex, Ochlerotatus* and *Culicoides* (a biting midge) species, as well as a the bridging vector between peridomestic and sylvatic cycles *Coquillettidia perturbans* (Kissling, 1960)(Service, 2004). Peak transmission can be found between March and September in the United States (Kissling, 1960). There are four lineages of EEE virus Group I occurring in North America and the Caribbean with the greatest health impact and primarily equine related in Central and South Americas caused by IIA, IIB, and III

(CDC, 2005). On average, 5 cases of human disease occur per year; between 1964 and 2004 approximately 220 human cases were confirmed (CDC.gov, 2007). EEE has a mortality rate of 50-75% of diagnosed cases, while 30% of those surviving infection remain with a neurological sequelae for life (Chonmaitree et al., 1989)

West Nile virus (WNV) and St. Louis Encephalitis virus (SLE) are both members of the Flavivirus genera also known by older CDC nomenclature as Group B viruses; they encompass 27 distinct viruses, including dengue, Japanese B and yellow fever. *Culex* species including *Cx. pipiens, Cx. modestus and Cx. univattatus* are all known vectors of West Nile virus for humans as well as birds, which may also be infected by certain ticks according to Service (2004). From 1964-2006 there were 27,573 cases of WNV in the U.S. reported to CDC, in 2006 among patients 33% had encephalitis symptoms, 65% had a milder form referred to as West Nile Fever and 59% had unspecified symptoms, SLE however from 1964-2006 had only 4,658 cases reported, fatalities among cases of SLE are generally approximately 5% of the infected, while those with life long neurological damage may include 10% of those surviving (CDC.gov DVBID, 2007).

Alphaviruses

Epidemiology and pathogenesis

Alphaviruses and Rubiviruses are the two genera that make up the family *Togaviridae* (Schmaljohn and McClain, 1996, Powers et al., 2001). Alphaviruses are present on all continents of the Earth with the exception of Antarctica (Powers, 2001). Due to their individual ecological cycles, amplifying and reservoir hosts, as well as their often highly specific hematophagous vectors, they remain relatively focused in their ideal niches such as swamps or woodlands, western or eastern North American geographies (Powers, 2001). There are seven acknowledged serocomplexes, or antigenic types of alphaviruses, three of these are of medical importance; they include Eastern Equine Encephalitis viruses (EEE) subdivided into North and South American, Western Equine Encephalitis viruses (WEE), and Venezuelan Equine Encephalitis viruses (VEE) (Passler and Pfeffer, 2003, Schmaljohn and McClain, 1996).

EEE can be found predominantly in a natural cycle between songbirds in fresh water swamps. Research by Unnasch et al. indicates that the cyclic behavior exhibited by this virus may be due to feeding by mosquitoes on naïve young of year (YOY) birds, mainly by the ornithophilic mosquitoes like Culiseta *melanura*, this research focused on the middle to eastern parts of the United States including almost the entire Atlantic coast from Florida to New Hampshire (Schmaljohn and McClain, 1996, Weaver et al., 1994, Unnasch et al., 2006). WEE, isolated from birds, horses, humans and other mammals can be found in both Canada and the western United States (Calisher, 1994). VEE unlike EEE and WEE does not appear to use birds as a reservoir host; it is believed that the cycle

includes a mosquito to small mammal and back to mosquito pathway (Schmaljohn and McClain, 1996). The last major epizootic of this disease was seen from 1969 to 1972 whereby it entered the United States from Mexico through Texas (Schmaljohn and McClain, 1996).

Alphaviruses in general produce a variety of disease processes and symptoms; these can include a fever, macular-papular rash, arthralgia, malaise, and arthritis in Old World viruses such as Ross River, Barmah Forest, Mayaro, Chickungunya and Sindbis, and encephalitides in the New World viruses such as EEE and WEE (Powers et al., 2001). They can infect a variety of creatures including rodents, reptiles, amphibians, fish, horses, humans and birds through the passage and multiplication within their arthropod vector with the exception of the salmonid viruses which include Salmon Pancreas Disease virus and Sleeping Disease virus isolated only from salmon and rainbow trout and not currently from any vector species (Powers et al., 2001). Each of these species have the ability to mount a specific immune response to the infective agent by way of the proteins protruding through the host derived phospholipid bilayer present surrounding the viral capsid protein. A specific immune response provides an opportunity to not only assess contact with the agent but in some cases the approximate time frame of contact. (Schmaljohn and McClain, 1996)

Classification and antigenic types

Classification of the alphaviruses has generally been based on the antigenic relationship of the members (Schmaljohn and McClain, 1996). The amino acid

sequences of the envelope glycoproteins E1 and E2 following PCR amplification is a more recent method used to identify and understand the evolutionary relationships of the alphaviruses (Bell et al., 1984, Schmaljohn and McClain, 1996). This technique originates from the original grouping that was based upon serological cross reactivity in hemagglutination-inhibition testing, complement fixation and the plaque reduction neutralization tests (Bell et al., 1984).

Immunological Response

Human immunity consists of two phases of response to an invading agent. These are the innate immunity for the early reaction and the adaptive or specific immunity for the later response. Of most importance for serological testing methods of acute disease is the response of the later phase, the adaptive response; the adaptive response itself is divided into two types, cell mediated and humoral immunities. Cell mediated immunity involves T lymphocytes while humoral immunity concerns the antibodies made by B cells. Antibodies contain binding sites specific to each invaders surface protein or other antigenic components. These immunoglobulins have the ability to target an invader for phagocytosis and destruction, neutralize its infectious ability, limit the toxicity of toxins and prevent entry of viruses into host cells.

There are different types of antibodies produced by B cells, these are different depending on the B cells state of activity, whether the cell is still naïve or has been activated by T lymphocytes and contact with a recognized antigen. IgM is a pentamer molecule and is the first immunoglobulin (Ig) molecule secreted from B cells while it is still a naïve cell, prior to isotype switching to IgG. IgM is a primary antibody response,

taking place when an antigen makes contact for the first time. After the immune response to the antigen is complete and the infection subsides these B cells will become memory cells and are kept for a period of time in case they are needed again. IgG is a secondary response, this Ig begins to be produced after continuous activation by the antigen and T lymphocytes. As the initial infection proceeds the cells begin to switch their specificities, IgG then becomes the prominent class over IgM in the case of most bacteria and viruses. IgG is a monovalent Ig and in humans has several different heavy chain isotypes. (Pier, 2004)(Janeway, 2005)

Because IgM is the primary antibody response to viral infection, quantification of it is an indicator of a new response to that infection, while IgG response alone simply indicates that at some point the individual has reacted to this invader before. IgM can also be seen as a subsequent response to the same (or in some cases cross-reactive) antigens, it is however usually in lower quantities than an initial response.

In non-vertebrate mammals, such as the chicken, Ig isotypes vary slightly from vertebrate mammals. Avian IgY is a homologue to human IgG with some slight variation; IgY contains four C_H domains while human IgG contains only three, it still however displays the same functional properties (Viertlboeck, 2007). Chickens also produce IgM and IgA, IgM being produced prior to IgG (IgY) as it is in all vertebrate species (Johnson, 2003). It is this early Ig that is the focus of the MAC-ELISA and the current Luminex protocols employed by the Florida Department of Health.

Eastern Equine Encephalitis virus

History

Eastern Equine Encephalitis (EEE) of the genus *Alphavirus* and the family *Togaviridae* appears to run in both sylvatic and peridomestic cycles (Figure 1) (Kissling, 1960). This virus is highly focalized due to ecological factors including the seasons, weather, the host/vector immunity and the density of populations. It was first isolated in 1933 from the brain of a horse, though the possibility of its presence in North America goes back to 1831 when a disease of similar clinical symptoms was documented after the death of 75 horses due to an encephalitic disease, though not confirmed (Calisher, 1994, Evans, 1977). The first human cases were confirmed in 1938 after the death of thirty children in the northeastern region of the United States following an epidemic in horses (Calisher, 1994). *Culiseta Melanura* is the vector responsible for the cyclic transmission among swampy and fresh and salt water marsh habitats (Calisher, 1994). This vector feeds primarily upon the wild bird populations in these areas and permits enzootics and epizootics that have the ability to move outward from the permanent foci of the virus, among the marshland wild birds (Calisher, 1994, Kissling, 1960). The intersection of the epidemic vectors into the scheme also takes place within the swampy marshlands. The epidemic vectors *Aedes sollicitans* and *Mansonia perturbans* were initially the main epidemic vectors in the United States; they are slowly being replaced however since the 1985 introduction of *Aedes albopictus* (Calisher, 1994).

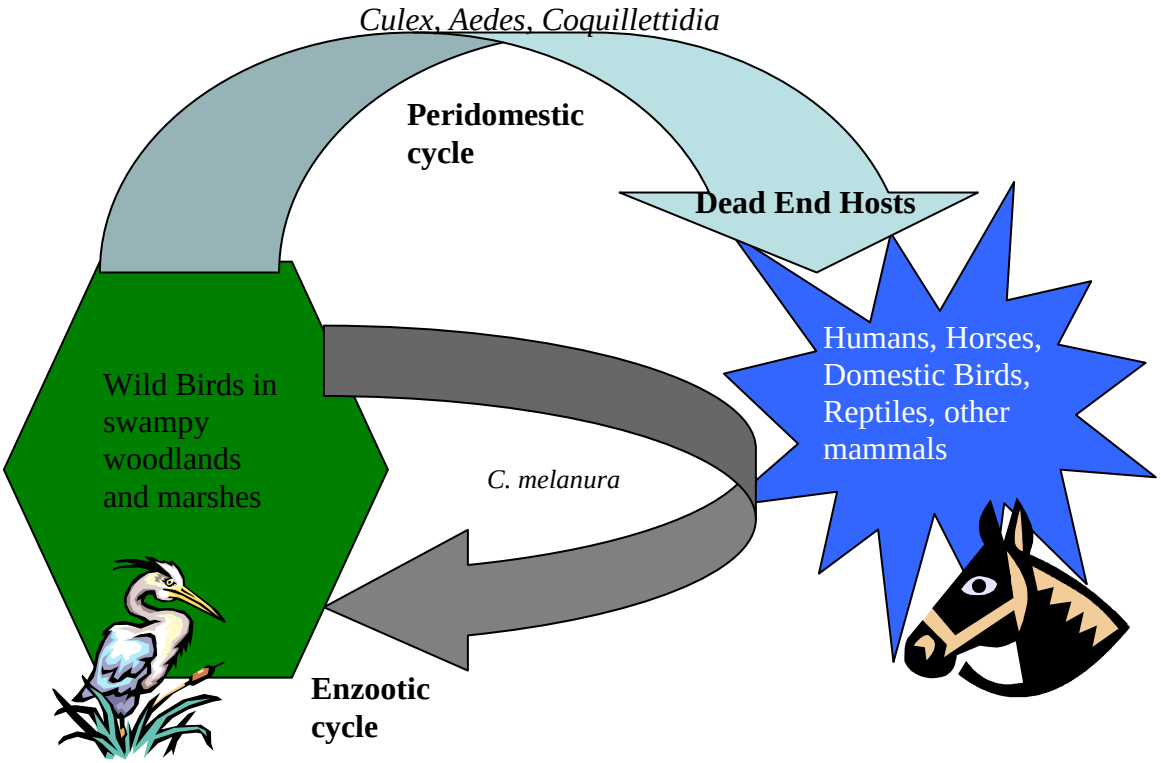


Figure 1. Diagram of the sylvatic and peridomestic cycles of Eastern Equine encephalitis.

The epidemic vectors are responsible for the transmission of the infection from wild birds in the marshes to human and animals outside this ecological niche including dead end hosts such as humans, horses, swine, deer, small mammals, and reptiles. These dead end hosts, while having the ability to become infected by the virus, rarely establish a viremia significant enough to permit transmission to naïve mosquito vectors; although it has been documented that horse to horse transmission can take place through *A. sollicitans* if the horse's viremic titers are found to be above normal (Kissling, 1960). Species of mosquito that are known to become infected and transmit EEE have been shown to require different viral titers for infection. Certain *Aedes* and *Psorophora* sp. have been shown to require a viral titer of are least $10^{3.0}$ LD_{50} per ml, several species of *Culex* have a requirement of at least $10^{8.0}$ LD_{50}, though *C. tarsalis* needs only $10^{2.5}$ LD_{50} per ml (Kissling, 1960). The percent efficiency of infection is directly related to the viremic titer and therefore changes differently based upon vector and host species.

Epidemiology

Eastern Equine Encephalitis virus can be subdivided into the North American, (also including the isolates from the Caribbean) and South American (also including isolates from Central America) subtypes (Calister, 1994, Passler and Pfeffer, 2003, Weaver et al., 1994). These two subtypes differ from each other in epidemiological, biological and genetically characteristic ways, thereby allowing isolation methods such as complement fixation (CF), hemagglutination-inhibition (HAI), enzyme-linked immunosorbent assay (ELISA), plaque reduction neutralization test and nucleotide

sequencing for distinction of the pathogen for research and diagnostic testing (Passler and Pfeffer, 2003, Strizki and Repik, 1994). In fact, it was Casals et al. who were able to show homogeneous reactions between virus located in North America and the Caribbean using kinetic HAI tests (Calisher, 1994).

The South American subtype appears to cause fatal infection in horses; however clinical symptoms in the rare human cases appear significantly less severe than its North American counterpart (Calisher, 1994, Passler and Pfeffer, 2003). Infection by the North American subtype while also rare in humans, compared to infection by some of those in the Flavivirus group, appears to have severe clinical manifestations for both horses and humans (Calisher, 1994, Passler and Pfeffer, 2003). The North American subtype is annually found in horses along the eastern coast, as far north as the southeastern portion of Canada and as far west as the upper Midwest United States (Calisher, 2003).

The continued study of West Nile virus has brought about another possible mode of transmission, organ and blood transplantation. The first report of WNV transmission in the blood supply occurred in 2002 (CDC, 2007). This then prompted the nationwide screening of the blood supply using minipool nucleic acid-amplification testing (MP-NAT) (CDC, 2007). Due to the extremely rare nature of the EEE virus in humans it is not likely that this mode of transmission will be of considerable significance. However it is important to keep vigil of the country's much needed blood and organ donation supply especially considering that many requiring its use may have immune compromise.

Host Vector and Transmission

While arboviruses are spread by a variety of arthropods, Eastern Equine encephalitis is primarily transmitted by mosquitoes. There are approximately 3300 species of mosquito in the world, all of which are contained within the family Culicidae (Service 2004). Three subfamilies that branch from this include the Culicinae, Anopholinae and the Toxorhynchitinae. EEE is primarily transmitted by members of the Culicinae subfamily, although Kissling and also Wellings both reported isolation of EEE virus from *Anopheles crucians* (Kissling, 1960, Wellings et al, 1972). In the sylvatic cycle the ornithophagic mosquito *Culiseta melanura* transmits the virus during the acquisition of a blood meal. According to research by Unnasch et. al nestlings and young-of-the-year (YOY) passerine birds provide a large majority of the meals for this endemic vector. These naïve young birds tend to form circulating viremia faster, and to levels equal or greater than those found in adult birds (McLean et al. 1995, Unnasch, 2006). In the peridomestic cycle, that which interacts with the birds, as well as humans and in limited areas horses, the primary bridging vectors includes several species of *Oclerotatus, Aedes, Culex* and *Coquillettidia,* again all members of the subfamily Culicinae (Kissling, 1960, Service, 2004).

As all mosquitoes, those in the subfamily Culicinae have a life cycle involving larvae, pupae and then adult phases. The eggs in this family are normally dark in color (brown or black) but can be laid either singly or in rafts depending on the genus (Service, 2004). All mosquitoes require water for egg deposit in some form, *Culiseta melanura* typically prefers fresh water, such as swamps or lakes, *Culex* prefers ground collections

of water as apposed to *Aedes* and *Oclerotatus*, which prefer smaller container habitats which include such things as tree-holes, tires, pock pools and plant axils (Service, 2004, Davis, 2005). Larvae also have distinguishing characteristics, *Culex, Oclerotatus* and *Aedes* all breath air directly from a siphon at the surface of the water in which they reside. *Coquillettidia* on the other hand, similar to the *Mansonia* genus, have siphons that provide them the ability to insert it into the roots of plants that float on the surface (Service, 2004). This has a significant impact on the methods of mosquito control for each type, those that reside on the surface can be reached by pesticide much easier than those submerged, however, those dependent on plants for air might alternatively be indirectly controlled by controlling the plant growth (Service, 2004). The pupae of each genus remains in the habitat of deposition, their breathing characteristics remain the same as during their larval stages (Service, 2004). Upon emerging from the pupal casing the adult mosquitoes vary widely in physical characteristics including color and pattern of their scales on wings, abdomen and legs. These very distinctive differences in appearance allow entomologists and those in mosquito control to identify the many varieties.

While it was long believed that infection of mosquitoes with an arbovirus caused no deleterious effects upon its host, it is now understood that infection with the virus may have an effects upon the hosts' fecundity, survival and obtainment of a blood meal (Moncayo, 2000). Moncayo et al. discovered that disseminated EEE infection had a significant impact on the length of survival (7-14 days) of *Cq. perturbans* and *Cs. Melanura* in their 20 day per os experiment, though there was little effect seen in *An. Quadrimaculatus* or *Ae. Albopictus, which* lived throughout the time of the experiment (Moncayo, 2000). The survival of the mosquito post extrinsic incubation period is vital in

determining vectorial capacity and therefore the time the virus can be transmitted through the vector as well as its rate of replication (Moncayo, 2000).

Clinical Features of EEE

Humans

There are an average of five cases of EEE each year in the United States, though this fluctuates by the year from 0-15. Florida, Georgia, Massachusetts and New Jersey have some of the highest case numbers seen by the CDC. While Human cases are very rare the sequelae post infection in many of those infected can be permanent and require the individual to be placed in permanent institutional care (Kelso, 1999) or lifelong disease related expenses nearing 3 million dollars per patient (CDC, 2005). Symptoms of EEE are usually seen within three to ten days of being bitten by an infected vector, it has an infection rate of 33% and if infected 50-70% of cases end in mortality and only 10% fully recover with no morbidity (Nandalur, 2007, CDC.gov) These statistics are based upon those who have had sera submitted for testing for viral antibodies; these numbers have not been fully assessed in a surveillance testing of the general population. These symptoms include: sudden onset of fever, vomiting, leukocytosis, hematuria, stiff neck, headache, malaise, and general muscle pain, and may progress to meningitis and /or encephalitis of the brain, seizures coma or paralysis. By the time symptoms are present virus is no longer found in the blood (Chonmaitree, 1989) therefore public health laboratories are usually called upon to test for antibodies to the virus in blood or cerebral

spinal fluid to determine if infection is recent (IgM) or from a previous period (IgG). Testing for these antibodies produced in the later phase of infection includes such techniques as hemagglutination inhibition (HAI) testing, MAC-ELISA, serum neutralization (SN) (Calisher, 1986) and complement fixation testing (CF). Confirmation of damage by EEE infection can be done with magnetic resonance imaging and computed axial tomography or CAT scan, provide evidence of edema, ischemia, and hypoperfusion in the early stage and proceeding to necrosis, vascular hemorrhage in brain and visceral organs and encephalitis (Paessler, 2004).

Horses and other ungulates

Horses are also a dead end host for EEE. Mortality in horses is between 75-90%. As in humans the disease can cause permanent neurological damage. In 2003 a record number of horses were infected with EEE, cases have risen nearly 3,000% in some parts of the United States. Clinical symptoms include a lack of coordination, loss of appetite, the grinding of teeth, inability to swallow, circling, involuntary muscle movements, rear end weakness, blindness, excitability and sensitivity to light and sound (aphis.usda.gov, 2003). These symptoms can then progress to further in-coordination, hyperesthesia, paralysis, coma and seizures which occur usually within 48-72 hours of clinical signs, eventually leading to death (aphis.usda.gov, 2003, Davis, 2005). Several clinical signs of EEE may be misinterpreted as rabies or toxicosis or vice versa, these include aimless wandering, circling, head pressing, staggering gait and difficulty swallowing, viral isolation or antibody testing can be used to differentiate the diagnosis from other

potential diseases such as rabies or some physical disease process such a stroke. MAC-ELISA is commonly used to test horse sera; a follow up paired sera for confirmation is also usually submitted to confirm diagnosis by PRNT. PCR is used to test for viral titer also in blood, though viremia is commonly transient, although commonly diagnosis is made upon necropsy (Poonacha, K.B., 1998) Recovery takes weeks to months of gradual improvement, though they may never fully recover pre-infection abilities (Davis, 2005). Those horses that do survive disease are often referred to as “dummy” horses due to the long term damage sustained to their brain, these horses can not be safely used to breed or ride (Jacob, 2003).

During the early phase of disease, when viremia is at its peak, is the most effective time to implement what little treatment is available for horses (Davis, 2005). This includes hyperimmune plasma or serum products infused immediately because by the time the neurological symptoms begin the viremic phase is most likely to be past (Davis, 2005). Truly vaccination is the best course of action. Vaccination for EEE, WEE and VEE in adult horses should be done bi-annually starting in early spring and early summer. In areas that experience endemicity however, the horses should receive boosters every six months (Davis, 2005). It has been shown that vaccination IgM should not interfere with the founding of the diagnosis, should the need arise; in other words the IgM level should never reach a four-fold rise in titer (Davis, 2005).

Pathological changes in the infected horse may include “neuronal degeneration and necrosis, vasculitis, and vascular thrombosis, gliosis, and neutrophilic infiltration” of the central nervous system including the cerebral cortex, thalamus, hypothalamus, and the anterior portions of the spinal cord (Poonacha, 1998, Franklin, 2002). One case report

indicated a colt with smooth muscle necrosis of the tunica muscularis of the small intestine post vaccination with a killed EEE vaccine, though the case is considered to be naturally occurring (Poonacha, 1998). Another case report indicated involvement of the bladder tissues when hemorrhagic tissue was found during necropsy also following 1 week post-vaccination (Franklin, 2002) Though these are the only mention found of such involvement outside the nervous system there is also a known case of myocardial involvement post infection in a pig (Poonacha, 1998).

Pigs, cattle, and goats exhibit similar symptoms as horses, however convulsions and paddling are added as more severe signs of pathology (Farrar, 2005). It is important to keep surveillance of this infection in horses and other barnyard animals in mind, due to their proximity to humans; disease in those unvaccinated indicates vectors present in the area are infected with the virus. Also important is the potential economic impact of the disease, racehorses, found commonly in Florida and other southern states cost an average of $95,000 to purchase and $35,000 dollars a year to train, this of course does not include those equines that are pets and still may be a considerable financial and personal loss for their owners. Another important consideration is the economic impact this disease may have upon agriculture. While human cases may be few, animal cases are known to be more common, this may impact food production in those animals raised for consumption such as pigs and cattle. Another consideration is the effect of infection in the long term for those animals that survive, is it possible that the animals or the products produced from surviving animals contains anything harmful to other animals of the industry or to the humans that consume them?

In Houston County Georgia in 2001 Tate et al. diagnosed EEE in a male white tailed free ranging deer (*Odocoileus virginianus*). Upon necropsy the deer was found to have histological finding similar to those found in EEE infected horses (Tate, 2005). In this same region the death and finding of the deer was following the death of two horses of the same virus (Tate, 2005). Antibody studies of deer in the region followed, 32% of the deer sampled were found to have antibodies to EEE (Tate, 2005). In one area the percentage reached as high as 55%, this indicates that deer are certainly exposed to the virus, however because there is little knowledge of symptoms or reports of outright disease they are probably not susceptible to severe infection (Tate, 2005).

Other Mammals

Other mammals documented to be susceptible to infection by EEE virus include, rodents, dogs, and bats. Studies in mice indicate that the virus spreads and multiplies through the peripheral tissues, into the blood stream and from there infects the CNS and brain tissue (Vogel, 2005). The symptoms found in mice include lethargy and ruffled fur initially, then after 4 days post-infection they exhibit to hunching, tremors, and prostration, which may then progress to death (Vogel, 2005). Hamsters and guinea pigs are also susceptible to infection by EEE and although it is not a mammal, inoculation of the spotted turtle (*Clemmys guttata*) has been shown to develop viremia (Smith A.L., 1980). The susceptibility of these small creatures of EEE has provided the opportunity to study vaccines in hamsters such as the work done by Cole and McKinney on a trivalent vaccine for EEE, VEE, and WEE (1971). Signs of infection in the hamsters initially

include pressing of their head against the cage, vomiting, lethargy, and anorexia; proceeding to central nervous system involvement after four days infection including stupor and coma then death (Paessler, 2004). These small mammals provide scientists with models in which to study the infectious disease process of EEE in an animal model with similar immunological response to humans it also allows the in-depth study of the pathogenesis of disease in humans in an economically feasible way.

In a twelve year span a veterinary clinic in South Georgia saw the deaths of 101 cases of dogs with neurological disturbances (Farrar, 2005). Symptoms included pyrexia, anorexia, and diarrhea, followed later by recumbancy, meningitis, nystagmus, depression and seizures (Farrar, 2005). The clinic notes no specific breed that was over represented, however all dogs were known to be housed outside primarily (Farrar, 2005). Upon necropsy the dogs were seen to have infiltrates in the gray matter of the brain predominantly in both their cerebral cortex and their midbrains; each diagnosis was confirmed by positive viral isolation by either tissue or blood samples (Farrar, 2005).

Bats are yet another mammal that has been shown to become infected by EEE. Colonial bats from Massachusetts, Connecticut, New Jersey and Georgia, are shown to be naturally infected by mosquitoes (Main, 1979). According to the work by Andrew Main at Yale University, it is believe the virus is spread to the host via the bite of the mosquito and not ingestion of the vector (Main, 1979). CNS involvement in bats is rare and therefore encephalitic symptoms are also rare, this may be due to the lack of neurotropism seen in Main's study of hibernating colonial bats (Main, 1979). While it has been thought that bats have the ability to be overwintering hosts, Main was able to detect viremia from hibernating bats only 24hr to 42 days from bats collected from caves and

mines in Massachusetts, Connecticut, New Jersey and Georgia (Main, 1979). These bats were bled and organ samples obtained following one week in an environmentally controlled cabinet in the lab (Main, 1979). Potential methods of transmission among the bat population include transplacental, through mother milk or possibly through urine (Main, 1979).. Another important finding of this research was the limited number of bats positive for EEE in their saliva, thereby making it unlikely that transmission could proceed by the bite of these animals unlike rabies (Main, 1979).

Fowl

Birds are the known reservoir hosts for EEE. All birds including those wild, as well as raised domestically are susceptible to infection, domestically reared fowl such as emus can have an infection rate of 65 percent with a mortality of 80 percent but can be as high as 100 percent, pheasants have a potential mortality of 25%-100% of those infected, turkeys considerably less at 5% mortality and ducks up to 60% can be lost due to infection with EEE (Helm, 2003). Signs of infection depend upon the species, but may generally include depression, bloody diarrhea, vomit, drowsiness, in-coordination, anorexia, blindness, partial paralysis and neurological involvement (Jacob, 2003, Helm, 2003). Emus in particular may become infected without vector involvement; their bloody feces can contain such high levels of viremia that it could be infectious to both other birds as well as humans (Helm, 2003). Supportive care is really the only treatment for infected birds, attempting to limit damage to the central nervous system and secondary infections.

Chickens tend to have greater viremia during infection when they are younger before the 14th day of life, a period prior to use by a sentinel chicken program (Guy, 2003, Byrne, 1960). The lethality of the infection decreases as the chicken ages (Guy, 2003). Signs in young chickens include depression, somnolence, paralysis, and death, though these are rarely seen (Guy, 2003). Myocarditis is attributed to be the most significant cause of death in chickens post EEE disease, though microscopic lesions are seen occasionally (Guy, 2003). Other findings on necropsy include necrosis of the liver, thymus, spleen, and bursa of Fabricius (Guy, 2003). Chickens are commonly used as sentinels for Arbovirus surveillance programs, the older the bird the less likely their infections will reach viremic levels sufficient to contribute to amplification in their immediate environment; they are relatively easy and inexpensive to maintain and bleed, and can be maintained relatively easily in populated areas where infection concerns may be greater than extremely rural populations (Moore et al. 1993).

Treatment and Prevention

Though various testing methods are now available for diagnosis of this viral disease, there are few if any treatments available to those infected. Pharmacologic supportive therapy for humans includes, antipyretics, analgesics, and anticonvulsants in addition to physical intervention such as ventilator support if patient becomes comatose (Nandalur, M, 2007). Although it is currently only in the research phase Ribavirin, pyrimidine derivatives and isoprinosine have been assessed for any attenuation of the infection in vitro, though in vivo results have been questionable (Nandalur, M, 2007)

Prevention is the best way to limit the spread of this disease. To do this people must be aware that mosquitoes are the vectors of transmission and by preventing bites from them, they are in fact protecting their health. Many of the Mosquito Control agencies of Florida and the Florida Department of Health all stress the five D's of prevention (http://www.co.bay.fl.us/bcpw/Diseases.html, (www.co.hernando.fl.us/mosquito/west_nile_update.htm) (Heshmati, 2004) and the one S These include avoiding being unprotected outside at dusk and dawn, the peak biting times of mosquitoes, dressing with clothing that provide coverage of the skin and therefore less skin accessible to biting, donning repellent with DEET, and draining of containers with standing water. The one S added by the Florida Department of Health includes the use of screens of windows to prevent mosquito entry into the home (Heshmati, 2004). As previously mentioned many of the genus's within Culicinae are container breeders, therefore removal or draining of a potential breeding container will decrease their numbers in the immediate vicinity of the container such as a residence if there is an open rain barrel.

Arbovirus Surveillance

Surveillance for arboviruses varies depending upon the state. Programs can include counting or viral detection in vectors, serological testing on sentinel or wild vertebrate hosts and case detection among domesticated animals such as horses as well as humans. A good surveillance system takes into account seasonal dynamics, ecology, meteorological data, and vector and vertebrate host surveillance. It is this last category

that is focused upon by the Florida Sentinel Chicken Program. According to the CDC Guidelines for Arbovirus Surveillance Programs in the United States by Moore et al. (1993), arbovirus vertebrate hosts for surveillance should have the following characteristics:

"1. Susceptibility to the monitored virus at rates that reflect virus activity in the surveillance area,

2. High Titer and long duration of antibody response,

3. Low morbidity and mortality (except in those species where high mortality is easy to detect),

4. Locally abundant population,

5. Locally mobile to increase exposure to and dissemination of virus,

6. Frequent exposure to vector species (could overcome lack of mobility),

7. Attractive to and tolerant of vector feeding,

8. Easily captured by conventional methods,

9. Ease in handling and obtaining blood specimens,

10. Age determination possible, at least young of year, or the regular multiple captures of tagged animals permits detection of seroconversions,

11. Relatively long-lived for multiple sampling of same animal".

With this information in mind there are several available surveillance hosts from which to choose. These include chickens, wild birds, equines, domestic and wild mammals, mosquito and lastly human case surveillance. Each meets the criteria listed above though each has its challenges. Chickens are relatively inexpensive to maintain and do not require large amounts of space, they must simply be placed

strategically so that infection can take place in areas commonly inhabited by people (Moore et al, 1993). Chickens can be used for WN, SLE, WEE, or EEE viruses though they have not been found useful in New Jersey for EEE (Moore et al, 1993). Use of equines is usually either a passive or rarely an active surveillance method. Due to the expense of raising and keeping horses it would not be economic to raise horses purely for the use of surveillance. Cases can however be quantified using passive surveillance of veterinarians. However in the cases of horses their vaccination status, transportation and delayed veterinary reporting may affect the data received and thereby effect the conclusions and activation of the warning system (Moore et al, 1993).

Florida Sentinel Chicken Program

The Florida Department of Health and Rehabilitative Services began the Florida Sentinel Chicken Program Arbovirus Surveillance Program in 1978 during a rural epidemic of SLE virus (Day and Stark, 1996). This system provides detection and early warning for areas, like Florida, where arboviruses at times become epizootic and spill over into the human populations causing epidemics. This system currently focuses primarily on West Nile virus (WNV), St. Louis Encephalitis virus (SLE), and Eastern Equine Encephalitis (EEE). Time placement of the chicken to their location is critical. A 1991 outbreak of EEE, due possibly to the late placement of chickens in mid June and therefore limited vector control, did not receive early detection or provide warnings and five human and an above-normal number of horses were infected (Day and Stark, 1996).

Chickens in a majority of the counties are kept from June through December though some year round, and are bled once weekly, or in some counties every other week, by the mosquito control personnel that keep them (Day and Stark, 1996). After baseline titers are drawn, 1.0ml of blood is drawn and sent to the Department of Health, Bureau of Laboratories in Tampa Florida for serological testing for antibodies to the viruses (Day and Stark, 1996). Once a chicken's sera is determined to be antibody positive, the chicken is re-bled for confirmation and if confirmed replaced with a new naïve chicken (Day and Stark, 1996). An increase in positive findings to 5-10% or more of the flock indicates viral presence and amplification in the vicinity of the flock; it is at this time that mosquito control is implemented to reduce the population of vectors in the area (Calisher et al, 1986).

Serological Antibody Detection Method

Arbovirus infection can be detected by a variety of serological assays. Serology determines infection past or present based upon the detection of an immune response by the host. Immunoglobulin G (IgG) antibody (Ab) is a monomer with a half life of 23 days, weighing in at 150kDa, it comes in four different classes 1-4 each varying only slightly in its characteristics; its main functions include opsonization, complement activation, antibody-dependent cell mediated cytotoxicity, and feedback inhibition of B cells (Pier, 2004, Abbas, 2005). In humans and other mammals, IgG is an indicator of a past infection, though when that past infection occurred cannot be determined. IgG has a higher affinity for its protein antigens than immunoglobulin M (IgM). IgM is indicative

of a recent infection. It usually appears in humans within 5-10 days of infection and while it has less affinity and specificity for its antigen that IgG, it exhibits greater avidity due to it pentamer or hexamer arrangement of binding sites (Pier, 2004, Abbas, 2005). IgM weighs 950 or 1,140 kDa depending on its arrangement and has a half-life of five days at 1.5mg/ml of blood in humans (Pier, 2004, Abbas, 2005). At the initial stimulation of B cells by infection and the subsequent activation of T-Cells which release important cytokines, IgM begins to be secreted and steadily increases, if this activation continues B cells begin what is called isotype class switching, thereby eventually secreting IgG. These cells are then kept as memory B cells in the event of a subsequent invasion by the same organism (Pier, 2004, Abbas, 2005). It is this process of reactions that serological testing allows us to investigate, in order to determine course of treatment for a patient or activation of epidemiological investigation during surveillance. The following are not exhaustive of the serological methods available for determination of infection. These are however the most commonly applied techniques in the Florida sentinel chicken surveillance program.

Hemagglutination Inhibition Test

The Hemagglutination test is an economical screening tool for the arboviruses in the Sentinel Chicken Program in Florida. Inhibition of agglutination by present antibodies indicates a positive reaction. Positive reactions are seen regardless of whether the immunoglobulin is IgG or IgM. IgM can be detected as early as four days post infection, while IgG by day seven (Calisher et al., 1986). IgM titers persist for 250 days

post infection, while its titer does drop initially it can then increase again over an eight month period, IgG seen one week after inoculation, peaks at three to four weeks and then subsequently declines (Calisher et al., 1986). Cross reactivity is commonly seen in this test between members of the same family; for example, West Nile Virus and St. Louis encephalitis, both from the Flavivirus family and Highlands J and EEE virus of the Alphaviruses.

At the Florida Department of Health the following steps are performed for the HAI testing. This process provides screening results within one week's time (samples on Monday or Tuesday and submitted by Wednesday have results by Friday) though this does not include confirmation by MAC-ELISA or SN.

Processing of sera of the Hemagglutination Inhibition testing at the Florida Department of Health follows briefly.

- Centrifuge vial at 800 x g for 10min,
- transfer supernatant into clean tubes,
- aliquot 100uL of serum into new tube set,
- place in ice bath and add 0.5mL of Protamine Sulfate (PSO_4), (Holden, 1966)
- add 6mL of Acetone and agitate for 5 minutes with wooden sticks,
- pour off acetone and vortex to break up particulates at the bottom,
- repeat last two steps then dry samples overnight.
- the next day add 1mL BABS, using sticks to scrape sides if necessary,
- let stand for 1 hour and then centrifuge for 10minutes at 800 x g,
- transfer samples to new vial and add 2 drops of washed goose erythrocytes,
- shake and hold for 20 minutes in ice bath,

- centrifuge at 800 x g for 10minutes,
- aliquot serum into microtiter well plates, add antigen, incubate overnight at 4ºC
- add goose erythrocytes, incubate at room temperature one hour and read for reactivity.

HAI while known to have few false positives it does however take time and dedicated personnel. This method has few places in which automation is beneficial and also requires a source of geese erythrocytes.

IgM Antibody Capture Enzyme-Linked Immunosorbent Assay

IgM Antibody Capture Enzyme-Linked Immunosorbent Assay or MAC-ELISA is a more rapid testing method used for confirmation of antibodies to Arboviruses following HAI. Time requirement is only two days as opposed to five with approximately four hours of hands of time for a 40-sample test (Johnson, 2005). IgM testing is chosen for arboviruses not only for the ability to capture antibody in a faster time period, but also IgM is less cross-reactive than IgG (Martin, 2000). The Florida Department of Health Bureau of Laboratories in Tampa briefly uses the following steps in their Sentinel Chicken sera testing (Martin, 2000).

- Add 75uL of anti-chicken IgM capture antibody to microtiter plate,
- incubate overnight at 4ºC,
- decant antibody and blot,
- add 200µL of blocking buffer and incubate for 30 minutes at room temperature,

- wash plate with washing buffer 5 times,
- add 50µL of chicken sera at 1:400 dilution, add 50µL of positive and negative control sera at 1:400 dilution to the plate,
- cover, incubate for 1hr at 37ºC,
- wash plate 5 times,
- add 50µL of viral antigen to appropriate wells,
- add 50µL of normal (negative control) antigen to appropriate wells,
- incubate overnight at 4ºC,.
- The following day wash plate 5 times,
- add 50µL of diluted anti-viral monoclonal antibody conjugated to (name of enzyme) to the wells,
- incubate 1 hr at 37ºC, wash plate 5 times twice,
- add 75µL TMB (3,3',5,5'-tetramethylbenzidine) to each well and cover to protect from light,
- incubate 10 minutes at room temperature,
- add 50µL of 1N H_2SO_4 to stop reaction,
- let stand for 1 min and the optical density (OD) is read at 450nm with a spectrophotometer plate reader

A serum is determined positive if the P/N is greater than to 2.2 and negative if less than 21.6. A result of equivocal is given if the result is in the range of P/N 1.6-2.2. Taking the OD of the test serum and antigen and dividing it by the OD of the negative control serum and antigen determines the P/N. A P/N of 2 indicates that the OD of the test sera is twice that of the negative sera. This testing method is one of the methods on

which the Luminex Microsphere assay is based, however instead of a solid surface (microtiter plate) the surface of reaction is the bead itself.

Plaque Reduction Neutralization Test

The Plaque Reduction Neutralization Test or PRNT is the most specific of the tests thus far discussed for the arboviruses. This test is used in confirmation of positive sera as well as if sera tests negative in the ELISA, following a positive reaction in HAI. Though cross-reactivity still occurs it is thought to be less of a problem in the PRNT though ambiguous results still do occur (CDC.gov, 2004). This test relies upon the actual virus itself for quantification of antibody; it must therefore be done in proper BSL-3 containment. This makes the test more technically difficult and restricts its use to those laboratories with the proper room, equipment and training.

Test serum is titrated and mixed with a known concentration of test virus. This serum virus mixture (SV) is incubated overnight at 4ºC and then Vero (African Green monkey kidney) cells are infected with the SV.. Plaques, areas of cell death occur is a mathematically predictable fashion directly related to the amount of virus plated. Seeing a decrease in the expected number of plaques versus controls indicates that antibody capable of neutralizing the virus exists in the sample.

Overall it may be two to three weeks before a final result is issued on a newly positive or borderline sample. This time delay may cause effects in mosquito control as well as the potential for further animal and human infection. It is a goal of this research to demonstrate that a Microsphere immunoassay for the presence of IgM antibodies to EEE virus will be a sensitive and specific and far more time efficient manner to continue testing for this important virus.

Microsphere-based Immunoassay

While the MAC-ELISA assays provide faster time to result than HAI, a still faster option is now available using new technology. The Microsphere based immunoassay combines flow- cytometry and ELISA technology into one. Instead of a microtiter plate as the solid phase of the assay Microspheres with two varying fluorescent dyes, which give up to 100 options of color identification to choose from, are used upon which can be bound antigen or antibodies. These beads, through varying protocols, can be used to quantify such things as antibodies, enzyme substrates, receptors, viral antigens, and cytokines by using bound fluorochromes to indicate bound anylate. Two of the main advantages of this relatively new technology are speed and the ability to multiplex. Assay of a 96-well plate takes between 30-40 minutes and it can be tested for up to 100 different analytes, limited only by the differing beads required to indicate the different substances. This large array of different beads allows the testing of different analytes all in the same well at the same time. In general a Microsphere assay must include a defined bead set, the anylate and a reporter fluorochrome such as R-phycoerythrin. (Bio-Plex, 2005)

The xMAP 5.5-micron polystyrene beads produced by Luminex Corp (Austin, TX) with a carboxylated surface can be bound to such things as proteins, oligonucleotides, polysaccharides, lipids, antibodies, small peptides, antigen or antibodies directly, permitting the testing of the before mentioned analytes (Kellar and Iannone, 2002). Using varying protocols, samples and controls are added followed by the reporter fluorochrome in order to determine the strength of signal in each well for each bead set.

The Luminex Bio-Plex system by Bio-Rad draws a sample using a syringe mechanism from each well and in singular file two lasers are beamed at the sample to excite the fluorescent pigment in the bead for classification and the fluorochrome reporter which determined the fluorescent intensity and further can be used in conjunction with a standard to quantify the samples.

The first laser is a red laser; also know as a classification laser. This excited the bead and allows determination of the two red fluorescent dyes within and therefore its specificity, which is determined by the researcher. The second laser is a green laser, which is also called the reporter laser. This is used to excite the reporter molecule and from this the mean fluorescent intensity of the sample is calculated.

The MIA protocol currently used by the Florida Department of Health for the Sentinel Chicken sera was designed by Logan Haller M.S.P.H (Haller, 2006) is based upon a duplex method designed by Johnson et al. (2005) at the DVBID laboratories of the Centers for Disease Control and Prevention for the detection of anti-West Nile and anti-St. Louis antibodies in human sera. Prior to any testing the carboxylated microspheres (Radix Biosolutions) must be bound to 6B6C-1 monoclonal antibodies; this process is completed to two different bead sets which will permit the differentiation of the two viral antibodies once antigen is subsequently bound the final product . Positive and negative viral antigens are required for the binding of specific IgM antibodies. For example, the protocol for West Nile testing requires the mixing of 580µL of WNV positive recombinant COS-1 Antigen with 350µL of the bead set 32 solution and 2870µL of running buffer or 160µL of WNV negative recombinant antigen, 200µL of the bead set 32 solution and 1640 of the running buffer. SLE testing requires 35µL of SLEV positive

SMB antigen, 350µL of the bead set 57 and 3115 of the running buffer, 20µL of the SLEV negative Normal mouse brain antigen is added to 200µL of bead set 32 and 1780 of the running buffer.

After binding antigen the beads can be used in capturing specific antibody in the chicken sera. Testing is done in a 96 well filter plate (Millipore cat # MABVN1250, lot# F5HN65106), which allows the reaction to occur and washing steps to be sequentially done and the supernatant vacuumed through the porous filter bottom (VWR cat# 16003-836) without the loss of the 5.5uM beads. The current WN/SLE protocol includes many wash and reaction steps.

Objectives

As the climate and landscape of our world changes, and people encroach further upon sylvatic habitats, vector borne diseases are becoming an ever-greater public health concern. Eastern Equine Encephalitis virus has a mortality rate of 30% of those cases diagnosed, while 30% of those surviving infection remain with neurological sequelae for life (CDC.gov, 2007).

The use of sentinel chickens for surveillance of arboviruses that are known to use birds as a reservoir host, such as St. Louis Encephalitis (SLE), West Nile (WN) virus, Eastern Equine Encephalitis (EEE) and Highlands J (HJ) virus, in Florida began with the Sentinel Chicken Arboviral Surveillance Network in 1978 (Day and Stark, 1996). This network enables the activation of an early warning system for citizens, as well as, county epidemiologists and those in mosquito control, allowing for a coordinated effort of disease prevention.

The current methods in use at the Florida Department of Health, Tampa Branch Laboratory include screening of sera for antibodies to these arboviruses of epidemiologic importance by way of the hemagglutination inhibition test (HAI), and confirmation by the IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) and Plaque Reduction Neutralization test if the MAC-ELISA proves to be negative. While

these tests combined are providing the results needed, the time to result can be a week or greater depending on the initial screening result in the HAI tests.

The Microsphere Immunoassay (MIA) provides the capacity for an improved time to result. In addition, it is expected that the EEE MIA assay will provide results both more specific and sensitive than the current protocols.

This research aims to demonstrate that the use of a Microsphere-based Immunoassay (MIA) for the detection of EEE IgM antibodies in chicken sera will provide accurate classification of the results, comparable to those of the ELISA assay, in a significantly reduced time frame from days to hours.

The specific aims of this research include:

1) determine if Luminex Microsphere Assay Technology can provide an accurate, more rapid detection method specifically designed to test for antibody to EEE in sentinel chicken sera,
2) determine if any tested samples collected from those sera routinely submitted and tested by HAI and shown negative in standard testing, indicate positive or equivocal result by the MIA method and therefore indicate a missed positive
3) determine the Sensitivity and Specificity, Positive and Negative predictive values of this new protocol as compared with MAC-ELISA as a reference standard.
4) determine if Highlands J virus will have an impact on the testing protocol and results of this test meant to be specific for EEE

Materials and Methods

Processing and submission of Sentinel Chicken Sera Samples

Serum samples are submitted from counties with chickens at sites maintained throughout Florida to the Florida Department of Health Bureau of Laboratories Tampa location weekly. Upon arrival at the laboratory, samples are aliquotted for screening using the hemagglutination inhibition antibody test (HAI) for flavivirus (WN and SLE) and alphavirus (EEE and HJ) antibodies (Figure 2). Those screening positive for either of these groups are then submitted for confirmation of IgM using the IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA). Following a negative result in the MAC-ELISA the samples are tested using the plaque reduction neutralization assay, which classifies the sample based on IgG. Following this process the samples are kept at 4ºC until tested using the Microsphere immunoassay (MIA). In 2007, 2,752 sentinel chickens were maintained in 282 locations throughout the state. Overall 47,803 sera were submitted for testing to the Florida Department of Health including 5,432 from the southern region, 24,383 from the northern and panhandle regions and 17,988 from the central region. From these samples 2,162 total sera were randomly selected for MIA analysis, 748 for the south region, 516 for the northern/panhandle region and 681 for the central region. For analysis of known positive HJ positive and EEE positive sera, an additional 314 samples were randomly chosen from sera collected during 2003-2007

(Table 2). In addition three wild bird sera were tested to confirm that this methods primary antibody cannot be used for bird species other than chicken.

Table 2. Total sera samples submitted during the 2007-year and samples tested in this research both regionalized by location.

	Sera submitted 2007	Tested in MIA
South	5,432	748
Central	17,988	681
North & Panhandle	24,383	516
Known positives	N/A	314
Total	47,803	2,291

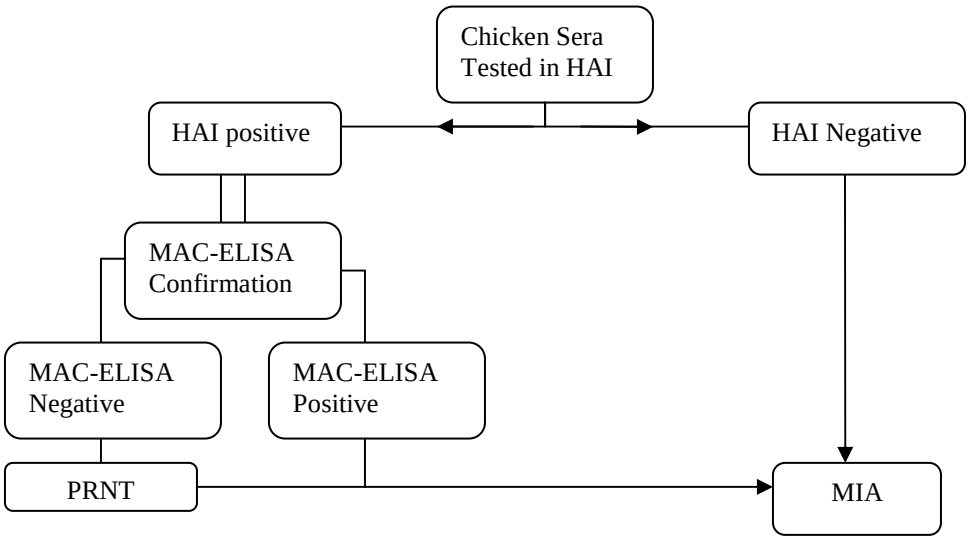


Figure 2. Flowchart for sentinel chicken sera testing for antibody to alphaviruses at the Florida Department of Health Bureau of Laboratories. Sera are aliquotted separately from the original sample for each test; all samples are stored at 4ºC. It is first tested for antibody to alphaviruses using the HAI method, which has the ability to detect both IgM and IgG. In order to confirm that this bird is newly infected a MAC-ELISA is performed to determine if IgM is present. If IgM is not found the samples are tested on a PRNT assay to detect any viral specific IgG in the sample. Following these standard testing methods, the samples were then assayed using the MIA that detects only IgM.

Sample Size and Specimen selection

Due to the regionalized patterns observed in EEE sentinel chicken data (Table 3), samples were drawn based on the region in which the site was maintained (Table 4). On a weekly basis sample numbers were randomly selected using Research Randomizer (http://www.randomizer.org/form.htm, 2008). Sera had been stored at 4ºC prior to testing. To determine the sample size needed for each region, the sample size calculator from Cameron and Baldock (1998) was used selecting 80% expected sensitivity and specificity, a level of significance or $\alpha = 0.05$, a power of 95% and the varying populations and percent of expected prevalence (Table 5).

Serum analysis

Sera for this study were taken from samples submitted for arboviral testing at the Florida Department of Health Bureau of Laboratories. Positive samples were primarily taken from year 2005 stored samples, though some were taken from 2003; these positives were chosen with regard to their MAC-ELISA P/N result to incorporate a wide range of values from equivocal (1.6 - 2.2) to low through high positive (18.6).

HAI testing had been performed on these sera using the method by Clarke and Casal (1958), after protamine sulfate and acetone extraction were performed to eliminate non-specific protein interactions (Holden, 1966). Sera both negative and positive for EEE had been held for testing following the HAI and MAC-ELISA (Martin, 2000) and or PRNT testing at 4ºC.

Table 3. Sentinel chicken seroconversion rates by region and state for 2002-2007. Stark, L.M. and Kazanis, D. (2002-2007) Arbovirus Surveillance: Annual Summary Report. Florida Department of Health, Bureau of Laboratories, Tampa.

Year	South	Central	North/Panhandle
2007	0	1.2	7.6
2006	0	0.1	5.0
2005	0.2	2.7	14.5
2004	0.2	1.0	7.8
2003	0	2.1	13.6
2002	0	1.1	4.9
2007 MASR 95% CI*	0.03-0.43	0.46-3.48	7.92-12.16

* Confidence interval of the Mean Annual Sero-conversion Rate

Table 4. Counties by region used for sample selection

Panhandle	**North**	**Central**	**South**
Alachua	Citrus	Brevard	Charlotte
Bay	Duval	Hillsborough	Collier
Escambia	Flagler	Indian River	Dade
Gulf	Nassau	Manatee	Glades
Jackson	Orange	Osceola	Hendry
Leon	Pasco	Pinellas	Lee
Walton*	Putnam	Sarasota	Martin
	Seminole	St. Lucie	Palm Beach
	St. Johns		
	Volusia		

* wild bird sera also submitted but not tested by MIA

Table 5. The sample size for each region as determined using the sample size calculator from Cameron and Baldock (1998). Sero-conversion means were taken from 2002-2006 arbovirus surveillance reports.

Stark, L.M. and Kazanis, D. (2002-2006) Arbovirus Surveillance: Annual Summary Report. Florida Department of Health, Bureau of Laboratories, Tampa.

Region:	Mean sero-conversion	Mean population	Number of samples recommended
South	0.03-0.43%	758	758
Central	0.46-3.48%	1008	673
North	7.92-12.16%	1265	479
Panhandle	7.92-12.16%	506	52
			1962

The process for MAC-ELISA as previously discussed, using antigen provided by CDC, was performed by technologists of the Florida Department of Health, Tampa Branch. Briefly lyophilized goat anti-chicken antibody (MP Biomedicals, cat# 64395, lot# 8155H) diluted at 1:1000 in buffer (0.015M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6) and placed in separate 96 well microtiter plates. Sera and controls diluted 1:400 in wash buffer were then added in duplicate and EEE Antigen (CDC, cat# M29603) was added at 1:800 dilutions in wash buffer. After an overnight incubation at 4ºC, monoclonal antibody to EEE virus conjugated to horseradish peroxidase (CDC, cat# VS2371) was added. After a one-hour incubation at 37ºC, the plate was washed and the enzyme substrate TMB (3,3',5,5'-tetramethylbenzidine) (Sigma, cat# T8665), was added. After 10 minutes, the reaction was stopped with 1N H_2SO_4 and the plates read for their optical density (OD) at 450 nm, using a Beckman Coulter AD340 spectrophotometer. Positive to negative ratios (P/N) was determined by dividing the mean OD of the test serum with the viral antigen by the mean OD of the negative control serum on the same plate. If the P/N is greater than or equal to 2.2 the serum is considered positive, if 1.6 to 2.2 it is considered equivocal and less than 1.6 it is considered negative. Equivocal and negative results are then confirmed using the PRNT method.

Samples that are negative for antibodies by HAI are not tested further in the MAC-ELISA therefore they were tested directly using the Luminex. For those samples chosen to be included in the calculation of the cutoff values, MAC-ELISAs were performed to determine IgM value. A sample of 720 sera testing both positive or negative in MIA as well as MAC-ELISA were then used in the determination of result cutoff using

Receiver Operator Characteristic (ROC) curves from Analyse-it software v2.0 (Analyse-It Software LTD. England, United Kingdom).

Microsphere-based Immunoassay

Addition of Antigen to Bead Sets

Three µl of normal suckling mouse brain (NSMB, CDC cat# M29714) or EEE+ mouse brain antigen (CDC, cat# M29603) and running buffer, phosphate buffered saline, with BSA, pH 7.4, was combined with bead set 15 coupled to 2A2C-3 monoclonal antibody to alphavirus (Radix Biosolutions, Georgetown TX) (Johnson (CDC, Personal Communication) (Table 6).

The mixture was placed in a 4ml brown Nalgene bottle (Nalgene, HDPE lot #2004-915) to limit light absorption and incubated on a labquake tube rotator at 8 RPM (VWR cat # 56264-302) for one hour at room temperature. This method gives a final concentration of 500 beads/µL. After antigen capture, the beads were kept at 4ºC for no more than one month (Johnson, 2005). Working dilutions of bead sets, primary and secondary antibodies were prepared on the day of experimentation, kept on ice and wrapped in foil to protect from ambient light bleaching of the bead sets and the reporter molecule prior to use.

Table 6. Volume of reagents used to create antigen coupled bead set 15 at 500 beads/µL with a total volume of 1000 µl.

Reagent	Volume in µL
Buffer	897
NSMB or EEE+ Antigen	3
Bead set 15 with 2A2C-3 antibody	100
Total volume	1000

Standardization of Microsphere-Based Immunoassay

Testing of human sera for IgM requires the depletion of IgG from the sample. To rule out the need for such treatment for chicken sera, multiple samples were subjected to IgG depletions using the Protein G sepharose 4 Fast flow (Amersham Biosciences #NC9354476) following the manufacturer's recommendations. Briefly, a slurry was made using one part Protein G sepharose (PGS) to three parts 20% ethanol. A filter plate was pre-wet with PBS for five minutes. After suctioning off the PBS, 80 µl of PBS plus 20 µl of PGS slurry were added and then the diluent immediately suctioned off (Multiscreen Resist vacuum manifold cat#MAVM0960R); 95 µl of PBS and 5 µl of neat serum were added. Mixture was resuspended by shaking with a Lab-line plate shaker platform (VWR# 57019-600) at 1100 RPM. A microtiter plate was placed below the filter plate to collect the now IgG depleted serum from the filter plate after vacuum filtration. In order to determine if this process was necessary in chicken sera MIA assay results of depleted and non-depleted sera were compared using an ANOVA.

Testing chicken sera as opposed to human sera offers some challenges. For example, in the research by Johnson et. al with human clinical samples, there was available a single anti-human IgM antibody conjugated to the detector molecule phycoerythrin (PE) commercially available, however, such a commercial product is not available for chicken antibody and therefore a two antibody approach with goat anti-chicken and porcine anti-goat conjugated to PE was used (Haller, 2004). This deviation from the original method designed by Johnson et al. provided an economic and feasible alternative.

The specially designed filter plate (Millipore cat # MABVN1250) was divided in two halves, the left containing the positive antigen bead set and the right containing the negative antigen bead set. In order to determine the best concentration for the bead set, using the least number of beads, three concentrations of bead set with buffer were tested and the data analyzed using student's t-test and ANOVA. Bead concentrations included a 1:5, 1:10 and 1:20 dilution of the stock solution tested at various serum concentrations (1:80, 1:160, 1:320, 1:400, 1:640, 1:1280) in duplicate, holding the primary and secondary antibody dilutions at 2µg/ml and 1µg/mL respectively, a dilution previously determined optimal for the WN/SLE test by Haller. These findings were then tested using an ANOVA followed by Tukey's multiple comparison tests.

Determination of the most appropriate dilution for the chicken sera using the least sample was accomplished in a similar manner, 24 known positive and negative sera were tested in duplicate and then compared at 1:80, 1:160, 1:320, 1:400, 1:640, 1:1280 between each concentration by ANOVA followed by a Tukey's multiple comparison test. These dilutions were performed holding the primary and secondary antibody dilutions at 2µg/ml and 1µg/mL respectively.

The optimal concentration of the primary goat anti-chicken IgM serum lyophilized (MP Biomedicals, cat# 64395) antibody and secondary porcine anti-goat IgG-phycoerythrin (PE) (R&D Systems, cat# F0106) antibody was done by testing 23 samples in duplicate using three different combinations, 4µg/mL of primary goat anti-chicken with 2µg/mL of porcine anti-goat PE, 2µg/mL of primary goat anti-chicken with 2µg/mL of porcine anti-goat PE and finally 2µg/mL of primary goat anti-chicken with 1µg/mL of porcine anti-goat PE all at 1:400 sera dilution factor; These were then evaluated using an

ANOVA followed by a column chart of the values and a Tukey's multiple comparison test.

In order to determine that antibody binding was giving true reading results, not aberrant noise and that incomplete test wells would not give false results, tests were run for a baseline of fluorescent response with positive and negative known samples with and without primary as well as secondary antibody (Table 7).

For the establishment of the cutoff values using ROC curves, 720 specimens were selected with both Luminex and MAC-ELISA results. The method of data transformation (P/N) method used in the ELISA was chosen to determine the cutoff values at the screening level. To determine the cutoffs, the positive Ag beads set Mean Fluorescent Intensity (MFI) value was divided by the Negative control sera MFI value of the plate, this was considered the transformed value for screening. The same cutoff value was applied to the confirmation testing which incorporated the division of the positive antigen bead set MFI divided by the negative antigen bead set MFI for that serum. This confirmation, which took into account the background reactivity of each sample, was critical to determine if the sera were indeed reactive to the antigen and not to something non-specific in the well. Results discussed later will show the importance of the negative antigen value when determining the final result conclusion for each serum.

Table 7. Combinations employed to confirm absence of reactivity in incomplete wells.

Sample	Primary	Secondary
None	Present	Present
Present	None	Present
Present	Present	None
Present	Present	Present

Microsphere-Based Immunoassay Protocol

Bead sets conjugated with positive viral or negative antigen as previously described were diluted 1:10 with running buffer; sera were diluted at a 1:400 in deep well plates, with running buffer. Primary and secondary antibodies were diluted with running buffer at 2µg/mL and 1µg/mL respectively; all working diluent dilutions were covered with aluminum foil and kept on ice for use the same day. To establish the most accurate assay with the least cost both a screening and confirmation protocol were designed.

In the screening protocol a 96 well filter plate was designed with a negative and positive control in duplicate, these controls are also found on the confirmation plate on both the positive antigen and negative (control) antigen sides. The goal of screening was to establish a preliminary reference for the number of sera with potential to have confirmed results. In this case the MFI values of each tested sera were divided by the negative control MFI values. The negative control serum used had been through the standard process on negative confirmation as well as the MAC-ELISA prior to use in this and other assays requiring a sera negative control.

The 96 well plate was divided into two halves for the confirmation protocol; one for the reaction with the positive EEE antigen (CDC, cat# M29603) (left) and one for the negative NSMB antigen (CDC, cat# 0006) (right) (Figure 3). This configuration permitted the assay of 44 sera samples per plate. To hydrate the filter, 100µL of running buffer was added to each well and left in place for five minutes; 50µL of each bead set (diluted 1:10) was added to its respective side and the plate washed twice with running

buffer. 50µL of sera (dilution 1:400) was then added and the plate, covered with tin foil to protect from ambient light bleaching, was placed on a Lab-line plate shaker platform (VWR# 57019-600) initially for 30 seconds at 1100rpm and then for one hour at 300 rpm. The plate was washed twice with running buffer, primary antibody 2µg/mL was added and the plate was incubated on the plate shaker for 30 seconds at 1100 rpm and then at 300 rpm for thirty minutes. After the plate was again washed twice with running buffer, secondary antibody was added at 1µg/ml and the plate was incubated at room temperature for 30 minutes on the shaker at 300 rpm after 30 seconds at 1100 rpm. After a final double wash, 100µl of running buffer was added and the plate shaken at 1100 rpm for 30 seconds. This completes plate preparation; it is placed into the Luminex Bioplex 100 suspension array system (Bio-Rad Labs (BioPlex) cat# 171-203060) for assay. The software returns the results in MFI (Mean Fluorescent Intensity).

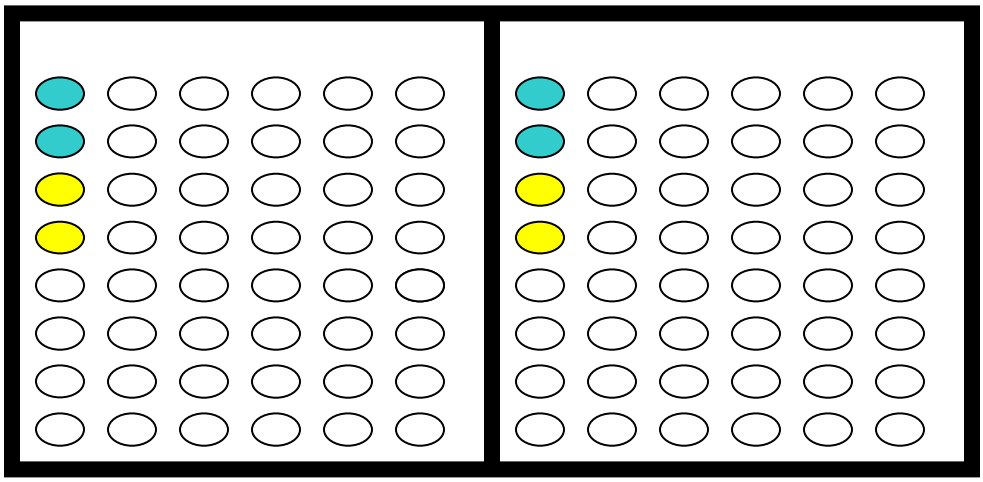
During setup of the instrument protocol, settings different than factory defaults were used, these were: 100 beads per region were read with a sample size per needle draw of 75 µl. The Override gates were placed at 5,000-10,500. Results were then selected to be automatically stored; worksheets were categorized by bead set.

Discernment of IgM Antibodies to EEE

Prior to each assay run, the Luminex system was turned on and the lasers warmed up for one half hour and the level of Bioplex sheath fluid (BioPLex, 20L cat#171000055) was checked to ensure testing could be completed without interruption or artifact due to

clumping or bubbles. After warm up, instrument calibration was performed and data relating to the samples and dilution were entered into the protocol screen.

Each bead set in a Luminex microsphere assay is read first by the red laser, also known as the classification laser. This laser quantifies the number and type of bead set in the sample indicating the number and percent of clumping of sample in the results. The green laser, also known as the reporter laser, quantifies the R-Phycoerythrin attached to the secondary antibody, porcine anti-goat. The intensity of the R-PE signal is directly proportional to the concentration of the PE that is bound to the chicken antibody/antigen /2A2C-3 antibody/bead complex. This value is then translated by the software into the Mean Fluorescent Intensity (MFI) of the samples



EEE positive Antigen Bead Set

NSMB Control (Negative) Antigen Bead Set

EEE positive sentinel chicken control sera

EEE negative control sera

Sentinel Chicken test sera

Figure 3. Confirmation Plate design used for testing of chicken sera for IgM antibodies. Original design for plate illustration taken from work by Haller (2006).

Classification of Luminex MFI Results

Classification of the results was based upon a transformation of the Mean Fluorescence Intensity (MFI) data. The transformed values for screened sera were calculated by using the standard method used also in the MAC-ELISA testing algorithm known as a P/N value. In brief the MFI of the negative control, tested on the positive bead set, was divided into the MFI of the sera tested on the positive antigen alone. The transformed value in the confirmation protocol varied slightly due to the addition of the testing of negative Ag bead sets. This negative antigen bead set provides an assessment of the reactivity of the individual sera against the proteins of the NSMB; it allows the calculated removal of non-specific reaction by the antibodies, this therefore takes into account any reaction other than the reaction to the viral antigen. By dividing the positive antigen bead MFI by the reaction to the negative antigen bead set the non-specific reactivity is removed and its value can be compared to the cutoff and assessed for final interpreted result.

Receiver Operating Curves (ROC) were employed to determine an appropriate cutoff value using this method. ROC curves were generated with the Analyse-It Software, a Microsoft add-in program (http://www.analyse-it.com). These tests allow a visual interpretation of the assay's ability to discern between positive (antibody detected) and negative (antibody not detected) populations, in this case chickens. By analyzing the area under the curve of the two normal curves produced by these different populations and following the corresponding listing of the true positive and true negative fractions

(sensitivity and specificity) the best cutoff value of 9.7 was determined and subsequently used for result determination.

In this instance 720 confirmation transformed values of positive and negative sera were compared against the final positive or negative results from the MAC-ELISA, the closest to a gold standard for IgM available at this time. These results were evaluated and the cutoff and test sensitivity and specificity, positive predictive and negative predictive power noted.

A total of 1,590 further samples were then evaluated using these cutoffs; 38 of these samples were known Highlands J (HJ) antibody positive. Testing of HJ positives were done to show that this other Alphavirus commonly cross-reactive in the HAI would not affect this test. In addition three wild bird samples were also assayed to re-affirm the specificity of the test to chicken antibodies.

Results

Due to the regionalization of EEE virus in Florida, sample size was determined using the mean sero-conversion rates and the mean populations of each testing region. Sample sizes were determined by the method of Cameron and Baldock to be 758 for the southern region, 673 for the central region, and 479 for the combined north and panhandle regions. The total number of samples tested from those found negative by HAI for each region included 681 for the central region, 748 for the south region, 516 for the northern/panhandle region, and 32 from samples taken in Alabama, in addition to this 314 samples known to be positive for WN/SLE, EEE, and HJ by HAI from the years 2003, 2005, 2006 and 2007 and 3 from wild birds.

Luminex Microsphere Immunoassay Technology

Protocol design for this Luminex assay required the evaluation of dilution factors for the sera, bead sets, primary and secondary antibodies. Prior to determining these values however it was first necessary to evaluate the need for IgG depletion of the chicken sera as this is a common practice in human sera. Cutoffs were then determined and all results were analyzed, those with Luminex results differing from the HAI and MAC-ELISA results were scrutinized. The sensitivity, specificity, positive and negative predictive values were also evaluated compared to both the HAI as well as the MAC-

ELISA results. Lastly due to the cross reactivity of the alphaviruses, sera known to be positive for antibody to Highlands J virus, sometimes found in the Florida sentinel chicken sera by the DOH, was also tested to indicate whether the antibodies to this virus might give a false positive for EEE in the MIA.

IgG Depletion of Sentinel Chicken Sera

Previous Luminex microsphere assays in humans for IgM have depleted the sera of IgG prior to testing (Johnson et al, 2005), while chickens technically have an immunoglobulin known as IgY with slightly different characteristics that IgG it was still important to determine if this immunoglobulin caused any interference in result. Therefore to determine if such IgG depletion steps are necessary in the testing of chicken sera, 40 sera were testing with and without depletion. The data was then transformed into a P/N ratio by taking the positive antigen test serum MFI and dividing by the positive antigen MFI of the negative control serum. This transformed data was then analyzed using an ANOVA table (Table 8). The F value was found to be significantly less than critical F and the P-value also indicated that the differences between the depleted and non-depleted sera were not significant. All samples tested following this determination were no longer treated to remove IgG.

Table 8. Single Factor ANOVA table for depleted vs. non-depleted chicken sera

Anova: Single Factor, transformed data for depletion vs. non-depletion of sera

Groups	*Count*	*Sum*	*Average*	*Variance*
transformed data depletion	40	1383.423	34.585	1413.998
transformed data no depletion	40	1331.205	33.280	1325.082

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	34.084	1	34.084	0.0248	0.875	3.963
Within Groups	106824.1	78	1369.540			
Total	106858.2	79				

Dilution Factors for Testing Protocol

Bead set dilutions were evaluated based on 24 samples at 1:5, 1:10 and 1:20 dilutions. An ANOVA of the data between dilutions indicates that since the F 1.35 is less than the F critical 3.12 and the p-value is 0.26 that the dilution of the bead set has no significance at the varying sera dilutions of 1:80, 1:160, 1:320, 1:400, 1:640 and 1:1280 (Table 9). In order to confirm the ANOVA finding a Tukey's multiple comparison test was done. The Tukey's multiple comparison test also known as the Tukey's honestly significant difference test or HSD is one available test for use in determining which means amongst a set of means that differ from the rest being compared. With only two groups being compared the t-test would be sufficient, however, when comparing more than one mean this method would be inappropriate. The Tukey HSD test like the t-test and ANOVA assumes that data from the different groups are from different populations, have normal distribution and the same standard deviation in each group (Table 10).

Table 9. Single Factor ANOVA table for bead set dilutions at sera dilutions of 1:80, 1:160, 1:320, 1:400, 1:640 and 1:1280. The F value of 1.35 < critical F 3.12 and therefore indicates that there is no difference between the bead set dilutions. The P-value also reflects this result..

Groups	*Count*	*Sum*	*Average*	*Variance*
Bead set 1:5	24	17104	712.6667	1116462
Bead set 1:10	24	16355.5	681.4792	977367.9
Bead set 1:20	24	7945	331.0417	299771.8

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2155334	2	1077667	1.350685	0.265825	3.129642
Within Groups	55052831	69	797867.1			
Total	57208164	71				

Table 10. Tukey's multiple comparison test for the bead set dilutions 1:5, 1:10 and 1:20, calculated from P/N transformed data with positive and negative antigen. These results indicate that there is no significant difference among the transformed values between the three different beads set dilutions. Therefore a 1:10 dilution was chosen to coincide with the dilution used in the WN/SLE testing currently in place to simplify future multiplexing efforts.

Dilution	**Difference between means**	**95% Confidence Limits**
Positive Antigen bead set		
1:5 - 1:10	66.6	-997.5 - 1130.6
1:5 - 1:20	768.8	-295.3 - 1832.8
1:20 – 1:10	702.2	-361.8 - 1766.3
Negative (NSMB) bead set		
1:5 – 1:10	4.2	-88.1 – 96.
1:5 – 1:20	5.5	-86.7 – 97.8
1:10 – 1:20	1.3	-90.9 – 93.6

Sera dilution for the MAC-ELISA and current WN/SLE microsphere assay are currently at 1:400. To evaluate if this dilution was also appropriate for the EEE microsphere assay, 24 serum samples, 144 separate observations of known positives and negatives, were assayed at varying dilutions. ANOVA resulted in an F value less than critical F 0.14 > 1.82 and a P-value of 0.98, thus it was determined that all dilutions were equivalent. A Tukey's multiple comparison test was then completed to confirm there was no point of dissimilarity between the dilutions. Tukey's HSD indicated that all sera dilutions were grouped together in the same Tukey grouping as well as all 95% confidence intervals included the value of zero. Based on the ANOVA and the Tukey's multiple comparison procedure results that no specific dilution was preferred the decision was to use the standard of 1:400 as is found in the MAC-ELISA. Before establishing this value however all samples for bead set, and primary and secondary antibody dilution were tested with these multiple dilutions and then evaluated based upon the 1:400 after dilution for the sera was determined.

Antibody dilution for the primary goat anti-chicken IgM and the secondary porcine anti-goat IgG-PE when evaluated by ANOVA indicated that the dilutions were not all equal, with a F value of 67.34 > F critical of 2.06. To indicate which treatment varied from the null hypothesis a Tukey's multiple comparison test was done. The Tukey test indicates significant differences in a variety of the different dilutions (Table 11a and Table 11b) it was however obvious from the groupings that the dilutions were divided by the strength of the secondary antibody. By evaluating the data individually in a column graph we can easily visualize the differences found in the Tukey examination (Figure 4 and 5). It can be seen that the point of saturation for the primary antibody can be found at

1µg/ml, this is indicated by the lack of change in signal strength at this dilution and it is in fact the secondary antibody that changes the outcome of the signal strength due to its saturation of the binding points found on the primary antibody. Looking specifically at the transformed MFI's on the Y-axis a value of 40 can be seen for the 2µg/ml of primary with 1µg/ml of the secondary. This is an acceptable value and indicating saturation by the primary antibody but less that saturation of the secondary antibody at that dilution. These dilutions permit easy differentiation of positive and negative reactions. This dilution was also deemed appropriate in the development of the MIA protocols for detection of antibodies to WN and SLE viruses in chicken sera. The future goal is to multiplex this assay with the WN and SLE protocols and it was this that therefore became a deciding factor in limiting the dilution of the primary and secondary to 2µg/ml of the primary and 1µg/ml of the secondary.

Table 11a. Tukey Comparisons for significance of difference between various Ab dilutions using P/N transformed MFI values. Values with significant difference at the 0.05 level are indicated by ***.

Dilution Comparison	**Between Means**	**Simultaneous 95% Confidence Limits**		
3 – 5	3.796	-12.352	19.944	
3 – 1	4.272	-11.876	20.419	
3 - 6	44.982	28.834	61.130	***
3 - 2	47.968	31.820	64.116	***
3 - 4	50.199	34.051	66.347	***
5 - 1	0.476	-15.672	16.623	
5 - 6	41.186	25.038	57.334	***
5 - 2	44.172	28.024	60.320	***
5 - 4	46.403	30.255	62.551	***
1 - 6	40.711	24.563	56.859	***
1 - 2	43.697	27.549	59.844	***
1 - 4	45.927	29.779	62.075	***
6 - 2	2.986	-13.162	19.134	
6 - 4	5.217	-10.931	21.365	
2 - 4	2.231	-13.917	18.379	
4 - 6	-5.217	-21.365	10.931	

Table 11b. Tukey comparison groupings for significance of difference between various Ab dilutions using P/N transformed MFI values.

Dilution Factor	Tukey Grouping	Mean	N	Dilution Num
2 µg 1 / 2 µg 2	A	92.520	7	3
1 µg 1 / 2 µg 2	A	88.724	7	5
4 µg 1/ 2 µg 2	A	88.249	7	1
1 µg 1 / 1 µg 2	B	47.538	7	6
4 µg 1 / 1 µg 2	B	44.552	7	2
2 µg 1 / 1 µg 2	B	42.321	7	4

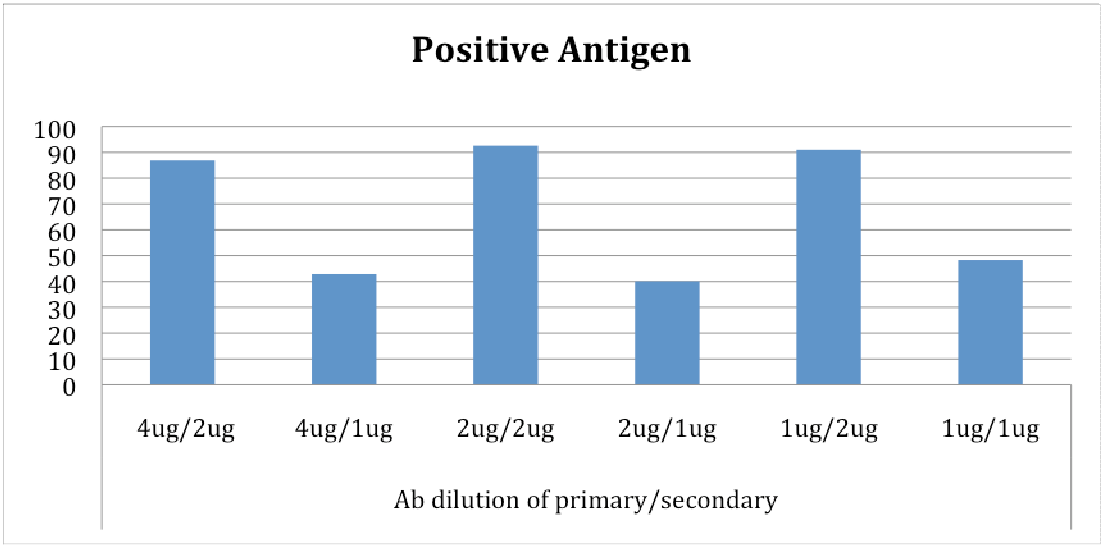


Figure 4. Column graph of primary and secondary antibody dilution with viral antigen positive bead sets. The Y-axis is the P/N transformed value while the X-axis shows the dilutions of primary and secondary antibodies analyzed. The testing of sera completed after analysis was kept at the value of 2µg/ml of primary and 1 µg/ml of secondary (2 µg 1 / 1 µg 2).

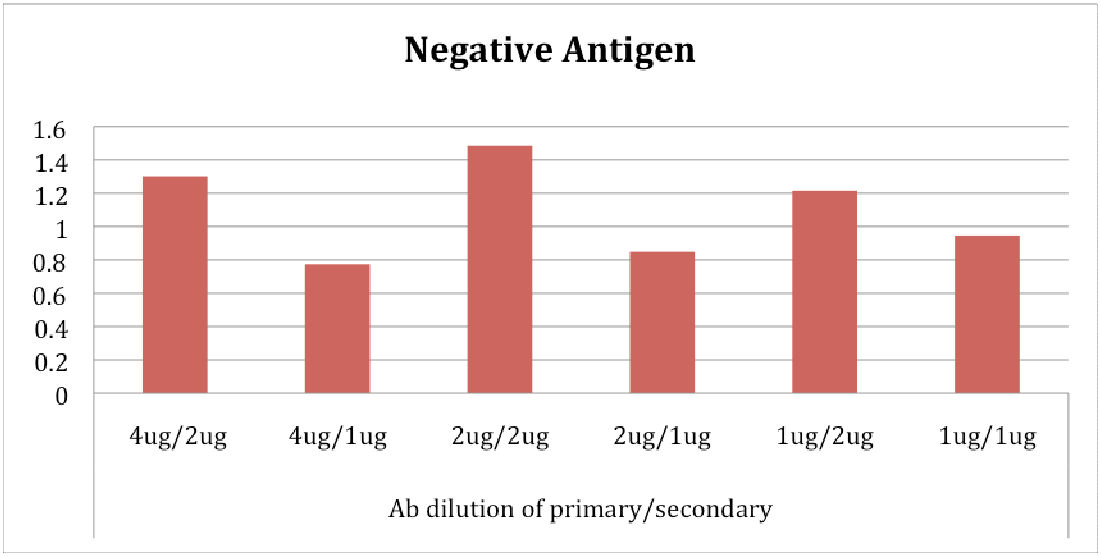


Figure 5. Column graph of primary and secondary antibody dilution with EEE negative control bead sets. The Y-axis is the P/N transformed value while the X-axis shows the dilutions of primary and secondary antibodies analyzed. The testing of sera completed after analysis was kept at the value of 2µg/ml of primary and 1 µg/ml of secondary (2 µg 1 / 1 µg 2).

Classification of the Microsphere–Based Immunoassay Result

To determine the cutoff values for the Luminex microsphere assay for EEE, 720 samples, 530 negative and 190 positive, were tested by HAI, MAC-ELISA, and then MIA. The positive or negative results from the MAC-ELISA were paired with transformed MFI values of the same sera and analyzed by the Analyse it software (Figure 6). Sera with equivocal IgM ELISA results were treated as negative since they have a P/N < 2. MIA data was transformed into a P/N value by dividing the Positive antigen MFI by the negative control value on the same plate. The resulting ROC plot illustrates a cutoff value for the transformed data of 9.7 with a sensitivity of 97%, specificity of 95%, a positive predictive value of 87% and negative predictive value of 99%. The confirmatory test data was also held at this same cutoff though transformation of this data also involves the division of the positive MFI value by the MFI value of the negative bead set for the same serum. Once calculations on all tested samples were complete, samples with disagreement between the microsphere assay and the HAI and MAC-ELISA results were noted and explanation for discrepancies sought.

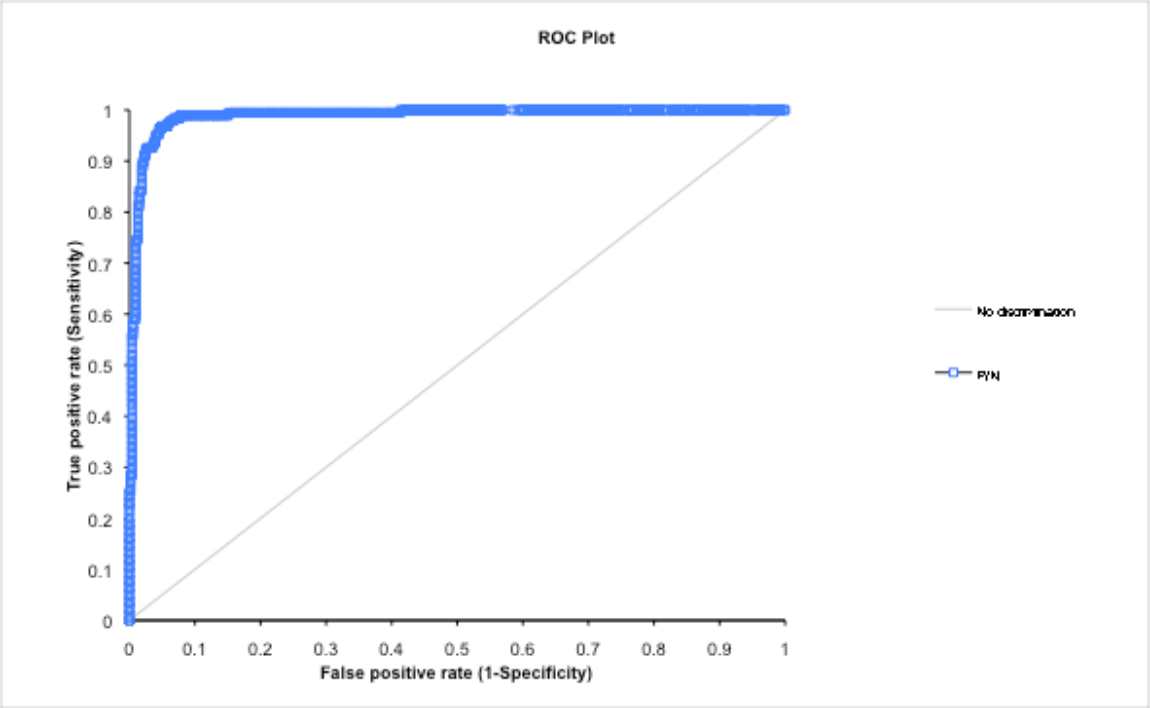


Figure 6. EEE ROC curve showing a visual representation of the cutoff value (9.7). The sensitivity can be seen on the y-axis and 1-specificity along the x-axis along the bottom. The sensitivity of the assay at a cutoff value of 9.7 is 97% while specificity is 95%, with a positive predictive value of 87% and negative predictive value of 99%

Detection of Antibodies to Eastern Equine Encephalitis

In order to gauge the accuracy of the MIA assay, HAI and MAC-ELISA testing was done in conjunction with MIA and the results compared. MIA assays were performed on 2,290 specimens including 720 also assayed with the MAC-ELISA. Of these samples, nine were included that were flavivirus positive to gauge any cross-reactivity that might stall future efforts of multiplexing with the WN/SLE test, 40 HJ positive sera were included to define any alphavirus cross-reactivity and three wild bird sera to show chicken specificity. When evaluated, 92 samples were found to have results from the MIA that disagreed with the HAI through the screening method and 75 that differed in the confirmation results (+Ag vs. –Ag). Results were analyzed to discern a possible explanation for the observed differences. A majority of the samples with HAI to MIA disparity had an ELISA value that supported the MIA result. Samples labeled "unconfirmed" are due to the lack of testing of the sample using the confirmation protocol. In other words sample was not available to run on a +Ag/-Ag plate which could then be compared to the P/N screening result. Sera that were equivocal on ELISA testing were also considered and 50% of those tested with the MIA were found to be classified positive while the other half were classified negative by this new method.

The MIA assay detected IgM in sera several weeks after the initial positive assay, the longest tested at four weeks post first positive ELISA result; these counted with the true or late positive group. Additionally, the MIA detected missed samples, that is, samples testing negative in HAI and ELISA but found to have MFI levels well above the cutoff level not due to high background. Comparing the MAC-ELISA results after

removal of the 14 MAC-ELISA equivocal, 40 HJ and 3 wild sera, 31 discrepancies were noted out of 706 with the screening method and 41 with the confirmation method. In order to evaluate if the MIA assay was incorrect in these instances, MIA results were compared to the true results (arrived at by interpreting the HAI, MAC-ELISA and PRNT together) and attempts were made to explain them.

When comparing the ELISA with the screening P/N value, 15 of the 31 samples were found to have true results of EEE positivity, this includes newly positive birds as well as sera that were from bird previously positive for antibodies (two to seven weeks). Four of the results could not be confirmed as positive due to the lack of specimen available to retest for using the confirmation protocol result by +Ag/-Ag transformation, three samples were equivocal on the ELISA and since MIA does not have this classification, positive or negative was assigned, but similar to the HAI two were positive and one was negative. Excitingly possibly one more HAI missed positive was found.

The ELISA vs. MIA confirmation testing with +Ag/-Ag had 30 samples where the MIA agreed with the HAI result: 17 were found to be true or late positives which the ELISA did not pick up on, due possibly to the low IgM levels; eight were equivocal on ELISA and were divided with 50% showing positive in the MIA to 50% showing negative results when tested by the MIA. Three of the samples appeared to have high negative antigen background and were therefore a false MIA positive and two 2005 samples were found negative by MIA, possibly because they were stored only at 4ºC for an unknown period of time and had lost some IgM in that time.

Analysis of the difference between the screening and confirmation testing was performed to evaluate reasons for discrepancies. Nine screening and confirmation tests

did not agree. Five were found to be positive with screening and negative upon confirmation, of these two sera had a true value result of EEE positive and two negative. Of those testing negative on the screening four were found to be positive after confirmation testing, all of these had a true value of positive, two of which were known to be greater than 2 weeks post-infection.

HAI Negative Samples Showing Luminex Positivity

Of the 1,976 HAI negative samples tested 28 were found to be positive by MIA screening methods. ELISA found eight of the 28 screening results negative, but ten of the samples, three of which, were also negative by ELISA, were found positive by the confirmation method. There was insufficient serum remaining for 22 of the samples to be tested by the confirmation method and therefore they could not be confirmed to have a valid positive. Of those that were tested by both the screening and confirmation methods, five had all testing methods with which to make an assessment of the reasoning behind the result (Table 12). One though screening positive, was found negative by confirmation; this may be due to either a strong background reaction or that the sera was truly negative and was caught with a strong positive screening result due to a low negative control sera. Two samples were found to be from a previously positive bird, MIA was more sensitive to the IgM in the sera than the ELISA and therefore was able to detect its existence. There are two positive sera that were previously mentioned to be HAI missed positives. The strong positive values (both markedly greater than the 9.7 cutoff) lead to the conclusion that since this test may be far more sensitive than HAI or

ELISA that these positives may previously have been missed. Both of these missed sera are from counties with multiple positive birds through the year 2007.

Table 12. Sera found negative by conventional methods, found positive in the MIA. There are varying reasons for this situation to occur. In two of these cases due to the strength of the signal shown in the MIA it is possible that positives have been missed using HAI and MAC-ELISA. In more instances than listed here sera greater than two weeks post infection (up to seven) were tested and all were found to be determined positive by either the screening or confirmation methods.

True Result	HAI Result	MAC-ELISA Result	MIA P/N value	MIA Screening result	MIA +Ag/-Ag	MIA Confirmation Result	MIA conclusion
Negative	Negative	Negative	20.4	Positive	2.5	Negative	Negative
Negative	Negative	Negative	15.4	Positive	11.8	Positive	EEE (old)
Negative	Negative	N/A	12.5	Positive	21.9	Positive	EEE
Equivocal	Negative	Negative	11.3	Positive	10.1	Positive	Low +
Negative	Negative	Negative	17.1	Positive	30.5	Positive	EEE

Assay Sensitivity, Specificity, Positive Predictive

Samples were first analyzed for results with HAI, MAC-ELISA and MIA to determine a cutoff value for the transformed data. The best cutoff value of 9.7 was determined by the Analyse it software (2007). Following the establishment of the cutoff value it was incorporated into data analysis to determine the result of the screening and confirmation transformed data values and from this the final conclusion was developed.

In order to assess the sensitivity, specificity, PPV, and NPV of the MIA screening and confirmation tests against the other testing methods, they were compared with the results found from the testing of samples by HAI as well as MAC-ELISA (Table 13). Sensitivity of the MIA was 65% to 67% when compared to the HAI test results for the confirmation and screening results respectively. Specificity however was 99% accurate in both the screening and confirmation methods. PPV was found at 88% and 95% for the screening followed by the confirmation and lastly the negative predictive values were 95% and 90% for the same methods.

The MIA proved to have a strong screening and confirmation sensitivity of 97% and 86% when compared to the MAC-ELISA, the current standard for IgM testing in sentinel chicken sera. The specificity remained high at 95% and 94%, and negative predictive value at 99% and 95%. Positive predictive value for the screening however remained the same as the comparison to HAI at 88% while the confirmation decreased to 84%.

Table 13 Sensitivity, Specificity, Positive and Negative predictive values for EEE MIA protocol vs. the HAI and ELISA methods. These values are calculated between the HAI and MIA screening and confirmation and the ELISA versus the screening and confirmation.

	HAI vs. MIA		ELISA vs. MIA	
	Screening	**Confirmation**	**Screening**	**Confirmation**
Sensitivity	0.67	0.65	0.97	0.86
Specificity	0.99	0.99	0.95	0.94
PPV	0.88	0.95	0.88	0.84
NPV	0.95	0.90	0.99	0.95

Testing Results of the cross-reactive Alphavirus Highlands J

Highlands J is an Alphavirus known to be cross-reactive with EEE antibodies. Highlands J positive samples from the north and central regions, collected in 2005 were analyzed to determine if the MIA assay could distinguish between antibody to this virus and antibody to EEE. Forty known HJ positive samples in total were run, all had tested positive for alphavirus in HAI and confirmed negative for EEE by ELISA and assigned a true result of positive for HJ through PRNT. Of these 40 samples, not one was found to be positive by the MIA assay through the screening method. There were, however, three that tested positive in the confirmation assay, while two were close to the cutoff at 11.1 and 16.3 one was found to be more than twice the cutoff at 20.5. Due to the need that both the screening and confirmation result combined be positive for a positive final result for the MIA both of the sera mentioned above were determined negative. This means that the MIA correctly determined through the combination of screening and confirmation the correct result in all 40 specimens known to be HJ positive. It is possible that the one serum that showed a P/N of 9.2 and a positive confirmation was in fact misidentified in 2005 and that after this 2 year time period the IgM has in fact diminished in the sample.

Discussion

This study was undertaken to develop and prove the efficacy of a microsphere immunoassay (MIA) for the quantification of antibodies to Eastern Equine Encephalitis virus in chicken sera. Counties that participate in the Florida Sentinel Chicken program, which has been in operation since 1978, submit sera weekly. Currently sera are tested using the HAI followed by the MAC-ELISA followed by then PRNT. This flow of testing and results provides preliminary HAI results the same week of submission and confirmatory results in 2-3 weeks. The MIA assay on the other hand takes a total preparation time of approximately three hours and a running time of 45 minutes per plate and requires only a minute amount of sample. This estimate doe not include the accession of the sera that is currently done for the HAI testing and would still be done prior to testing with this method. However with the inclusion of automation working together with the technicians of the laboratory this still has the potential to decrease time to result greatly, which would then impact the speed at which individual counties could implement actions to address the mosquito vector and warn residents. Of even greater importance in the implementation of this method is the increase in the sensitivity of the method which will increase true positives and decrease false positives and therefore decrease the amount of time and cost necessary for confirmatory testing.

Johnson et al (2005) previously designed a study for the detection of WN and SLE viruses in human sera. This was followed by the work by Logan Haller (2006) for an MIA assay for antibodies to the Flaviviruses WN and SLE for the Florida Sentinel Chicken program sera at the Florida Department of Health. Haller was able to adapt the human sera assay reagents and dilutions to establish a sensitive and specific test for WN and SLE. EEE is a serious disease for humans as well as horses and up until this study antibodies to it in chicken sera had not yet been tested for using the MIA assay.

The first aim of this research was to determine if the MIA assay technology could be implemented and shown to be a more rapid and still accurate method of testing for antibodies to EEE. The research began by determining if depletion, such as in the work by Johnson et al. (2005) with human sera, was necessary. This was found to not be the case in both the research by Haller (2006) as well as in this current method.

Dilutions of the reagents involved in the MIA method were also tested for the best result with least background, using the least quantity of reagent. Initially three microliters of antigen or NSMB were mixed with the bead set 15 with 2A2C-3 coupled antibodies (Radix Biosolutions) and 100µl of running buffer (Johnson, personal communication). Sera were found to produce a sufficiently strong signal-to-noise result when assayed at a 1:400 dilution in running buffer. Thus four microliters of sera were combined with 1,600 µl of running buffer. The optimal dilution of Luminex bead set 15 with positive and negative antigen was a 1:10 dilution with running buffer prepared on the day of test run, to a volume sufficient for 50µl per well. Haller (2006) used a two-antibody combination in order to bind the reporter molecule (R-PE) to the captured chicken antibody which provides the quantification of bound chicken IgM. This is in contrast to the work by

Johnson et. al (2005) who was able to use a single antibody, directly linked to the R-PE for assay of human sera. The same two-antibody approach was also used in this assays development. The primary goat anti-chicken IgM and the secondary porcine anti-goat IgG-PE were found to be at their best at 1µg/1ml, the primaries point of saturation, and 2µg/ml the dilution with the strongest signal paired with the primary.

The results of the clinical methods by Johnson et al (2005) incorporated the use of historical data in the statistical analysis. Haller (2006) noted that due to a differing reaction of chickens to the negative antigen proteins this same calculation system would not be applicable. As was done by Haller (2005) cutoff values using ROC curve analysis was performed using 319 known positive and 63 negative samples for the WN and 44 known positive and 64 negative samples for the SLE. The raw MFI values were transformed by division of the positive bead set MFI by the negative control antigen for screening and also division of the positive bead set MFI by the negative antigen bead set for the confirmation. These transformed values were evaluated for the best balance of sensitivity and specificity using Analyse it software (2007). These transformed values, after the MIA test is fully multiplexed, can be run concurrently with the WN and SLE protocols and a database of historical data can then be accrued and possibly applied to a different calculation scheme in the future.

Of the samples that were noted to have different MIA results from their HAI and ELISA counterparts, two sera were found to be potentially missed positive specimens by the current testing methods. This is a very exciting finding, it indicates that due to the tests sensitivity and specificity more samples submitted may be found positive and therefore a more thorough intervention on the behalf of human health can occur. While in

this research only two birds were found positive with no previous indication of positivity through HAI testing, it is anticipated that if this test is run weekly on all specimens many more will be potentially found. Both samples came from counties that had previously positive birds. Nine sera were found positive in the MIA that was determined to have come from birds with a positive EEE serum prior to this serum sample. One sample was in fact found positive by MIA four weeks post the original positive HAI result. Three samples older than two weeks (convalescent sera), while found positive by the HAI, were negative by the ELISA but positive by the MIA. This illustrates that highly sensitive nature of the MIA assay and the ability to confirm even small levels of IgM in the samples. It is likely that these birds had, in fact, proceeded on to secretion of the more specific IgG antibody and that the IgM was in a declining phase, and while it was not enough for the ELISA to detect, the MIA still found it possible to quantify. These results in five of the cases were fairly close to the cutoff value for the screening assay, indicating this was a very low level of IgM.

In the current testing protocols of the Florida Department of Health, HAI testing is used as a tool for screening thousands of sera weekly for IgM or IgG, there is no distinguishing between the two immunoglobulins in this test. IgM indicates recent infection while IgG indicates a later infection. This is of vital importance when determining the risk posed by the disease to humans and livestock in a particular area. The evaluation of the sensitivity, specificity, PPV and NPV against the true value gives a great deal of information about the ability of each test to detect the different antibody types. The true value result is comprised of the combined results of several tests. When samples are negative for HAI this sera is considered to be negative and is not further

evaluated, if in fact the result for the HAI is positive, samples are then tested in the MAC –ELISA followed by the PRNT (when necessary), the results of each test are evaluated and the final "True Result" is determined. IgM is the first indicator serologically of an infection, but it can be a week to several weeks before the chicken may develop the neutralizing antibodies that will be detected in the PRNT. At the point where neutralization antibodies come into titers high enough for detection, the IgM antibodies may begin to diminish.

The sensitivity of the HAI to the true value indicated that in the testing for EEE, 99% of the samples that were termed true positives were detected. The specificity of the test was 98% for the EEE virus (Figure 7). The positive and negative predictive value of this test was found to be 90% and 99% accurate (Figure 8). The sensitivity of this test turned out to be greater than all other testing methods, however it must be noted that all sera marked negative are not further tested and this biased these results. Not all sera were tested by MAC-ELISA or PRNT, but all were tested by HAI. The specificity however proved to be less than all other methods as was the positive predictive value the negative predictive value was on par with the P/N result calculation but above both the MAC-ELISA and the positive and negative antigen results.

When comparing the results from the MIA against the HAI the sensitivity and specificity were found to be 65% and 67% for the screening and confirmation respectively, specificity climbs to 99% for both screening and confirmation (Figure 9), PPV drops to 88% and 95% and NPV to 95% and 90% respectively (Figure 10). The drastic decrease is the sensitivity of the MIA vs. the HAI is due to the fact that the MIA is selecting for IgM only, whereas the HAI may be positive if either IgM or IgG is present.

In addition the HAI has varying levels of activity that is categorized by the reaction to the varying dilutions of antigen on microtiter plates. These reactions can be labeled "R",1:10,1:20, or 1:40", these varying decrees of reactivity are directly related to the inhibition of the agglutination reaction occurring in each well. This is not an exact measurement as is the quantification by use of the Microsphere assay. It is sensitive to interpretation. It is likely that if stratified by the inhibition titer of the HAI the sensitivity and specificity would show the varying degrees to which the true result compares with the varying dilution results. HAI requires great skill and experience to complete and properly categorize the result dilution. The MIA requires no specifically trained person the read the results, only to run the test protocol and plug results into the Excel file.

The MAC-ELISA is used as a confirmatory test for the samples found to be positive in the HAI testing. The basic theory behind the MAC-ELISA and the MIA are similar. Antibodies in the sera are captured in the ELISA by antigen bound to well of the microtiter plate while the MIA uses a very movable and fluid 5.5-micron bead with carboxylated surfaces upon which many different substrates can be bound. This flow cytometry based, fluorescent detecting technology allows even great binding space due to the fact that the bead itself can be bound from any direction all along its surface as apposed to a microtiter plate that only has one surface exposed for binding of the antibodies holding the antigen. The sensitivity found for the MAC-ELISA vs. the "true result" came to be 80%; this indicates that of all of the negative samples tested in MIA that go on to be tested in PRNT, the ELISA gives the correct result with 80% accuracy (Figure 7).

The MAC-ELISA is a very labor-intensive IgM only testing method; its results may also vary due to pipetting error or the use of different reagent lots from one test to another. It is also only used if the sample is shown to be HAI positive at some point, except in the case of this research where 720 samples were run regardless of HAI result. The MAC-ELISA test was the highest, however, in specificity against the true result at 99% accuracy over all samples tested on the MAC-ELISA. In other words of all of the samples tested in this research the HAI had a specificity of 99% when compared with the true result, again this is testing both IgG and IgM and is therefore not directly comparable to the MIA which tests only IgM. This indicates that of those samples tested, it was extremely reliable in indicating the true result that was elucidated from combination with PRNT and MAC-ELISA. The PPV and NPV against the true result were also high at 99% and 90% respectively (Figure 8).

A striking finding after calculating the true result vs. testing method statistics was the similarity of the MAC-ELISA sensitivity, specificity, PPV and NPV to those of the confirmation (Ag+/Ag-) MIA results. Due to the fact that both of these testing methods are based on IgM and both can be considered confirmation testing methods due to the final calculation of positive antigen divided by background reaction this was not entirely surprising but was an exciting find.

When comparing the MAC-ELISA to the MIA we see that the sensitivity of the screening and the confirmation of the MIA is 97% and 86%, the specificity 95% and 94% (Figure 9), the PPV decreases significantly to 88% and 84% while the NPV increases to 99% and 95% respectively (Figure 10). This is in line with the fact that the MAC-ELISA

is also specific for IgM. The Discrepancy between these tests may be due to the fact that the MIA has the capability to detect lower levels of IgM than the MAC-ELISA.

The comparison of the “true result” to the P/N MIA result indicated that the sensitivity or 83.6% of the samples were identified correctly by the MIA without additional testing; the specificity on the other hand indicates 98.5% were correctly assigned their result value (Figure 7). This is in line with the fact that the true result is based upon both IgM and IgG antibody identification. In some cases it was seen that extremely late positives (up to seven weeks post first detection) were not always identified with the screening P/N calculation method, this was not unexpected and is probably due to the decrease in IgM over time, they were commonly identified by the confirmation method, but due to the lack of positive result with the screening method the overall result was considered negative. The capability of the screening method by itself to positively predict the outcome was at 87%, which was less than both the ELISA and confirmation MIA, but above the value of the HAI test (Figure 7); the NPV was found to be 98% (Figure 8).

The confirmation results as previously stated was found to have the same statistical strength as the MAC-ELISA. This testing method assures that it will correctly identify samples potentially positive by screening with 80% sensitivity and a specificity of 99%(Figure 7). Samples that were up to seven weeks post initial positive were correctly identified with this calculation method in most cases due to the reactivity to the negative antigen bead set (NSMB protein antigen with no viral antigen which then accounted for any reaction in the well to this protein instead of the viral antigen). The PPV and NPV were both 92% (Figure 8).

In order to take a closer look at the statistical similarity between the ELISA and the confirmation results, a comparison was done to see if sample identification numbers that were classified as negatives in both tests, but that had a true bird result of positive for EEE, were in fact the same sample numbers (Table 14). It was hypothesized that the same samples in both sets was the reason the statistics were so similar. Forty-five samples tested equally tested in both methods were found to be negative with a true bird result of positive for EEE. While all of the samples analyzed in ELISA and MIA corresponded to each other and were therefore run in both testing protocols, Twenty-seven of the samples were identified as contributing to the discordance in both testing methods, where forty-nine were found not to be the same in the two tests. In other words an analysis of the discordant results (false positives and negatives by the MIA and MAC-ELISA vs. the true result) in the MAC-ELISA and the confirmation of the MIA had only 27 samples in common that contributed to the discordant results, while 49 of the samples contributing to the discordant values in each testing method individually and did not account for the similar statistical result.

Finally in order to get a proper look at the actual Final MIA result, which is considered positive if both the screening and the confirmation methods positive but negative if either test gave a negative result, a final result was calculated and compared to the true result. The sensitivity and specificity in this final result were also very close to the MAC-ELISA results, the sensitivity was found to be 78% while the specificity was at 99% (Figure 7). The PPV was a strong 98% while the NPV came out at the 93% level, actually slightly higher than the MAC-ELISA at 90% (Figure8). This final result will be the actual result reported after a screening process followed by a confirmation test if the

screening test is found to be negative. With historical information it may be noted at a future date that the sensitivity of this test is probably actually higher than reported in this work, it may be seen to fluctuate through time when factors such a sero-prevalence and submitted sera increase. In the calculation of these final results it is possible that some of the old positives (greater than one week post original positive determination) may have been included and it is possible that due to the sensitivity of this test some of those found positive for EEE by the old method including PRNT (IgG) were not in fact still positive for IgM (Table 14).

Following the analysis of the MIA final result with the true result, the MIA was compared against the MAC-ELISA the current gold standard for IgM. The sensitivity and specificity in this case were 84% and 97%. This indicates a great improvement in the sensitivity of this test if only IgM positives and negatives are compared. The PPV and the NPV were also robust at 92.9 % and 93.6% leading to the conclusion that this test overall is an strong test that once multiplexed will provide fast and accurate results for the surveillance of the common arboviruses of Florida through the sentinel chicken program.

Highlands J is also an alphavirus with cross-reactivity to the antibodies for EEE. Forty samples were tested that were known to be positive for antibody to Highlands J virus. Of these, not one serum tested positive by the screening method. Three did however test positive when using the positive to negative antigen bead set (confirmation) transformation method, though again not in the screening method and since a final result of positive requires both the screening and confirmation to be positive the final result by the MIA in these samples is actually negative. This indicates that 100% of the known HJ positive samples were correctly identified and classified.

Table 14. Comparison of testing methods to the true value in 2X2 tables.

HAI vs. True Value	**True value**		
HAI	EEE	Negative	Grand Total
Negative	2	1970	1972
Positive	242	25	267
Grand Total	244	1995	

Elisa vs. True Value	**True value**		
ELISA Result	EEE	Negative	Grand Total
Negative	45	423	468
Positive	191	2	193
Grand Total	236	425	

MIA Screening vs. True Value	**True value**		
Screening result	EEE	Negative	Grand Total
Negative	40	1965	2005
Positive	203	30	233
Grand Total	243	1995	

MIA Confirmation vs. True Value	**True value**		
Confirmation	EEE	Negative	Grand Total
Negative	45	1000	1045
Positive	191	10	201
Grand Total	236	1010	

MIA Final Result vs. True Value	**True value**		
MIA Final Result	EEE	Negative	Grand Total
Negative	47	1007	1054
Positive	170	3	173
Grand Total	217	1010	

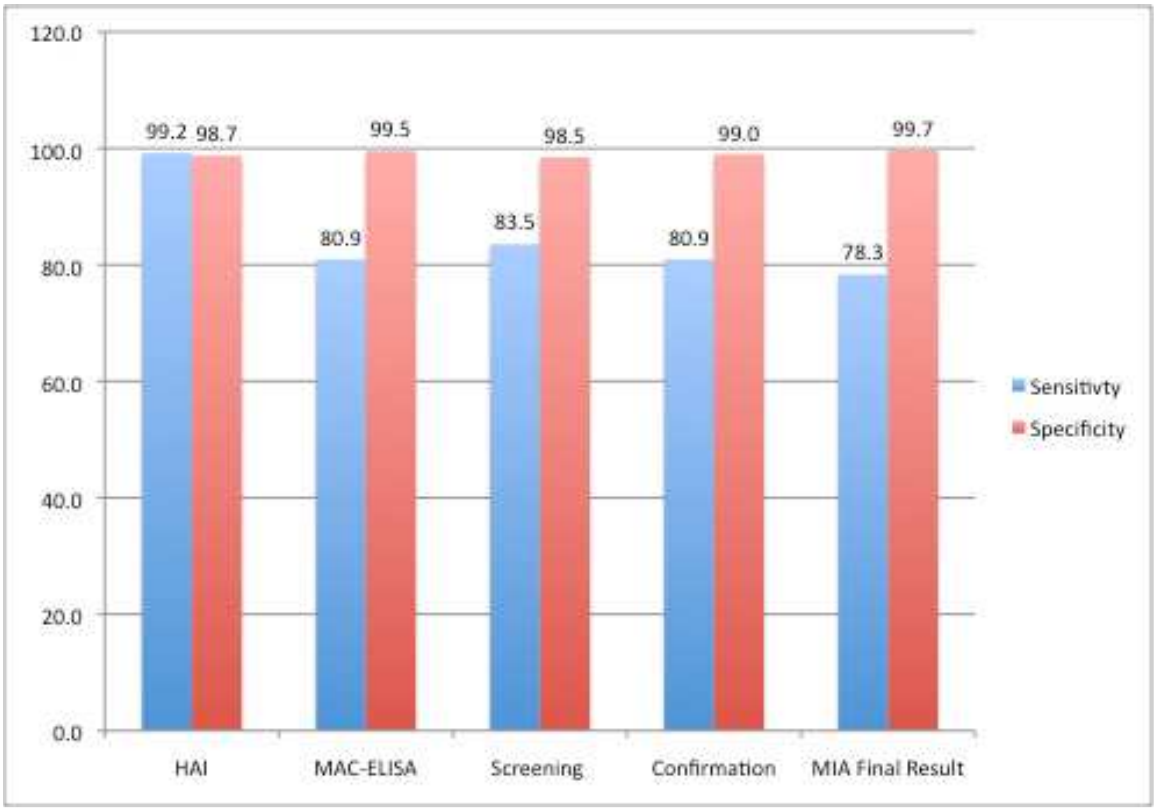


Figure 7. Comparison of sensitivity and specificity for the Hemagglutination Inhibition test (HAI), MAC-ELISA, and the Microsphere-based Immuno assay (MIA) for the detection of antibodies to Eastern Equine Encephalitis virus (EEE) compared to the true value results (combining both IgM and IgG testing methods).

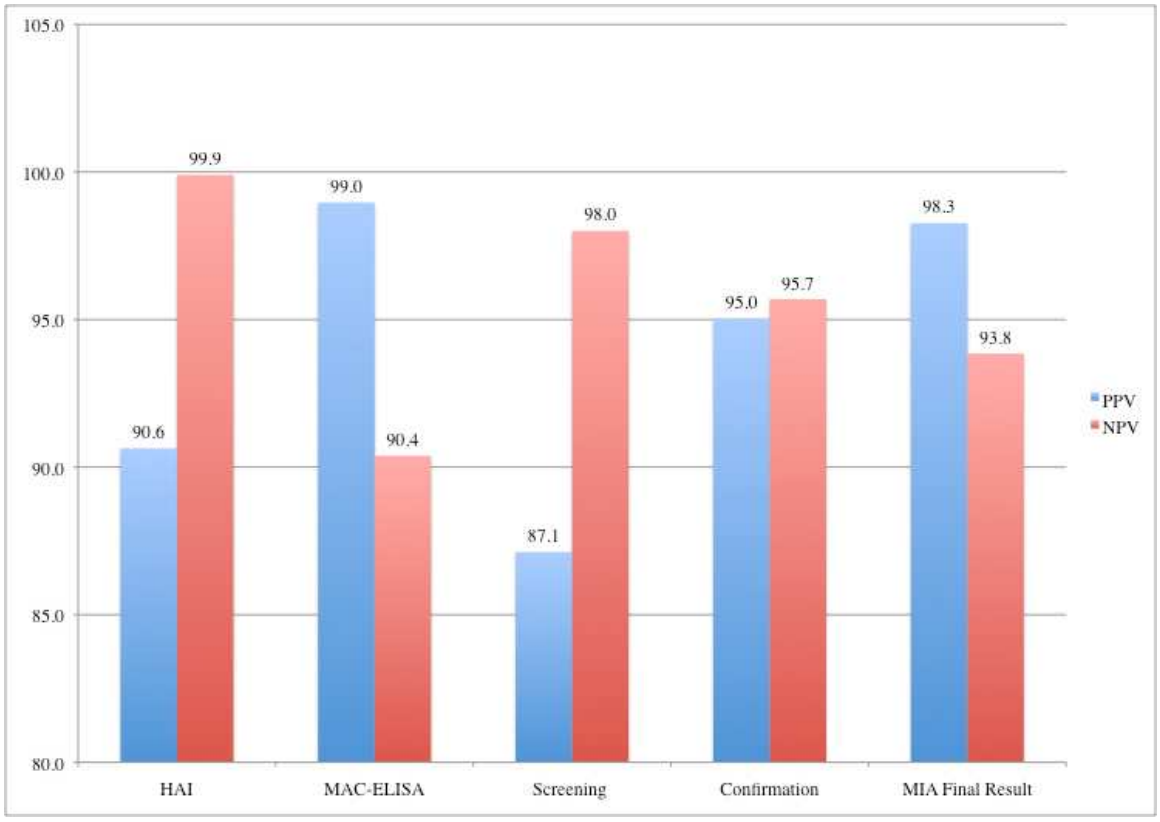


Figure 8. Comparison of Positive and Negative predictive values (PPV, NPV) for the Hemagglutination Inhibition test (HAI), MAC-ELISA, and the Microsphere-based Immuno assay (MIA) for the detection of antibodies to Eastern Equine Encephalitis virus (EEE) compared to the true value results (combining both IgM and IgG testing methods)

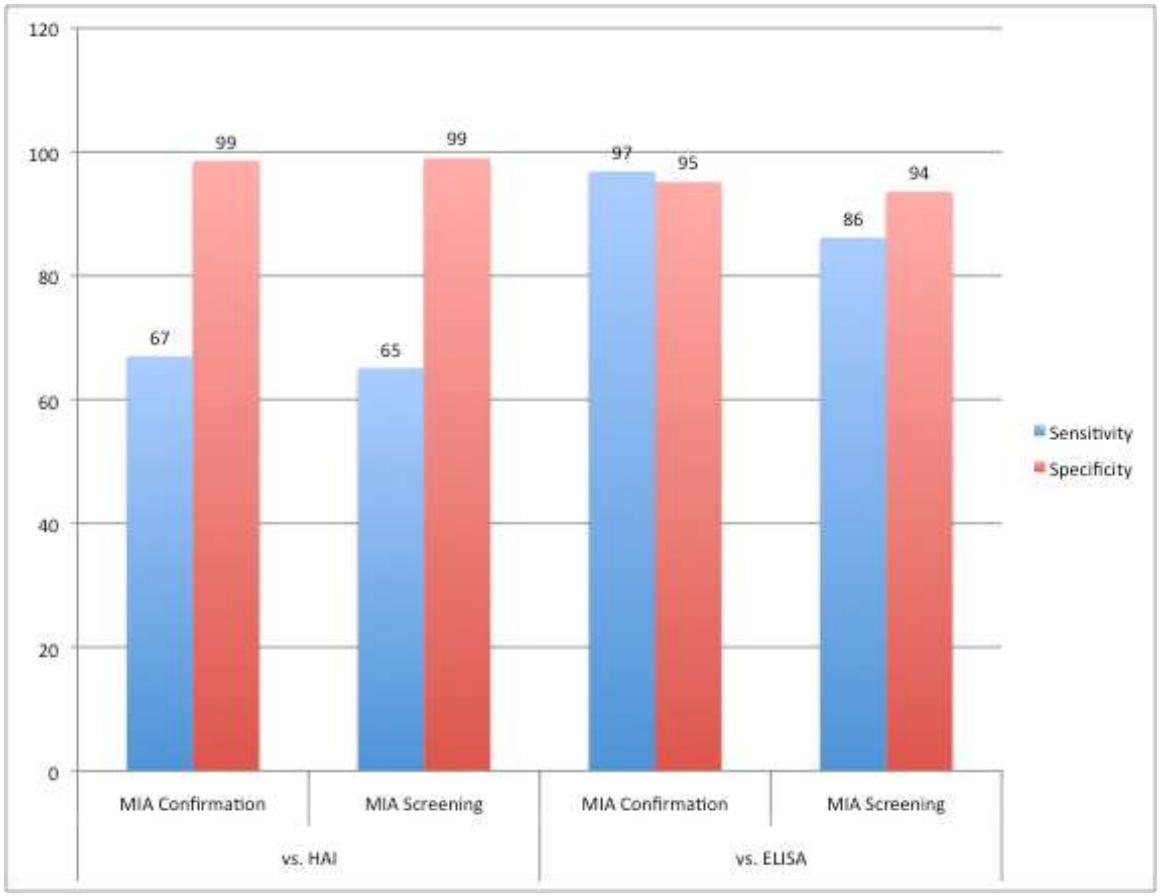


Figure 9. Comparison of sensitivity and specificity for the Hemagglutination Inhibition test (HAI), MAC-ELISA, against the screening and confirmation results of the Microsphere-based Immuno assay (MIA) for the detection of antibodies to Eastern Equine Encephalitis virus (EEE).

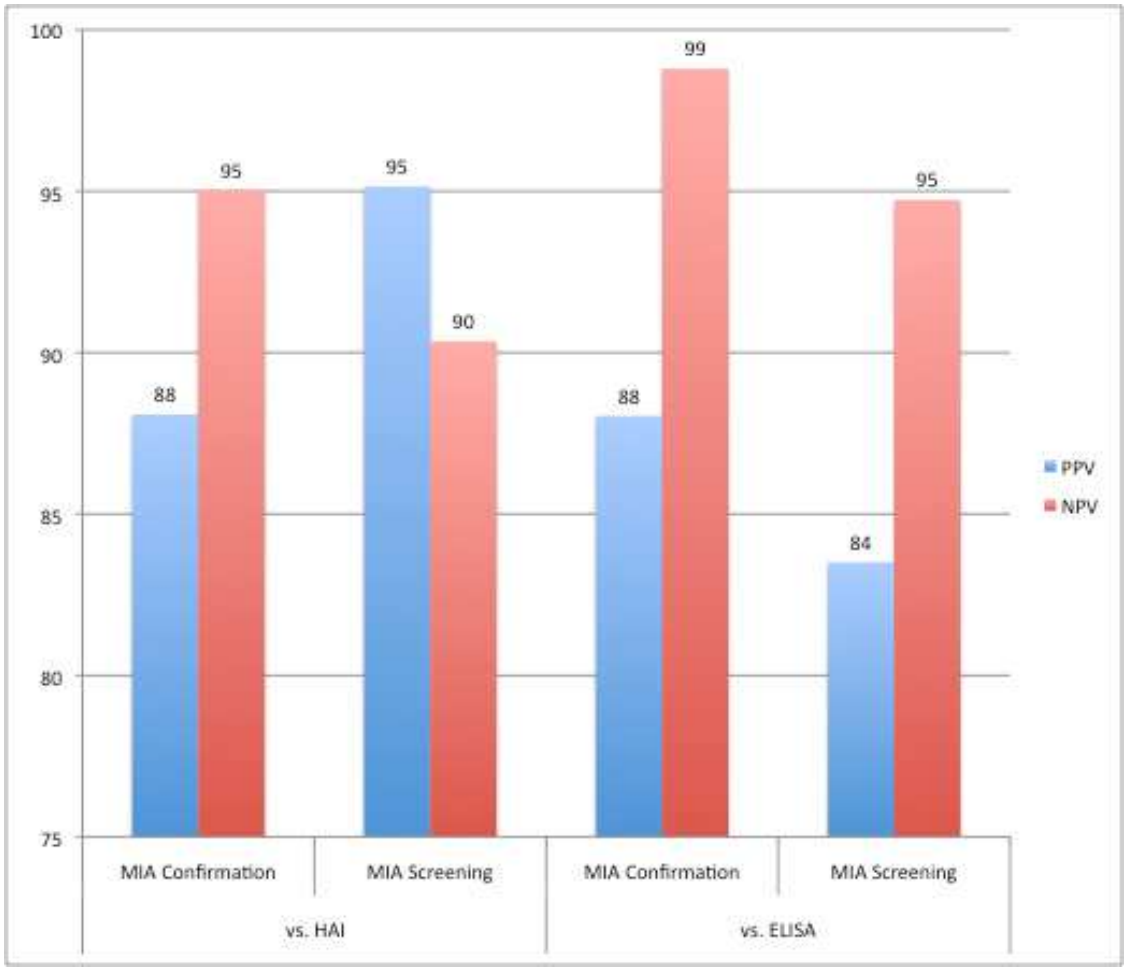


Figure 10. Comparison of Positive and Negative predictive values (PPV, NPV) for the Hemagglutination Inhibition test (HAI), MAC-ELISA, against the screening and confirmation results of the Microsphere-based Immuno assay (MIA) for the detection of antibodies to Eastern Equine Encephalitis virus (EEE).

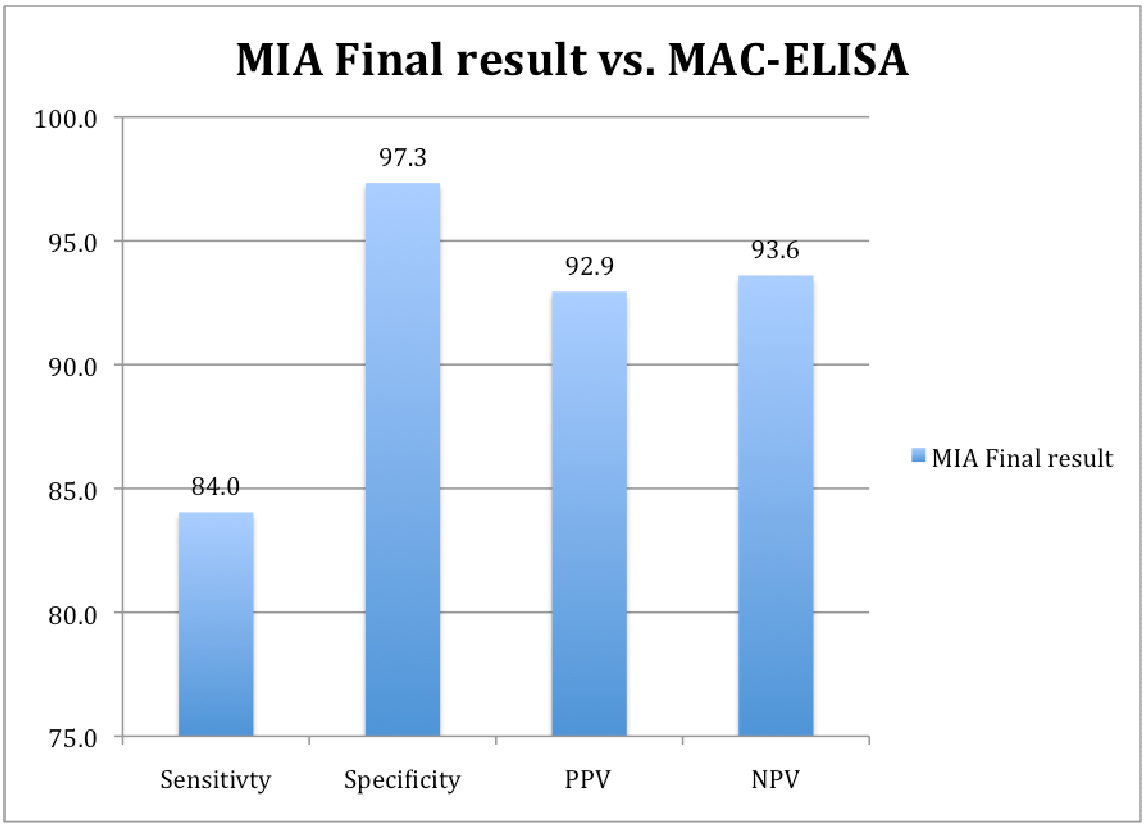


Figure 11. MIA final results incorporating both the screening and confirmation techniques for one final result vs. the IgM ELISA, the current gold standard for IgM detection in sentinel chicken sera.

Summary and Conclusions

The specific aims of this research were to show that the Luminex Microsphere assay technology can provide an accurate and rapid detection method for IgM antibodies to sentinel chicken sera. Secondly, samples that were differing in their response to HAI and MAC-ELISA when compared with MIA were analyzed to evaluate if positives were in fact missed by the current methods. Thirdly the sensitivity, specificity, PPV and NPV were evaluated for the HAI, MAC-ELISA and MIA against the true result as well as in comparison of the HAI and MAC-ELISA to the MIA itself. Lastly, to determine if the Highlands J virus cross-reactive antibodies to EEE has any impact upon the testing results. These aims are all vital to show strong support for the efficacy of this testing method.

The accuracy of the MIA protocol reagents has been shown through individual testing of all reagents and analysis to find the most advantageous dilution, thereby increasing accuracy and decreasing cost. This test is in fact more sensitive that the MAC-ELISA and takes a great deal less time. The HAI results can take days until a final result is received, the ELISA also takes a period of at least two days, the MIA on the other hand takes only hours to screen (thereby limiting the need for HAI to instances where IgG is being sought) and only hours to confirm (thereby limiting the need for ELISA). Samples found to be old positives from convalescent sera of up to seven weeks indicate that the MIA has the ability to correctly classify them with the confirmation screening. While

samples less than five weeks post-infection were frequestly also seen positive in the screening method the confirmation showed the ability to be able to detect them even if the MAC-ELISA could not. This test however does not work on wild bird sera and thus wild birds will still need to be tested using the HAI and PRNT methods. In the future it will be important to generate and store data to increase the utility of this testing algorithm. Only through time can data generated by this method produce the historical baseline needed for interpretation of the health implications of the demonstrated seroconversion rates.

The analysis of samples with discrepant results between the HAI, ELISA and MIA unearthed two sera that would otherwise have been labeled negative by the current HAI, MAC-ELISA method. While the sera themselves were selected to be true negatives the MIA indicates that there is a low level of IgM present (both samples with a P/N of less that twice the cutoff value). The confirmation tranformation however indicates a much stonger reaction both being greater than twice the cutoff value. Future research that may clarify such discrepancies could include the testing of antibody titers of naïve chickens innoculated with the virus drawn at interval periods to assess the strength of the response in time in both the HAI, MAC-ELISA as well as the MIA. In that case the birds would have a known innoculation of the virus and evaluation can be made if in fact the HAI and MAC-ELISA are indeed missing potential positives as the MIA suggests. Another suggestion for further research includes the analysis of results in the MIA and MAC-ELISA by stratification of the inhibition tire of the HAI. Using this technique evaluation could be made of the result received in confirmatory testing in relation to the strength of the titer reported (“R” vs. 1:40).

Alphaviruses are known to be extensively cross-reactive. Highlands J commonly co-circulates through the bird population with EEE. This alphavirus however is not a pathogen to humans, it causes no disease process of which we are currently aware. However it can cause a positive result for alphavirus antibodies in the HAI method. It is not possible to delineate between a bird infected with HJ or EEE based solely on this testing method; MAC-ELISA does however have the ability to differentiate, for it tests only for EEE reactivity. MIA screening methods correctly classified 100% of the HJ positive sera to be negative for EEE. While the confirmation calculations alone showed three positive results, these sera were determined to have a final MIA result of Negative due to the fact that the screening protocol showed no reactivity and a positive in both the screening as well as the confirmation method is necessary for a final result of positive. It is clear that when this protocol is correctly implemented with the screening method first there should be no reason cross-reactivity will effect the result.

Overall the implementation of the Microsphere assay for Eastern Equine encephalitis in sentinel chickens will save the laboratories both time and money in the quest for results and infromation to archive and evaluate. Future projects should include the multiplexing of this assay with the WN and SLE protocol designed by Logan Haller (2006) and evaluation of data archived after implementation of this testing method in a high throughput situation. This data can be evaulated toward the goal of more difined cutoff values or re-evaluation of the data transformation methods.

References Cited

Abbas, A.K. and Lichtman, A.H. (2005). *Cellular and Molecular Immunology*. (5th ed.) Philadelphia: Elsevier Saunders.

Alan L. Schmaljohn and David McClain (1996).Virology: Alphaviruses. In Baron Samuel, M.D., ed. Medical Microbiology. *(*4th ed.) The University of Texas Medical Branch at Galveston.

APHIS veterinary services Fact Sheet; Eastern Equine Encephalitis. (2003) www.aphis.usda.gov.

Arboviruses. (1999, February 16). Retrieved December 15, 2007, from http://www.tulane.edu/~dmsander/WWW/335/Arboviruses.html

Bell, J.R., Kinney, R.M., Trent, D.W., Strauss, E.G., and Strauss, J.H. (1984). An Evolutionary Tree relating eight Alphaviruses, based on amino-terminal sequences of their glycoproteins. *Proc. Natl. Acad. Sci.*, 81:4702-4706.

BioRad Laboratories. (2005) BioPlex Manager Software 4.1 User guide. p187-

Byrne R.J. and Robbins, M.L. (1960). Mortality patterns and antibody response in chicks inoculated with eastern equine encephalomyelitis virus. *Journal of Immunology* 86:13-16.

Calisher, C.H. (1994). Medically Important Arboviruses of the United States and Canada. *Clinical Microbiology Reviews*, 7(1), 89-116.

Calisher, C.H., Fremount, H.N., Vesley, W.L., El-Kafrawi, A.O., and Al-Deen Mahmud, M.I. (1986). Relevance of Detection of Immunoglobulin M antibody Response in Birds Used for Arbovirus Surveillance. *Journal of Clinical Microbiology*. 24(5):770-774.

Centers for Disease Control and Prevention (2007).West Nile Virus Transmission Through Blood Transfusion - South Dokota 2006. *MMWR Morb Mortal Wkly Rep* 56(04):76-79.

Centers for Disease control and Prevention (2004). West Nile Virus: Information and guidance for clinicians. http://www.cdc.gov/ncidod/dvbid/westnile/clinicians/pdf/wnv-clinicaldescription.pdf.

Centers for Disease Control and Prevention (2005). Eastern Equine Encephalitis – New Hampshire and Massachusetts, August – September 2005. *MMWR Morb Mortal Wkly Rep55(25):697-700.*

Chamberlain R.W. and Sudia, W.D. (1961). Mechanism of Transmission of Viruses by Mosquitoes. *Annu. Rev. Entomol*. 6:371-390.

Chonmaitree, T., Baldwin, C.D., and Lucia, H.L. (1989). Role of the Virology Laboratory in Diagnosis and Management of Patients with Central Nervous System. *Clinical Microbiology Reviews*, 2(1):1-14.

Davis, E. (2005) Cause and Prevention: vector-mediated encephalitis: vaccinations, insect management can help stifle viral impact. *DVM Newsmagazine.* 36(3):1-8.

Day, Jonathan F., & Stark, L.M. (1996). Transmission Patterns of St. Louis Encephalitis and Eastern Equine Encephalitis Viruses in Florida: 1978-1993. *Journal of Medical Entomology*. *33,* 132-139.

Evans, Borton, Hintz, Van Vleck (1977). The Horse. W.H. Freeman and Company. New York.

Farrar, M.D., Miller, D.L., Baldwin, C.A., Stiver, S.L., and Hall, C.L. (2005). Eastern Equine Encephalitis in dogs. *J. Vet. Diagn. Invest.* 17:614-617.

Franklin, R.P., Kinde, H., Jay, M.T., Kramer, L.D., Green, E.N., Chiles, R.E., Ostlund, E. , Husted, S., Smith, J., and Parker, M.D. (2002). Esatern Equine Encephalomyelitis virus infection in a horse from California. *Emerging Infectious Diseases* 8: 283-288.

Gould, E.A., Higgs, S., Buckley, A. and Gritsun, T.S. (2006). Potential Arbovirus Emergence and Implications for the United Kingdom. *Emerging Infectious Diseases* 12(4): 549-554.

Guy, J.S. and Malkinson M. (2003). Section I Viral Diseases: Arbovirus Infections. In Y.M. Saif (Ed.), Diseases of Poultry. Blackwell Publishing.

Helm, J.D. (2003). EEE and Emus and Pheasants. Clemson University, Livestock Poultry Health Programs. www.clemson.edu/LPH.

Heshati, H. (2004). Florida Department of Health Brevard County – Advisory for West Nile Virus. Tallahassee, FL.

Holden, P., Muth, D. and Shriner, R.B.(1966). Arbovirus Hemagglutination-Inhibition in Avian Sera: Inactivation with Protamine Sulfate. *American Journal of Epidemiology*, 84(1):67-74.

ICTVdB Management (2006). 00.026. Flaviviridae. In: *ICTVdB - The Universal Virus Database*, version 3. Büchen-Osmond, C. (Ed), Columbia University, New York, USA

Jacob, J. (2003). Viral Encephalitis: The role of birds. IFAS extension: University of Florida Factsheet PS-50.

Janeway, C.A., Travers, P., Walport, M., Shlomchik, M.J. (2005). Immunobiology (5th ed.) New York: Garland Science

Johnson, A.J., Cheshier, R.C., Cosentino, G., Masri, H.P., Mock, V., Oesterle, R., Lanciotti, R.S., Martin, D.A., Panella, A.J., Kosoy, O., and Biggerstaff, B.J. (2007). Validation of a Microsphere-based Immunoassay for Detection of an anti-West Nile Virus and anti-St. Louis Encephalitis Virus Immunoglobulin M antibodies. Clin. Vaccine Immunol. 14(9):1084-1093.

Johnson, A.J., Noga, A.J., Kosoy, O., Lanciotti, R.S., Johnson, A.A., and Biggerstaff, B.J. (2005). Duplex Microsphere-based Immunoassay for the detection of anti-West Nile Virus and anti-St. Louis Encephalitis Virus Immunoglobulin M antibodies. Clinical and Diagnostic Laboratory Immunology 12(5):566-574.

Kellar KL and Iannone MA (2002). Multiplexed microsphere-based flow cytometric Assays. *Experimental Hematology* 30:1227-1237.

Kelso, K. and Trahan, B. (1999) Infectious Disease Epidemiology Annual Report 1999. Louisiana Department of Health and Hospitals Office of Public Health.

Kissling R.E. (1960). The Arthropod-borne viruses of Man and other Animals. *Annu. Rev. Microbiol.* 14:261-282.

Kuno, G. and Chang G.J. (2005). Biological Transmission of Arboviruses: Reexamination of and New Insight into Components, Mechanisms, and Unique Traits as well as Their Evolutionary Trends. *Clinical Microbiology Reviews,* 18(4):608-637.

McLean RG, Crans WJ, Caccamise DF, McNelly J, Kirk LJ, Mitchell CJ, Calisher CH. (1995) Experimental infection of wading birds with eastern equine encephalitis virus. *J Wildl Dis* 31:502–508.

McLintock, J. (1978) Mosquito-virus Relationships of American Encephalitides. *Ann. Rev. Entomol., 23-17-37.*

Moncayo, A.C., Edman, J. D. and Turrell, M.J. (2000). Effect of Eastern Equine Encephalitis virus on the survival of Aedes albopictus, Anopheles quadrimaculatus and Coquilletidia perturbans. *J. Med. Entomol*. 37(5): 701-706.

Moore, C.G., McLean, R.G., Mitchell, C.J., Nasci, R.S., Tsai, T.F., Calisher, C.H., Marfin, A.A., Moore, P.S., and Gubler, D.J. (1993). Guidelines for Arbovirus surveillance programs in the United States. Center for Disease Ceontrol and Prevention: Division of Vector-Borne Infectious Diseases.

Nandular, M. and Urban A.W.(2007). Eastern Equine Encephalitis. Retrieved February 25th, 2008, from emedicine from WebMD. http://www.emedicine.com/med/topic3155.htm

Olleveant, N.A., Humphris, G., Roe, B. (1999) How Big is a Drop? A volumetric assay of essential oils. *J. of Clin. Nursing (*8):299-304.

Paessler, S., Aguilar, P., Anishchenko, M., Wang, H., Aronson, J., Campbell, G., Cararra, A., and Weaver, S. (2004) The Hamster as an Animal Model for Eastern Equine Encephalitis and its Use in Studies of Virus Entrance into the Brain. *Journal of infectious Disease*. 189:2072-2076.

Passler, S. and Pfeffer, M. (2003). Detection of Antibodies to Alphaviruses and Discrimination between Antibodies to Eastern and Western Encephalitis Viruses in Rabbit Sera using a Recombinant Antigen and Virus-specific Monoclonal Antibodies. *J. Vet. Med* 50: 265-269.

Pier, G.B., Lyczak, J.B. and Wetzler, L.M. (2004). *Immunology, Infection, and Immunity.* Washington D.C.: ASM press.

Poonacha, K.B., Gregory, C.R., and Vickers, M.L. (1998). Intestinal lesions in a horse associated with eastern equine encephalitis virus infection. *Vet. Pathol*. 35:535-538.

Powers, A.M., Brault, A.C., Shirako, Y, Strauss, E.G., Kang, W, Strauss, J.H., & Weaver, S.C. (2001). Evolutionary Relationship and Systemics of the Alphaviruses. Journal of Virology. *75*, 10118-10131.

Service, M. W. (2004). Medical Entomology for Students. Third Ed. (pp. 65-70) New York, New York: Cambridge University Press.

Smith, A.L. and Anderson, C.R. (1980). Susceptibility of two turtle species to eastern equine encephalitis. Journal of Wildlife Disease 16(4):615-617.

Unnasch, Robert S., Sprenger, Tonya, Katholi, Charles R., Cupp, Eddie W., Hill, Geoffrey E., and Unnasch, Tjomas R. (2006). A dynamic transmission model of eastern equine encephalitis virus. *Ecol Modell.* 192, 425-440.

Viertlboeck, B.C, Schweinsberg, S., Hanczaruk, M.A., Schmitt, R., Du Pasquier, L., Herberg, F.W. and Go¨ bel, T.W. (2007). The chicken leukocyte receptor complex encodes a primordial, activating, high-affinity IgY Fc receptor. *PNAS*. 104(28):11718-11723.

Weaver, S.C., Kang, W., Shirako, Y., Rumenapf, T., Strauss, E.G., and Strauss, J.H. (1997). Recombinational History of Molecular evolution of Western Equine Encephalomyelitis Complex Alphaviruses. Journal of Virology 71(1):613-623.

Wellings, F.M., Lewis, A.L., and Pierce, L.V. (1972) Agents encountered during arboviral ecological studies: Tampa Bay Areas, Florida, 1963 to 1970, American Journal of Tropical Medicine and Hygiene 21(2):201-213.

Bibliography

http://www.who.int/mediacentre/factsheets/fs207/en/

http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/cchf.htm

http://www.ars.usda.gov/research/publications/publications.htm?seq_no_115=160736

http://www.tulane.edu/~dmsander/WWW/335/Arboviruses.html