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Development of an ELISA for Eastern Equine Encephalitis Virus that can Differentiate Infected from Vaccinated Horses

Andrea Bingham

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Development of an ELISA for Eastern Equine Encephalitis Virus
that can Differentiate Infected from Vaccinated Horses

by

Andrea M. Bingham

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

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Keywords: DIVA, nonstructural protein, serological diagnosis, horses, cloning

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DEDICATION

I would like to dedicate this thesis to Dr. Christy Ottendorfer who has been the best teacher and mentor I have ever had. Without her help I would have never gotten where I am today. She became my mentor both academically and professionally, and later helped me to become a volunteer in Dr. Unnasch’s lab. Within a couple of months, I became a part-time employee eventually receiving a full-time position as a research assistant, and I continue to work there today. She inspired me to apply for admission into the Ph.D. program and I hope to one day join her at the CDC.

I would also like to thank my parents for their positive support and always being there for when I needed reassurance. They helped me achieve everything that I currently have and have provided me with a foundation for everything to come. Without their encouragement, I would have never gotten this far. I would also like to thank everyone who has helped me get through the thesis process emotionally, especially Collin, Amanda, and Jenn. Thank you guys so much for all the distractions, support, and for putting up with all of my craziness. You are the best friends anyone could ask for.
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ABSTRACT

Eastern Equine Encephalitis virus (EEEV) causes a fatal mosquito-borne virus that is vaccine preventable for horses. The conventional serological tests measure antibodies to the structural proteins of EEEV which are also found in the vaccine. This makes it difficult to differentiate infected and vaccinated animals (DIVA). Detection of antibodies to non-structural proteins (NSPs) is a theoretical strategy that would allow you to survey natural infections among vaccinated populations. This test would also allow for more accurate representations of the natural infection rate, vaccination rate, and help identify vaccine failures. The potential uses of the NSPs of Eastern Equine Encephalitis virus as diagnostic antigens were examined in this study. Each of the four NSP encoding genes of EEEV strain FL93-939 was separated into two parts, inserted into expression vector pDEST17, and expressed in Escherichia coli strain BL21-AI. Recombinant forms of the protein were used as an antigen for an indirect IgG ELISA to measure the serological response of horse sera to the NSPs. Serum samples collected from infected, vaccinated, and unvaccinated horses were tested for NSP antibodies. A decrease in the optical densities (ODs) for the vaccinated horse sera was seen when using the NSPs compared to whole EEEV antigen. However, the ODs for the vaccinated horses were lowered to the same level as those infected, leaving no quantitative difference between the two. The use of the IgGa secondary antibody decreased the ODs even more for the vaccinated samples, but it was still impossible to differentiate the infected and vaccinated
sera due to the samples’ ODs being below the cutoff point. The IgGa ELISA however, was the only ELISA where the infected samples were consistently above the vaccinated samples. Based on the results of the study, it was not possible to accurately differentiate between infected and vaccinated animals. Future research should be conducted in other ways to use the NSP recombinants for the DIVA strategy. This could include the use of an IgM ELISA or microsphere immunoassay (MIA), using different IgG subtypes for the assays, using epitope mapping to develop a new recombinant protein, or the development of a DIVA vaccine.
CHAPTER ONE:
INTRODUCTION

Eastern Equine Encephalitis virus (EEEV), which causes a mosquito-borne disease, belongs to the genus *Alphavirus* in the family Togaviridae. The virus infects horses, humans, birds, and other animals and is transmitted by mosquitoes of the genera *Aedes*, *Coquillettidia*, *Culex*, and *Culiseta* (Centers for Disease Control and Prevention (CDC), 2010; Calisher, Fremont, Vesely, El-Kafrawi, & Mahmud, 1986; Hetrick, Yancey, Hansen, & Byrne, 1960; Hildreth, Beaty, & Maxfield, 1984). EEEV causes a severe disease that has a high mortality rate of approximately 30-35% in humans, and 80-90% in horses (CDC, 2010).

Descriptions of EEEV disease in horses were recorded as early as 1831 in Massachusetts when 75 horses died of encephalitic symptoms (Hanson, 1957). The virus was not isolated until after outbreaks that occurred in Delaware, Maryland, and Virginia in 1933 (Giltner & Shahan, 1933). The virus was isolated from birds and from *Culiseta melanura* mosquitoes in 1951 (Kissling, Rubin, Chamberlain, & Edison, 1951; Chamberlain, Rubin, Kissling, & Edison, 1951). The first human case was recognized in 1938 (Fothergill, Dingle, & Farber, 1938).

The distribution of EEEV ranges throughout North, Central, and South America and the Caribbean. There are two antigenic variants; North American and South American. The North American variant, which is considered to be more pathogenic,
includes the North American and Caribbean isolates. The South American variant however, is comprised of isolates from Central and South America (Casals, 1964; Brault, Powers, Chavez, Lopez, & Cachon, 1999). In the United States, cases occur more frequently in the Atlantic and Gulf Coast states, with approximately six human cases and 200 horse cases per year (CDC, 2010 and United States Department of Agriculture (USDA), 2010). The majority of cases occur in Florida, Georgia, Massachusetts, and New Jersey.

Florida has had more human and horse cases than any other state. From 1964-2010, Florida has identified 71 human cases (CDC, 2010). From 2003-2010, Florida has had 702 horse cases out of 2,208 cases (32%) nationwide (USDA, 2010). The average number of cases in Florida per year is considered to be 1-2 for humans and 70 for horses (Florida Department of Health (FL DOH), 2010). There are more human cases than horse cases due to mosquitoes having greater access to horses, and horses have few defense mechanisms against the mosquitoes. The majority of EEEV cases occur between May and August, but Florida’s climate allows for possible transmission year round. In 2010, there were 5 human cases and 93 horse cases, which represents the highest number of human cases that Florida has had since recording began in 1964 (FL DOH, 2010). The years 1978, 1980, 1991, and 2005 also had five cases each (CDC, 2010). While 93 horse cases are above average, the years 2003 and 2005 had the highest numbers with 212 and 150 respectively (USDA, 2010). These years were considered to have higher numbers of horse cases due to a lack of vaccination or inconsistent vaccination. Most of the infected horses were younger than three years of age and were not vaccinated. Those that had
been previously vaccinated did not receive the recommended number of vaccinations per year (Long, 2005).

EEEV is transmitted by mosquitoes with birds as the reservoir host. The virus is maintained in a cycle between *Culiseta melanura* and avian hosts often found in swampy and forested areas; this mosquito almost exclusively feeds on birds (Nasci & Edman, 1981; Hassan, Cupp, Hill, Katholi, & Klingler, 2003; Edman, Webber, & Kale 1972). In order for the disease to be spread to horses and humans, another mosquito species must create a bridge between the infected birds and the uninfected mammals. These bridge vector mosquitoes must have more catholic feeding habits (feeding on both mammals and birds), such as *Aedes vexans* and *Coquillettidia perturbans* (Chamberlain, 1958; Vaidyanathan, Edman, Cooper, & Scott, 1997). Horses and humans are considered “dead end” hosts because the concentration of virus in their bloodstreams, or viremia, is not sufficient to infect and transmit the virus to mosquitoes.

The clinical signs and symptoms of EEEV infection vary between species. In horses the incubation period is usually 3-10 days but it can last up to three weeks. Most horses die within 2-4 days after symptoms appear (Merck Veterinary Manual, 2010). After becoming infected, there is local viral replication in fibroblasts. However, younger animals also have increased viral replication in the osteoclasts of developing bone, which causes higher-titer viremia, higher incidence of neuroinvasion, and fulminant encephalitis; this could possibly explain why younger humans and horses have more severe disease and more deaths (Sellon & Long, 2007; Vogel, Kell, Fritz, Parker, & Schoepp, 2005). The illness begins with a fever that lasts 24-48 hours. The first stage of symptoms involves nervous signs such as sensitivity to sounds and periods of
hyperexcitability and restlessness. The symptoms from the next stage result from brain lesions and involve drowsiness, drooping ears, aimless wandering and circling, loss of coordination, head pressing, inability to swallow, grinding of the teeth, and irregular gait. The final stage before death involves paralysis ranging from difficulty raising the head to complete paralysis (Merck Veterinary Manual, 2010; Sellon & Long, 2007). Currently, there is no effective treatment for EEEV and all therapies given to horses are considered supportive.

An EEEV case for both humans and horses is considered confirmed if it meets one of the following criteria:

1) four-fold or greater change in virus-specific serum antibody titer, or 2) isolation of virus from or demonstration of specific viral antigen or genomic sequences in tissue, blood, CSF or other body fluid, or 3) virus-specific immunoglobulin M (IgM) antibodies demonstrated in CSF by antibody-capture enzyme immunoassay (ELISA), or 4) virus-specific IgM antibodies demonstrated in serum by antibody-capture ELISA and confirmed by demonstration of virus-specific serum immunoglobulin G (IgG) antibodies in the same or a later specimen by another serologic assay (e.g., neutralization or hemagglutination inhibition)” (CDC, 2010).

The most definitive way to diagnose EEEV infection is through virus isolation. This can only be done with fatal horse cases. The virus can be isolated from horse brains after death unless the clinical symptoms started more than five days before death (World Organization for Animal Health (OIE), 2010). Virus isolation requires that the tissue be inoculated into susceptible cell cultures which are monitored for cytopathic effect (CPE). It is also possible to diagnose EEEV through intracerebral inoculation of suckling mice and by detection of specific nucleotide sequences using reverse transcriptase-polymerase chain reaction (RT-PCR) (Sellon & Long, 2007). According to the CDC (2010), those
horses that are antemortem may be diagnosed by testing serum or cerebrospinal fluid (CSF) to detect virus-specific IgM and neutralizing antibodies. The initial tests performed are the IgM antibody capture enzyme linked immunosorbent assay (ELISA), the microsphere immunoassay (MIA), and the IgG ELISA. If these results are positive for serum samples, then further tests such as the hemagglutination inhibition (HI) and plaque reduction neutralization (PRNT) tests are performed in order to confirm the diagnosis of EEEV infection.

The most effective way to prevent EEEV infection in horses is through vaccination. The EEEV vaccine is a formalin inactivated, adjuvanted, whole virus product (Minke, Audonnet, & Fischer, 2004; Sellon & Long, 2007; OIE, 2010). The vaccine is currently only available for horses because the risk of becoming infected with EEEV is lower for humans and there is no commercial demand for the vaccine. Currently there are no live vaccines for EEEV. Early forms of the vaccine were prepared from virus that had been propagated in embryonated chicken eggs and then inactivated with formalin (OIE, 2010; Maire, McKinney, & Cole, 1970; White, Berman, & Lowenthal 1971; Randall, Mills, & Engel, 1947). The current vaccine is prepared from virus propagated in cell culture and inactivated with formalin to a final concentration of 0.05% (OIE, 2010). The EEEV vaccine is found in monovalent or multivalent forms, where it is usually combined with West Nile virus (WNV), Western Equine Encephalitis virus (WEEV), Venezuelan Equine Encephalitis virus (VEEV), equine influenza virus, equine herpesvirus, or tetanus toxoid (Sellon & Long, 2007).

Because the EEEV vaccine is a killed virus vaccine having low immunogenicity, animals need an annual booster dose in order to maintain neutralizing antibodies (Minke,
The primary immunization requires three doses of the vaccine 3-6 weeks apart. Those areas that have low incidence of EEEV and the mosquito vectors are active for less than six months only require the annual booster; in those areas where EEEV is endemic, like Florida, horses should be vaccinated more than once per year. Horses can be vaccinated up to three times per year whereas foals and yearlings must be revaccinated up to three times per year in their first three years regardless of risk areas, and whether or not the mare was previously vaccinated. This is due to maternally derived antibodies having an inhibitory effect on serological response when foals are given the vaccine (Sellon & Long, 2007). One study looked at the serological response of previously vaccinated horses to revaccination (Waldridge, Wenzel, Ellis, Rowe-Morton, & Bridges, 2003) and found that some horses had low to undetectable levels of antibodies within six months of vaccination, thus supporting the need for multiple vaccinations per year.

EEEV vaccine for animals is relatively inexpensive and cost ranges from $7-$26 depending on the brand, and how many other diseases it prevents (Equine Vaccine Product Chart, 2011). Even though the vaccine is not expensive, it is not readily used as increasing amounts of EEEV cases are occurring in areas where activity has previously been low or non-existent. A similar situation could exist in Florida. Researchers and veterinarians believe that the economic problems of the past few years have affected vaccine rates for horses (Parker, 2010). Horse owners cannot afford to pay for all the things that their horses need, therefore they leave out vaccinations. Veterinarians in Michigan found a decrease in vaccination rates of approximately 15% per year for the last three years (Parker, 2010).
Unfortunately, data on accurate vaccination rates are not available for Florida. The conventional serological tests associated with EEEV measure antibodies to the structural proteins of EEEV. Antibody response against infection is usually directed against these virion surface proteins (International Committee on Taxonomy of Viruses, 2006). Therefore, tests for serum samples will be positive for both infected and vaccinated animals, regardless of the antibody (IgM or IgG) targeted. This is why two serological tests must be positive for horse serum in order to confirm the case. Therefore, it is necessary to develop a diagnostic test that can differentiate infected from vaccinated animals (DIVA). The DIVA strategy involves the use of nonstructural proteins (NSPs) as theoretical markers of natural infection among vaccinated populations (Suarez, 2005). The premise behind this strategy is that inactivated vaccines only induce antibodies to structural proteins when the vaccine is fully purified. This test would allow the survey of natural infections for those horses that would normally not be properly diagnosed, thus allowing for the calculation of a more accurate natural infection rate. Possible problems with diagnosis can stem from not enough serum samples present to complete two serological assays before death or for those horses where virus cannot be isolated in the brain after death due to the duration of symptoms past five days. It would also show the importance of vaccination and allow for the calculation of accurate vaccination rates. Also important would be its ability to demonstrate vaccine failures when combined with traditional testing.

Several studies have used NSPs to differentiate infected and vaccinated animals for a variety of diseases. No study conducted thus far has been targeted for EEEV. The majority of DIVA research has been conducted on avian influenza (Zhao, Jin, Li, Tan, &
Wang, 2005; Tumpey, Alvarez, Swayne, & Suarez, 2005; Suarez, 2005). Research has also been conducted on Japanese Encephalitis virus in humans and horses (Konishi & Kitai, 2009; Konishi, Shoda, Ajiro, & Kondo, 2004), Rift Valley Fever virus in humans, sheep, and goats, (McElroy, Albariño, & Nichol, 2009; van Vuren, Potgieter, Pawska, & Dijk, 2007), bluetongue virus in sheep and cattle (Barros, Cruz, Luís, Ramos, & Fagulha, 2009), foot and mouth disease in cattle (Diego, Brocchi, Mackay, & Simone, 1997), and bovine herpesvirus in cattle (Parreño, Romera, Makek, Rodriguez, & Malacari, 2010). Research using this strategy to differentiate between primary and secondary dengue infections in humans was also conducted (Shu, Chen, Chang, Yueh, & Chow, 2003).

All of these studies involved cloning and expressing the recombinant protein and then performing an indirect IgG ELISA. The process of cloning in the Gateway system (Invitrogen) uses the lambda recombination system. DNA fragments can be moved between vectors that contain the lambda derived recombination sites (att) in the genome. Proteins catalyze the insertion of the DNA into the bacterial chromosome at these sites. The first step of gateway cloning is to insert the gene into an entry clone. This entry clone is a plasmid that will contain the gene and is flanked by these att sites. In entry clones, these sites are called attL sites. This DNA can then be transferred by recombination from the entry clone into a destination vector. The destination vector has attR sites, leading this reaction to be called an LR recombination reaction. The destination vector has a gene, ccdB, which is located between the attR sites and prevents the growth of Escherichia coli colonies that do not have the recombinant plasmid DNA. The destination vector can then be transformed into a bacterial cell line optimized for
recombinant protein expression. Once expressed, the protein is purified and can be used in the ELISA.

An ELISA is a diagnostic test that is able to detect the presence of an antibody or antigen in a sample. In an indirect ELISA, the antigen of interest (NSP) is directly immobilized on a solid surface. Horse serum is added, which may contain the primary antibodies that will bind to the antigen. An enzyme labeled secondary antibody is added that has specificity for the primary antibody. A substrate can then be added that is acted upon by the enzyme and a change in color results. Due to nonspecifically bound materials being washed away between each step, this indicates that the secondary antibody had bound to the primary antibody. Stronger color changes in the substrate indicate a higher concentration of the primary antibody in the sample. A spectrophotometer can quantify the color strength and a cutoff value for the test can be calculated (Crowther, 2001; Wardley & Crowther, 1982). The indirect ELISA is highly sensitive, inexpensive, rapid, and is more suitable for testing large numbers of specimens due to its simplicity when compared to other diagnostic methods. However, it does have a problem with false positives due to the possible cross-reactivity of components of the antigen with the secondary antibody as well as non-specific reactions, and it can only detect one pathogen at a time (Crowther, 2001; Wardley & Crowther, 1982).

Another important aspect of the DIVA strategy is the nonstructural proteins that are used. Four NSPs are found in the EEEV genome, NSP 1-4. The NSPs are encoded at the 5’ end of the genome (Sellon & Long, 2007). NSP 1 is thought to be involved in capping of viral RNAs and initiating negative strand RNA synthesis. The capping function is necessary since the viral RNAs are synthesized in the cytoplasm and host
capping enzymes are only found in the nucleus. The N-terminal section of NSP 2 is associated with a helicase for RNA replication and the C-terminal section is associated with a protease that processes the NSPs. NSP 3 is important for minus strand and subgenomic 26S mRNA synthesis. NSP 4 is an RNA dependent RNA polymerase and transcribes the subgenomic 26S mRNA. Subgenomic mRNA synthesis allows for compacting of genetic material into a shorter amount of genetic material by jumping to the end of the template during transcription. The 26S mRNA encodes the structural proteins (International Committee on Taxonomy of Viruses, 2006 and UniProt Knowledgebase (UniProtKB), 2011).

The hypothesis for this study is that the NSPs for EEEV can be used to create an indirect IgG ELISA diagnostic test that can differentiate between infected and vaccinated animals. The creation of such a test would eliminate the need for two diagnostic tests in order to confirm a case of EEEV. It would also allow for more accurate representations of the natural infection rate, vaccination rate, and help identify vaccine failures. With the above average horse cases of 2010 and previous years, it would be important to have a reliable and rapid method of screening potential cases without dealing with vaccine interactions.
CHAPTER TWO:
MATERIALS AND METHODS

Primer Design and PCR

Primers were designed from the FL93-939 Eastern Equine Encephalitis virus (EEEV) strain using the OLIGO 4.05 primer analysis software (National Biosciences Inc.). Strain FL93-939 was isolated in Vero cells from a 1993 Florida pool of Culiseta melanura mosquitoes (GenBank accession number AF159554). Two primer sets were designed for each of the four non-structural proteins (NSPs) based on their respective sequences (Table 1). In order to enable directional cloning in the Gateway System, the four nucleotide sequence CACC was included at the 5’ end of each of the forward primers. The nucleotide sequence of each NSP was compared with NSP gene sequences of representative EEEV strains from GenBank in order to analyze the homology between strains (Table 2). The NSP genes from the FL93-939 strain shared 98-99% of sequence identity with the NSP genes from four other strains from North America.

The eight NSP gene segments were amplified by a polymerase chain reaction (PCR) with these primers and the cDNA of EEEV. The cDNA was prepared from 3µl of infected vero cell culture that had been passaged twice, following the iScript cDNA Synthesis protocol (Bio-Rad, catalog no. 170-8896). The incubation of the reaction mix consisted of 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. PCR for the
NSP gene segments was undertaken with an initialization step of 95°C for 4 minutes, followed by 35 cycles (40 seconds at 95°C, 40 seconds at 55°C, 1 minute 20 seconds at 72°C), and with a final extension at 72°C for 7 minutes. The annealing temperatures for NSP 2A, 2B, and 4A differed from the rest, and were 65°C, 53°C, and 62°C respectively. PCR products were verified by multicapillary electrophoresis using the QIAxcel system (Qiagen). This produced the blunt-end PCR product necessary for cloning. The overhang added to the forward primer matches the overhang in the cloning vector, which helps to stabilize the PCR product in the correct orientation.

### TABLE 1. Primer sequences developed for EEEV NSPs

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>5’-3’ Sequence (Forward)</th>
<th>5’-3’ Sequence (Reverse)</th>
<th>Product Size (bp)</th>
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<tr>
<td>NSP 1A</td>
<td>CACCATGGGAGAAGTTCATGTTG</td>
<td>CTAATTATCCACTTTCCGCTAAA</td>
<td>880</td>
</tr>
<tr>
<td>NSP 1B</td>
<td>CACCAAGTAATATTTTCTGTGGGTCAAA</td>
<td>CTATGCTCTCTGCTCTGCACTG</td>
<td>875</td>
</tr>
<tr>
<td>NSP 2A</td>
<td>CACCGGCAGCGTGGAGACACCTAG</td>
<td>CTAAGATACGATGGCTCAACGCTTT</td>
<td>1293</td>
</tr>
<tr>
<td>NSP 2B</td>
<td>CACCTGGATCAAGACGGTACACG</td>
<td>CTATCTGCCAGCTCTAGTTGAGT</td>
<td>1074</td>
</tr>
<tr>
<td>NSP 3A</td>
<td>CACCGCACCTGCTATGAGATGTTG</td>
<td>CTAACCCCTGTAATCCGTAATT</td>
<td>879</td>
</tr>
<tr>
<td>NSP 3B</td>
<td>CACCCCTACACCATCCGTCTCT</td>
<td>TCAATTCGAGTGCCTCCGCA</td>
<td>871</td>
</tr>
<tr>
<td>NSP 4A</td>
<td>CACCTACATTTTCTCATCCGAGAC</td>
<td>CTATCTTTAGTGGTTTGCCC</td>
<td>894</td>
</tr>
<tr>
<td>NSP 4B</td>
<td>CACCGGGACAAAAACATACTGAAGA</td>
<td>CTATAGAGGGTTATGGGAGCACC</td>
<td>906</td>
</tr>
</tbody>
</table>

### TABLE 2. Homology analysis of NSP genes from FL93-939 strain with other EEEV strains

<table>
<thead>
<tr>
<th>EEEV Strains with Homology (%)</th>
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<tbody>
<tr>
<td>NJ/60</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
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</table>
Gateway Cloning Procedure (Invitrogen)

The amplified fragments of cDNA derived from the PCR were cloned into the pENTR D-TOPO vector (Invitrogen, catalog no. K2400-20) based on the manufacturer’s protocol. The plasmid was then transformed into *E. coli* One Shot TOP10 cells (part of Invitrogen kit). Cells were grown at 37°C on Luria-Bertani (LB) agar plates (BD, catalog no. 244510) containing 50µg/ml of kanamycin overnight. Colonies that formed were tested by PCR to confirm the presence of the plasmid and were subsequently sequenced to confirm that the gene was cloned in the correct orientation. The gene is usually inserted in the correct orientation 90% of the time, but in this study only six of the eight genes were in the correct orientation. Further attempts at trying to get those two genes to enter in the vector correctly were unsuccessful; therefore, NSP 2A and 4B were not included in the rest of the study. Successful *E. coli* clones were grown in LB broth (BD, catalog no. 244610) with 50µg/ml of kanamycin (Invitrogen, catalog no. 11815-016) overnight. The plasmid DNA was then isolated by a plasmid miniprep (Pure Yield Plasmid Miniprep System, Promega, catalog no. A1222).

An LR recombination reaction was then performed by mixing the DNA from the entry clone with the destination vector following manufacturer’s instructions. The destination vector used in this study was pDEST17 (Invitrogen, catalog no. 11824-026). This vector adds a 6xHis N-terminal tag to the product. The plasmid was then transformed into *E. coli* Library Efficiency DH5α cells (part of Invitrogen kit), creating an expression clone. Cells were grown at 37°C on LB agar plates containing 100µg/ml of ampicillin (Invitrogen, catalog no. 11593-027) overnight. Colonies that formed were
tested by PCR to confirm the presence of the plasmid and were subsequently sequenced to confirm that the gene was cloned in the correct orientation with the 6xHis tag. Those that did clone successfully were grown in LB broth with 100µg/ml of ampicillin overnight. The plasmid DNA was then isolated by a plasmid miniprep.

In order to express the recombinant protein, the DNA was transformed into E. coli BL21-AI One Shot cells (part of Invitrogen kit). Cells were grown at 37°C on LB agar plates containing 100µg/ml of ampicillin overnight. Colonies that formed were tested by PCR to confirm the presence of the plasmid. Those that did clone successfully were grown in LB broth with 100µg/ml of ampicillin overnight. These cultures were used to inoculate one liter of fresh LB medium containing 100 µg/ml of ampicillin and were allowed to grow until the optical density (OD$_{600}$) was approximately 0.4. Protein expression was induced by adding L-arabinose to a final concentration of 0.2%. The incubation was continued for four hours and the cells were harvested by centrifugation at 10,651 × g for 10 minutes at 4°C. The quality of the induction was confirmed by running the pre and post induction samples on an SDS-PAGE gel.

**Protein Purification**

The cell pellet was resuspended in 25 ml of native lysis buffer (20mM NaH$_2$PO$_4$ (pH 7.8) (sodium phosphate monobasic, Fisher Scientific, catalog no. BP329-500), 500mM NaCl (Fisher Scientific, catalog no. S640-10), 1mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, catalog no. P7626), 200µM N-α-Tosyl-L-Lysine cloromethyl ketone hydrochloride (TLCK, Sigma-Aldrich, catalog no. T-7254)) with 2
mg/ml lysozyme (Sigma-Aldrich, catalog no. L-6876) and incubated on ice for 30 minutes. After going through three freeze thaw cycles, the addition of benzonase nuclease (EMD Chemicals, catalog no. 70746-3), and sonication on ice, the extract was centrifuged at $26,703 \times g$ for 30 minutes at 4°C. Batch purification was then performed on the supernatant.

Batch purification involves the in-solution binding of Ni-NTA His·Bind resin (Novagen, catalog no. 70666-3) to the supernatant. The histidine tags found on the protein bind to the nickel cations that are immobilized in the resin. The His·Bind resin has a binding capacity of 5-10 mg protein per milliliter resin. One milliliter of resin was used for the 25 ml of lysate. The mixture was incubated on a roller for one hour and then loaded onto a gravity flow column per manufacturer’s instructions (Novagen). Fifty drop fractions were collected after the addition of the wash buffer (300 mM NaCl, 50 mM sodium phosphate buffer (pH 8.0), 20 mM imidazole (Sigma-Aldrich, catalog no. I-0125)) until the $\text{OD}_{280}$ fell below 0.1. The elution buffer (300 mM NaCl, 50 mM sodium phosphate buffer (pH 8.0), 250 mM imidazole) was then added and 50 drop fractions were collected until the $\text{OD}_{280}$ rose above 0.1 and were continued until the ODs started to go back down. The protein concentration of each of the purified fractions was determined by a Bradford Protein Assay (Bio-Rad, catalog no. 500-0001).

Approximately 500 µg of each protein was collected from the 50 drop fractions that were considered to be the most purified.
Indirect IgG ELISA Procedure

For this study, an indirect IgG ELISA was developed to test horse serum for the presence of the NSPs. Thirty vials of horse serum were received from the Department of Infectious Diseases and Pathology in the College of Veterinary Medicine, University of Florida, Gainesville, Florida. These samples fell into three categories: clinically ill with EEEV, vaccinated against EEEV, or non-vaccinated (negative).

The concentration of coating antigen and optimal dilution of sera were determined by checkerboard titration using whole EEEV antigen. This same antigen was then used to test all of the sera provided in order to find those samples that were strongly positive from the infected and vaccinated horses. These most reactive samples were then used in an assay with the NSPs to determine if they could elicit a different response between the infected and vaccinated samples. Based on the results of these tests, further checkerboard titrations were performed.

Wells of a 96-well flat bottom microtiter plate (Immuron IIHB, Fisher Scientific, catalog no. 1424561LC) were coated with 50 µl of each NSP diluted to 1 µg/ml in coating buffer (carbonate-bicarbonate buffer, pH 9.6). The plate was stored at 4°C overnight. Following six washes with PBS/0.05% Tween-20 (Amresco, catalog no. M147) wash buffer, the plate was blocked with 200 µl blocking buffer (wash buffer with 2.0% casein (Sigma-Aldrich, catalog no. C-6554)) for one hour at 37°C. Six rounds of washes were carried out before the addition of 50 µl of horse sera diluted 1/500 in sample/antibody buffer (wash buffer with 0.5% bovine albumin (Fisher Scientific, catalog no. Bp1605)) for one hour at 37°C. The plate was washed six times and 100 µl of
horseradish peroxidase (HRP) conjugated rabbit anti-horse IgG antibody (Jackson Immuno Research, catalog no. 308-035-003) diluted 1/5000 in sample/antibody buffer was added for 30 minutes at 37°C. The final ELISA performed used HRP-conjugated goat anti-horse IgG antibody (Bethyl Laboratories Inc., catalog no. A70-124P) diluted 1/10,000. After six more washes, 100 µl of SureBlue TMB Microwell Peroxidase Substrate (Kirkegaard and Perry Lab Inc., catalog no. 52-00-01) was added. Following incubation at room temperature for five minutes, the reaction was stopped by adding 100 µl of 1M HCl (Sigma-Aldrich, catalog no. H-1758). Absorbency measurements were performed at 450 nm using a microplate reader (GeneMate, UniRead 800). The cutoff value was calculated from the result of three samples of non-vaccinated sera performed in duplicate for each NSP or serum dilution using the formula mean optical density + 3 standard deviations. A serum sample was considered positive when its OD value was greater than the cutoff value.
CHAPTER THREE:  
RESULTS

Cloning, Expression, and Purification

In order to investigate the applicability of eight non-structural protein (NSP) segments for a DIVA test, each NSP gene segment from strain FL93-939 were subjected to the Gateway Cloning procedure. Each gene segment was amplified by PCR and verified by multicapillary electrophoresis. As seen in Figure 1, all of the designed primers produced a band that was approximately the correct size for each NSP. The bands of 2A, 2B, and 4A were faint, but further testing found appropriate annealing temperatures for these NSPs that provided a stronger band.

FIGURE 1. PCR showing result of designed primer sets for each NSP with EEEV cDNA
Each of the NSP gene segments was cloned into an entry vector, pENTR D-TOPO, and those found to be in the correct orientation after sequencing were cloned into the pDEST17 expression vector. The goal of this process was to achieve soluble expression of His-tagged recombinant proteins in *Escherichia coli*. Induction was achieved by L-arabinose and yielded the expected NSP products when SDS-PAGE analysis was performed (Figure 2). The NSPs ranged in size from 33KDa to 43 KDa after the addition of the 2.6 KDa His-tag. The bands that differed from the uninduced protein (lane 8) represent the unpurified NSPs and are referred to in Figure 2 by arrows.

FIGURE 2. SDS-PAGE gel showing the expression of protein after induction

Use of a protocol for purification of His-tagged proteins under native conditions (as described in Materials and Methods) was successful in purifying the NSPs. As seen in Figure 3, the NSPs eluted most efficiently in the 8\textsuperscript{th}-11\textsuperscript{th} fractions, corresponding to the 2\textsuperscript{nd}-5\textsuperscript{th} fraction after the addition of elution buffer. The graph shows the characteristic curve found during protein purification. The imidazole in the wash and elution buffers
bound to the nickel cations and disrupted the binding of the histidine tags. Therefore, any untagged proteins were removed by the wash buffer, resulting in a high optical density (OD\textsubscript{280}). The target protein was subsequently removed by the elution buffer, represented by the peak found in the graph. The yield of purified protein was approximately 500 µg for each NSP fraction that was considered to be the most purified. These fractions were then verified by an SDS-PAGE gel (Figure 4). The His-tag purified proteins are indicated by arrows in this figure.

FIGURE 3. Optical densities at 280 nm for the NSP fractions from the column
FIGURE 4. SDS-PAGE gel showing His-tag purified proteins

**Evaluation of Indirect IgG ELISA**

A protocol was developed (as described in Materials and Methods) to perform an indirect IgG enzyme linked immunosorbent assay (ELISA) for horse sera. The thirty horse sera samples have previously been described (page 16) and represent vaccinated, infected, and non-vaccinated horses. The concentration of coating antigen and optimal dilution of sera were determined by performing a checkerboard titration using whole EEEV antigen on two randomly chosen samples each of vaccinated, infected, and non-vaccinated horse sera in duplicate (Figure 5). The optimal amount of NSP for coating ELISA plates was determined to be 1 µg/ml. The optimal serum dilution was determined to be 1:500 because it was the best at discriminating positive and negative sera while minimizing false positives. The cutoff value was established to be 0.176 (mean OD value of known negative samples + 3 standard deviations) and is represented by a dotted line in the figure. Figure 5 shows that EEEV antigen interacts with both vaccinated and infected
horses but has a stronger reaction with those vaccinated. The second infected horse serum tested (not shown), I-4, didn’t exceed the cutoff value for any dilution.

FIGURE 5. Indirect IgG ELISA protocol tested with checkerboard titration and EEEV antigen. This graph shows the samples that had the strongest reaction with the EEEV antigen. The reactive infected serum is from sample I-1 and the vaccinated serum is from sample V-3. Two samples of each type of sera were used in duplicate at each dilution.

The EEEV antigen was also used to test all thirty samples of horse sera in order to find those samples that were considered to be strong positives for the infected and vaccinated horses. Three samples each of infected and vaccinated sera were used. These most reactive samples were used to test the expressed NSPs to see if they could elicit a different response between the infected and vaccinated samples. Figure 6A shows these samples with NSP 1A, 1B, 2B, and 3B as coating antigens. Figure 6B shows the same for NSP 3A and Figure 6C shows NSP 4A. The ODs for the vaccinated and infected sera decreased when using the NSPs in comparison to the EEEV antigen. The ODs for the vaccinated samples showed the largest decrease. However, the difference between the infected and vaccinated sera for the NSP ELISAs was minimal and not significant. The cutoff values for Figure 6A, 6B, and 6C were 0.204, 0.107, and 0.163 respectively and are represented by dotted lines in the figure. The short solid horizontal lines represent the mean OD for each group. NSP 1A was the only antigen where the mean OD of the
infected samples was above the mean of the vaccinated samples, but half of the infected samples fell below the cutoff value. NSP 1B and NSP 2B were found to have the strongest reactions with the infected samples, but NSP 2B was the only antigen where all of the infected samples tested were above the cutoff value. Therefore, NSP 2B was used for the remaining ELISAs.

![Indirect IgG ELISA for NSPs with reactive infected and vaccinated sera.](image)

**FIGURE 6.** Indirect IgG ELISA for NSPs with reactive infected and vaccinated sera. (A) ELISA using NSPs 1A, 1B, 2B, and 3B as coating antigens. (B) ELISA using NSP 3A as coating antigen. (C) ELISA using NSP 4A as coating antigen. Three samples of each type of sera were assayed in duplicate for each NSP.

A second checkerboard titration was performed using NSP 2B with the hope that decreasing the sera concentration would increase the difference between the infected and vaccinated samples (Figure 7). The serum dilutions ranged from 1/100 to 1/10,000. This
ELISA was performed with the two most reactive sera samples and each sample was assayed in duplicate. The cutoff point was found to be 0.169. Only the 1/100, 1/250, and 1/500 dilutions had positive results for the vaccinated samples. The 1/100 dilution of sample I-25 sample was the only infected sample to be positive in the entire plate. Again, there wasn’t much of a quantitative difference between the infected and vaccinated samples.

![Graph showing ELISA results](image)

**FIGURE 7.** Indirect IgG ELISA for NSP 2B with checkerboard titration and reactive vaccinated and infected sera. This graph shows the samples that had the strongest reaction with the EEEV antigen. The reactive infected serum is from sample I-25 and the vaccinated serum is from sample V-28. Two samples of each type of sera were used in duplicate at each dilution.

A final checkerboard titration with the same serum samples and dilutions used in Figure 7 was performed using HRP-conjugated goat anti-horse IgGa antibody (Figure 8). The previous ELISAs used a secondary antibody consisting of whole IgG, which would interact with all subtypes of IgG found in horses. This new antibody was used in an attempt to target the IgGa subtype that is found in infected horses (Wagner, 2006; Cunha, McGuire, Kappmeyer, Hines, & Lopez, 2006; Nelson, Schram, McGregor, Sheoran, & Olsen, 1998; Mizukoshi, Maeda, Hamano, Iwata, & Matsumura, 2002). The theory
behind this would be that the infected horse sera would interact more strongly with the IgGa antibody than the vaccinated sera would. The cutoff point was found to be 0.098. The results of this ELISA demonstrate a decrease in the OD values for the vaccinated samples when compared to the previous assays. There was little change between the infected samples for this ELISA and the previous ones. The use of the IgGa antibody caused the vaccinated and infected samples to be even more similar, bringing them to the same values with no quantitative difference. This was the only ELISA where the infected samples were consistently above the vaccinated samples, but the majority of the dilutions fell below the cutoff point. The 1/100 dilution of sample I-1 was the only infected sample to be positive in the entire plate. This dilution was also positive for the vaccinated sample.

FIGURE 8. Indirect IgGa ELISA for NSP 2B with checkerboard titration and reactive vaccinated and infected sera. This graph shows the samples that had the strongest reaction with the EEEV antigen. The reactive infected serum is from sample I-1 and the vaccinated serum is from sample V-28. Two samples of each type of sera were used in duplicate at each dilution.
CHAPTER FOUR:
DISCUSSION

Non-structural proteins (NSPs) are theoretical antigens for use with a corresponding immunoassay to differentiate naturally infected animals from animals vaccinated with an inactivated vaccine (DIVA). The potential uses of the NSPs of Eastern Equine Encephalitis virus (EEEV) as diagnostic antigens were examined in this study. An indirect IgG enzyme linked immunosorbent assay (ELISA) was developed to detect EEEV antibodies against these NSPs.

The results of this study demonstrated the successful cloning and expression of the NSPs of EEEV. A decrease in the optical densities (ODs) for the vaccinated horse sera was seen when using the NSPs compared to whole EEEV antigen. However, the ODs for the vaccinated horses were lowered to the same level as those infected, leaving no quantitative difference between the two. The use of the IgGa secondary antibody decreased the ODs even more for the vaccinated samples, but it was still impossible to differentiate the infected and vaccinated sera due to the samples’ ODs being below the cutoff point. The IgGa ELISA however, was the only ELISA where the infected samples were consistently above the vaccinated samples.

One explanation for the difficulty in using an ELISA targeting NSPs to detect antibodies is that infected horses may have a low antibody response. One study found that serum specimens from horses infrequently exhibited any IgM or IgG antibody titers.
because most horses would die before a significant immune response could be generated (Farrar, Miller, Baldwin, Stiver, & Hall, 2005). Another study found that horses require 5-7 days post infection to produce detectable EEEV titers (Sahu, Alstad, Pedersen, & Pearson, 1994). According to the Merck Veterinary Manual (2010), most horses have an incubation period from 3-10 days but can last up to three weeks. Most also die within 2-4 days after the onset of symptoms, leaving little time to produce sufficient amounts of antibodies. Another possible explanation is that the inactivated vaccine may not have been fully purified. One study found that partially purified vaccines may have NSPs as contaminants from the lysed cells (Tumpey et al., 2005). Yet another explanation could be the cross-reactivity between the IgG subtypes found in horses. The whole IgG secondary antibody used in this study would interact with all subtypes. The IgGa subtype found in infected horses, although specific, could have had the problem of a low antibody response affecting its result. (Wagner, 2006; Cunha, McGuire, Kappmeyer, Hines, & Lopez, 2006; Nelson, Schram, McGregor, Sheoran, & Olsen, 1998; Mizukoshi, Maeda, Hamano, Iwata, & Matsumura, 2002).

Future research should be conducted in other ways to use the NSP recombinants for the DIVA strategy. An IgM ELISA could be performed since IgM antibodies are usually detectable in acute sera. IgM antibodies though, can also be produced by recent vaccination (Sahu et al., 1994; Waldridge et al., 2003). Research should also be performed regarding the different IgG subtypes. It is possible that the use of IgGb or IgG(T) could be beneficial. There have been few studies conducted on the IgG subtypes of horses. One study that looked at equine influenza found an increase in IgGa and IgGb after acute infection and an increase in IgG(T) after vaccination (Nelson et al., 1998).
Another study on the parasite *Babesia equi* found that IgGa and IgGb increased during acute infection and IgG(T) was detected after resolution of acute parasitemia (Cunha et al., 2006). A study conducted on equine herpesvirus type 4 showed an increase in IgGa and IgGb after acute infection, low levels of IgG(T), and that the IgGb antibody was sustained in recovered horses (Mizukoshi et al., 2002). No published studies have looked at how the different IgG subtypes respond to EEEV infection, but current work by the University of Florida showed increased IgGa in infected horses and increased IgGb in vaccinated horses (M. Long, personal communication, February 9, 2011).

It is also possible that the ELISA that was developed in this study could be further manipulated to give the desired result, especially in regards to the IgGa subtype. IgGa antibody might be recognizing an epitope within the 2B protein for the infected samples. An epitope mapping assay would allow the binding sites of the antibody to be identified on the antigen. A recombinant protein containing this region of the 2B protein could then be used in an ELISA. Theoretically, this antigen would react similarly or better with the infected samples than the original antigen and the vaccinated samples would react less. The use of this region of the protein rather than the entire recombinant could improve the specificity of the serological diagnosis. One study focusing on sheep brucellosis found that using the immunogenic region of the protein was more effective at distinguishing vaccinated, infected, and negative sheep than the whole protein (Seco-Mediavilla, Verger, Grayon, Cloeckaert, & Marin, 2003). Improvements in already developed DIVA tests were also seen when using epitope mapping for foot and mouth disease virus (Muller, Wilkins, Foord, Dolezal, & Yu, 2010) and classical swine fever virus (Qi, Zhang, Shen, & Chen, 2009).
Another possible future research strategy would be to replace the ELISA with a fluorescent microsphere immunoassay (MIA). An MIA test has been used with NSPs for the DIVA strategy with West Nile virus (Balasuriya, Shi, Wong, Demarest & Gardner, 2006; Wong, Boyle, Demarest, Woodmansee, & Kramer, 2003) and avian influenza (Watson, Reddy, Brahmakshatriya, & Lupiani, 2009; Deregt, Furukawa-Stoffer, Tokaryk, & Pasick, 2006). The MIA test is similar to the ELISA test except the NSP is coated on microspheres and is covalently linked to the carboxylated surface of the microspheres rather than a microtiter plate. The microspheres act as the solid phase for the detection of antibodies. The MIA can also be used as a multiplex assay and is considered to be more sensitive than traditional immunoassays (Watson et al., 2009). Some disadvantages of MIA are that it can cost twice as much to perform the assay in comparison to an ELISA, and not all diagnostic laboratories would have access to the equipment. After the blocking, wash, and addition of serum and antibody steps, the reaction is detected by a Luminex 100 flow instrument and recorded as the median fluorescence intensity.

Yet another way to perform the DIVA strategy is to create something known as a DIVA vaccine. A DIVA vaccine involves a marker vaccine and a companion diagnostic test. The vaccine carries at least one antigenic protein or epitope less than the corresponding wild virus and can be a subunit, gene deleted, vector or DNA vaccine. The companion test would measure antibodies against the protein that is absent in the vaccine. Currently there have been no DIVA vaccines created for horses, but a vaccine for equine viral arteritis is in development (Minke et al., 2004; Balasuriya, Heidner, Davis, Wagner, & Hullinger, 2002; Castillo-Olivares, Wieringa, Bakonyi, Vries & Davis-Poynter, 2003). DIVA vaccines have been created for classical swine fever (van
Oirschot, 2003; Dong & Chen, 2007), pestivirus infections in swine (Langedijk, Middel, Meloen, Kramps, & de Smit, 2001), pseudorabies in swine (Elbers & Stegeman, 1996), porcine reproductive and respiratory syndrome virus (Lima, Kwon, Ansari, Pattnaik, & Flores, 2008), and bovine herpesvirus (Bosch, Kaashoek, Kroese & van Oirschot, 1996). Currently, DIVA vaccines for pseudorabies virus, bovine herpesvirus, and classical swine fever virus are the only marker vaccines that are commercially available (van Oirschot, 2003). The DIVA vaccine would also be able to differentiate between infected animals and maternally derived immunity. The DIVA vaccine is especially useful for the movement of horses between endemic and non-endemic areas. This makes this type of vaccine especially marketable to those that have horses that live in or travel to the southeastern United States. It would also be helpful with emergency vaccination programs during epidemics (Minke et al., 2004). The DIVA vaccine could potentially be helpful in correcting some of the issues associated with this experiment and provide a better way to vaccinate horses against EEEV because it would be simple to differentiate the infected and vaccinated animals.

Although this study produced negative results, it is important to realize that it doesn’t signify the end of this research. With the many future research directions for this strategy, it is still potentially possible to develop a test for EEEV that can differentiate infected and vaccinated animals. If this test were developed, it would not only be able to perform the DIVA strategy and the benefits that go along with it, but would also provide additional public health benefits; it would allow for faster diagnosis and reporting of cases, which can lead to faster responses by mosquito control institutions during epidemics as well as allowing diagnostic laboratories more time and resources to
diagnose other diseases. The benefits of a DIVA test are certainly worth the efforts of trying to optimize the results of this study in the future.
LIST OF REFERENCES


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