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Characterization of Unidentified Viruses from Florida

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by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Public Health
Department of Global Health
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Dedication

To Mary and Steve Dyer,

Who's endless supply of

biscuits and Thai food

has made everything that much better.

Acknowledgements

Thank you to my major advisor, Dr. Unnasch, for his guidance, time and advice. Sincere thanks to Dr. Christy Ottendorfer. This thesis would not have been possible without her guidance. Thank you to Gregory White for his technical guidance and endless patience. Special thanks to Dr. Stark for providing several of the isolates used in this study and for her expertise in Virology and Public Health. I would like to thank Dr. Azizan for her continual support during my time at USF. Thank you to everyone in the TRU lab for their assistance and contributions to this research.

Finally, I thank my parents for instilling in me confidence and curiosity.

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Jessie L. Dyer

Abstract

Public Health and clinical laboratories occasionally obtain viral isolates that cannot be typed by routine methods. Therefore, the sequence-independent, single primer amplification (SISPA) technique was adapted to rapidly identify and characterize viral isolates of unknown etiology. A panel of known (West Nile virus and St. Louis encephalitis virus) and unknown viral isolates (environmental samples collected in Florida) were used to develop and refine the SISPA technique. Selectivity for viral genomic sequences was obtained through enriching viral particles by centrifugation, removal of cellular debris by filtration and removal of host genomic material by benzonase application. The SISPA method successfully amplified the panel of known viruses and a previously unknown environmental viral isolate. The previously unknown environmental viral isolate was determined to be closely related, if not identical, to Flanders virus, a member of *Rhabdoviradae*. A Flanders virus specific RT-PCR assay identified a total of five previously unknown environmental viral isolates as Flanders virus. Unidentified viral isolates were obtained during arbovirus surveillance efforts in Florida, either from the Florida Department of Health program (BOL-Tampa) during 2005 – 2009, or collected during an ongoing project at the University of

South Florida studying the ecology of arthropod-borne encephalitis viruses at sites located in Florida. In a concurrent study, SISPA was successfully used to characterize an unidentifiable virus isolate related to members of the *Bunyaviradae* family which was designated as Infirmatus virus. Natural mosquito population (10,557 mosquitoes) collected in Florida was screened for Flanders virus and members of *Bunyaviradae* to determine infection prevalence. Although Flanders virus was not detected in this population, Infirmatus virus was identified in 14 mosquito pools with the highest infection prevalence in *Cx. quinquefasciatus* mosquitoes. The SISPA technique was successful for the genetic identification of unknown viral isolates and application of this method to samples with suspected or unidentified viral etiologies may be used to enhance public health surveillance of emerging or re-emerging viruses in Florida.

Introduction

Global epidemic arboviral activity has increased during the 20th century. Many of these epidemics were caused by viruses that were either thought to have been controlled and no longer a public health threat, or not considered of public health importance (Gubler 2002). Recent identification of previous unknown and re-emerging arboviruses has included the resurgence of Dengue fever in the United States in 2009 and the introduction of West Nile virus to North America in 1999 (Nash, Mostashari et al. 2001; CDC 2010).

Public health and clinical diagnostic laboratories occasionally obtain environmental samples that fail to be typed by common cell culture, serological methods (such as hemagglutination inhibition and complement fixation assays) or nucleic acid tests. In addition, agents collected during an outbreak may be misdiagnosed based on the presentation of similar clinical findings or cross-reactive test results. This may occur when closely related viruses circulate in the same area such as in the case of West Nile virus and St. Louis encephalitis virus (Calisher, Lazuick et al. 1980; Pesko and Mores 2009). Novel viruses that may cross species barriers, such as Influenza A subtype H5N1, and the emergence of antibiotic resistant bacteria, such as Vancomycin-resistant *Enterococcus faecium* (VRE) have also challenged the scientific community, clinicians, and public health professionals to rapidly respond to identify, treat and prevent/control these new pathogens (Jones, Patel et al. 2008). As a result, rapid diagnostic

techniques for clinical and field samples of unknown etiology are needed to safeguard public health.

Viruses are obligate intracellular parasites of virtually all living organisms (Levine 2001). Viruses have either DNA or RNA as their genetic material and can be single or double stranded (Clark 2005). All viruses possess a capsid in which the viral nucleic acid is enclosed (Clark 2005). The capsid is constructed of identical subunits designated capsomers and can be assembled into different shapes, such as helical and icosahedral (Clark 2005).

Historically, viral taxonomy was classified by disease, clinical symptoms or characterized in regard to their size which can range from 20 nm to 450 nm in diameter (Levine 2001). Currently, molecular techniques have allowed for the reclassification by the comparison of genes and genomic sequences. The International Committee on the Taxonomy of Viruses (ICTV) has developed an internationally agreed upon taxonomy and nomenclature for viruses based on the hierarchical levels of order, family, subfamily, genus and species (Condit 2001). Viral strains can be classified in subtypes based on antigenic characteristics.

Arboviruses (arthropod-borne viruses) are globally distributed and typically found in tropical areas where the climate permits year-round transmission by cold-blooded arthropods (Gubler 1996; Gubler 2002). These viruses are of considerable public health importance due to their ability to cause epidemics and produce viremia in humans (Gubler 2002). Arboviruses require a minimum of two hosts and blood sucking arthropods to complete their lifecycle (WHO 1985).

Arboviruses are taxonomically diverse and belong to eight families and fourteen genera. Only a small percent have been documented to cause disease in humans. The arboviruses that are medically important for humans belong to three virus families: the *Bunyaviridae*, *Flaviviridae*, and *Togaviridae* (Gubler 2002). As a result of the extensive arbovirus surveillance program in Florida, several arboviruses, such as Highlands J, Tenesaw, Tamiami, and Keystone virus, have been identified (Lewis, Hammon et al. 1965; Jennings, Lewis et al. 1970; Bigler, Lassing et al. 1975). The identification of new arboviruses is a reminder that health professionals must remain vigilant for the emergence or re-emergence of infectious diseases.

The prevention and control of arboviral disease depends upon identifying and monitoring vertebrate host and vector species involved in spring amplification, and monitoring the sequence of events and forces that lead to epizootics or epidemics (Moore 1993). Molecular detection and virus isolation methods are frequently used to identify arbovirus circulation in the mosquito population (Bae, Nitsche et al. 2003; Ayers, Adachi et al. 2006; Re, Spinsanti et al. 2008). On the other hand, transmission rates to sentinel animals may be monitored using serological assays to detect exposure (Nemeth, Dwyer et al. 2009; O'Brien, Meteyer et al. 2010). Many states maintain surveillance programs and perform risk assessments to alert the public and implement control measures when arbovirus activity is high (Moore 1993). Surveillance programs are essential in monitoring the levels of virus activity, vector populations, infections in vertebrate hosts, human cases, weather, and other factors to detect

or predict changes in the transmission dynamics of arboviruses (Moore 1993). Due to the complex life cycles of arboviruses, simultaneous data collection is needed in order to quantify arbovirus activity. Florida has utilized sentinel chickens to detect arboviral activity throughout the state since 1978 for endemic viruses (Nelson, Kappus et al. 1983).

Historically, 2 to 4 day old suckling mice were the primary host system used for recovering virus from mosquito (Bond, Hammon et al. 1966). Currently, pool screening, which is when adult mosquitoes are sorted by species and sex and placed in one tube, is a commonly utilized method in monitoring for arbovirus infections in field collected mosquitoes (Armstrong, Borovsky et al. 1995; Lanciotti, Kerst et al. 2000; Hadfield, Turell et al. 2001; White, Kramer et al. 2001).

Mosquito pool screening also provides information on the possible vector of a virus. Many studies have used this knowledge to base their studies on specific mosquito species known to have a role in disease transmission (Ortiz, Wozniak et al. 2003; Chisenhall, Vitek et al. 2008). This approach elucidates the mosquito component of the complex lifestyle that all arboviruses maintain. Surveillance programs often base mosquito screening on known vectors of disease. Surveillance agencies effectively prevent arbovirus transmission through mosquito abatement and medical alerts. Medical alerts often result in lower operating costs when compared to costs associated with the hospitalization and life-long debilitation of an encephalitic arbovirus case (Villari, Spielman et al. 1995).

Public health and clinical diagnostic laboratories utilize cell culture, serological methods (such as hemagglutination inhibition and complement fixation assays) or nucleic acid tests for virus isolation and identification (Blackmore, Stark et al. 2003). Current molecular techniques apply a form of the polymerase chain reaction (PCR) in which specific nucleic acids sequence of the template is required and are commonly species specific (Yandoko, Gribaldo et al. 2007; Re, Spinsanti et al. 2008) [Figure 1]. Identification is sometimes not possible when a sample cannot be amplified using a standard PCR screening process for endemic viruses or agent specific primer sets requested by the submitter (based on clinical symptoms of the suspected causative agent, such as encephalitis). Thus, a broad assay, in which no knowledge of the template is required, is needed for detection of viruses with unknown etiology. Virus titers may be high or low in clinical and environmental samples, depending on the type of virus and source (tissue, water) of the sample. Virus titer in mosquito pools may also vary depending on field sites and year collected due to variation in the viral strain or susceptibility of the mosquito to the virus (Nasci and Mitchell 1996).

Molecular assays are frequently used for viral detection, as they are sensitive and may pick up trace amounts of the agent. In certain cases, concentration of viral particles may be necessary for detection with downstream assays. For example, ultracentrifugation has been used with success to concentrate viral particles and allow for specific viral amplification (Djikeng, Halpin et al. 2008). Relatively large viruses (greater than 0.2 µm), such as herpes virus, have been successfully purified using a cesium chloride gradient designed

to capture known groups of DNA viruses (Breitbart and Rohwer 2005) or by using a sucrose cushion to purify viral particles (Braham, Iturriza-Gomara et al. 2009) which require specialized equipment. However, ultracentrifugation and gradient methods are too complicated, time consuming and costly for routine application in a public health laboratory.

Consequently, virus isolation remains the gold standard technique for viral diagnostics. Once the viral isolate is purified from the original source, it is inoculated into a susceptible host, such as an animal model (*in vivo*) or cell system (*in vitro*). Replication of the virus can be detected by observed clinical findings or by observation of morphological changes in a cellular system known as cytopathic effect (CPE) (Condit 2001). The virus may then be isolated by harvesting tissues from an animal model or by harvesting culture fluid from an infected cell line.

Previous studies have used a sequence-independent, single-primer amplification (SISPA) technique in clinical and public health laboratories to characterize unidentified viruses from environmental and patient samples (Reyes and Kim 1991; Djikeng, Halpin et al. 2008; Re, Spinsanti et al. 2008). An overview of the SISPA methodology can be found in Figure 2. SISPA involves the directional ligation of a linker/adapter oligonucleotide onto both ends of a target population of either double stranded DNA or double stranded cDNA

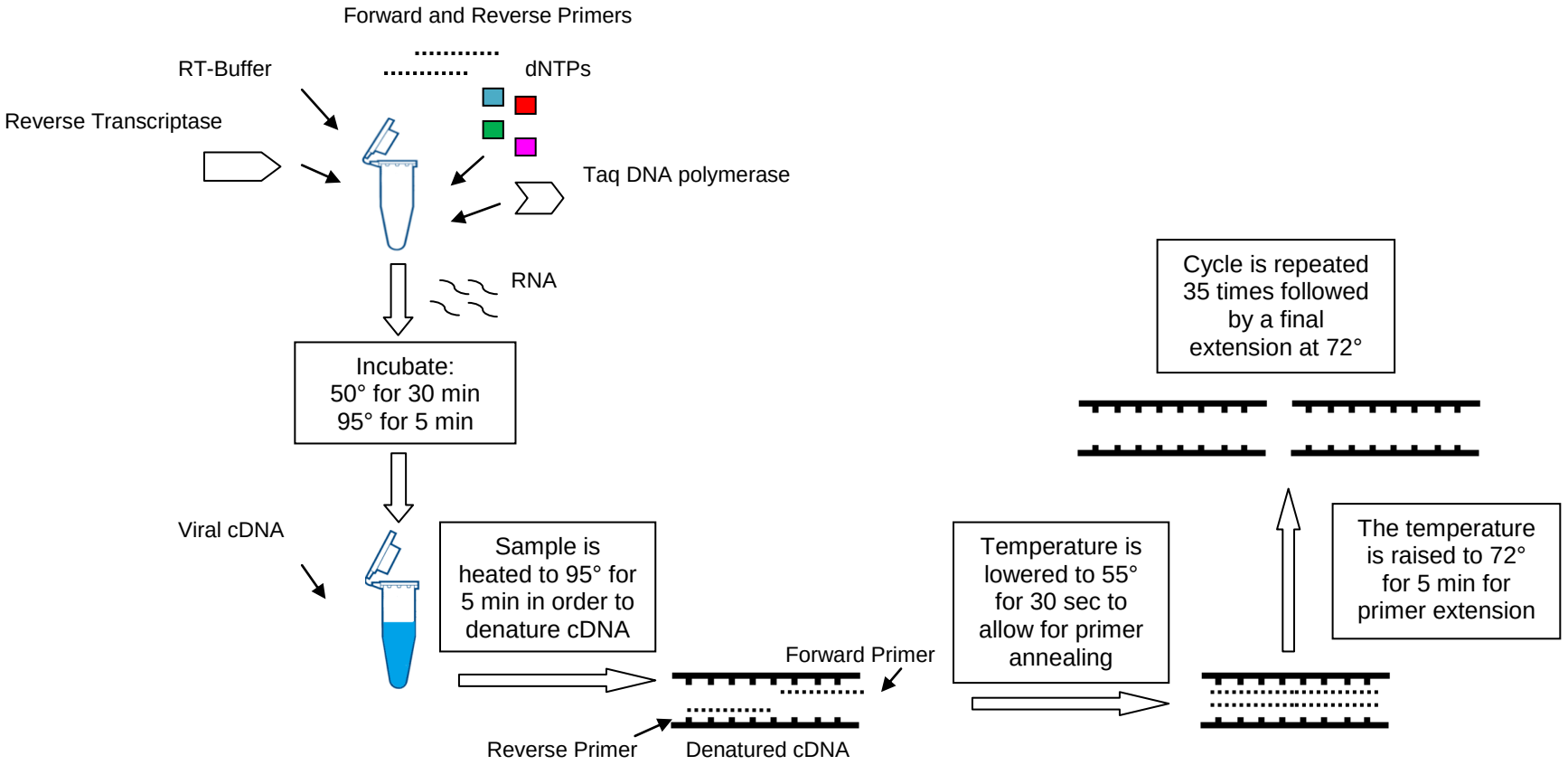

Figure1: Schematic representation of RT-PCR.

The reverse transcriptase polymerase chain reaction (RT-PCR) involves two steps. In the first step, RNA is reversed transcribed into its complementary strand (cDNA) using the enzyme reverse transcriptase and utilizing a pair of primers which are complementary to a region on the RNA and cDNA. The second step is the same as conventional PCR in which there is a denaturation, annealing and extension cycle. During denaturation the sample is heated until the cDNA separates into single strands. In the next cycle, the temperature is lowered to allow for primer annealing to the complementary strand. In the last step, the primers are extended through Taq DNA polymerase and the incorporation of nucleotides. The PCR cycle is repeated approximately 35 cycles. Each cycle makes a copy of the target sequence and the number of copies increases exponentially.

(Clem, Sims et al. 2007). Primers specific to the linker adapter molecule are used for PCR. The common sequence allows for the amplification of all nucleic acids in the sample, regardless of sequence content (Allander, Emerson et al. 2001). Earlier applications of SISPA have been successful for the identification of viral nucleic acids from both DNA (Woodchuck hepatitis virus, enterobacteriophage M13, hepatitis B virus) and RNA (enterobacteriophage MS2, bovine leukemia retrovirus, hepatitis C virus) templates (Reyes and Kim 1991; Allander, Emerson et al. 2001; Djikeng, Halpin et al. 2008). SISPA works efficiently on viruses purified from a number of sources, including bacterial growth media, plasma, serum, fecal material, and allantoic fluid (Reyes and Kim 1991; Allander, Emerson et al. 2001; Djikeng, Halpin et al. 2008; Victoria, Kapoor et al. 2008).

The original formulation of SISPA (Reyes and Kim 1991) involved aspects of two previously described methods. It was based on a technique referred to as "primer-directed enzymatic amplification" for cloning cellular mRNA (Akowitz and Manuelidis 1989), as well as the cloning of DNA dissected from specific regions of a chromosome (Johnson 1990). These methods were developed to make cDNA libraries from small amounts of mRNA (Akowitz and Manuelidis 1989) or involved the digestion of chromosomal DNA by the restriction enzyme *Mbo*I (Johnson 1990). The original formulation of SISPA (Reyes and Kim 1991) adapted the previously described methodology to include the directional ligation of an asymmetric adapter onto both termini of blunt-ended cDNA so that the common end sequence of the adapter is amplified in subsequent PCR using a

single primer. Furthermore, restriction endonuclease sites were located in the adapter to facilitate the cloning of SISPA products. Animal or cell culture models may result in contamination of the virus sample with host factors. By definition, viruses must exploit host cell molecules and processes (Knipe 2001). This in turn may lead to sample contamination of host factors such as genomic DNA, cellular RNA or by inhibitory substances found in cell culture media.

Usually, host contamination does not interfere with downstream molecular assays, such as virus gene specific PCR. In contrast, host contamination has been shown to impact SISPA application since this method is designed to amplify any nucleic acid present (Reyes and Kim 1991; Ambrose and Clewley 2006; Braham, Iturriza-Gomara et al. 2009). As a result, it is necessary to minimized host contamination of viral filtrates to enhance cloning efficiency and specificity.

For example, DNAse I is an endonuclease that nonspecifically cleaves DNA which allows for the removal of contaminating genomic DNA from RNA samples. Previous studies, (Allander, Emerson et al. 2001; Clem, Sims et al. 2007), have shown that the removal of host contaminants by filtration and the treatment of samples with DNAse I have resulted in an increase in sensitivity of the amplification of viral genomic sequences. These studies have indicated that DNAse I treatment can degrade most of the host genomic DNA and not affect viral nucleic acids, which are protected by stable viral capsids (Allander, Emerson et al. 2001).

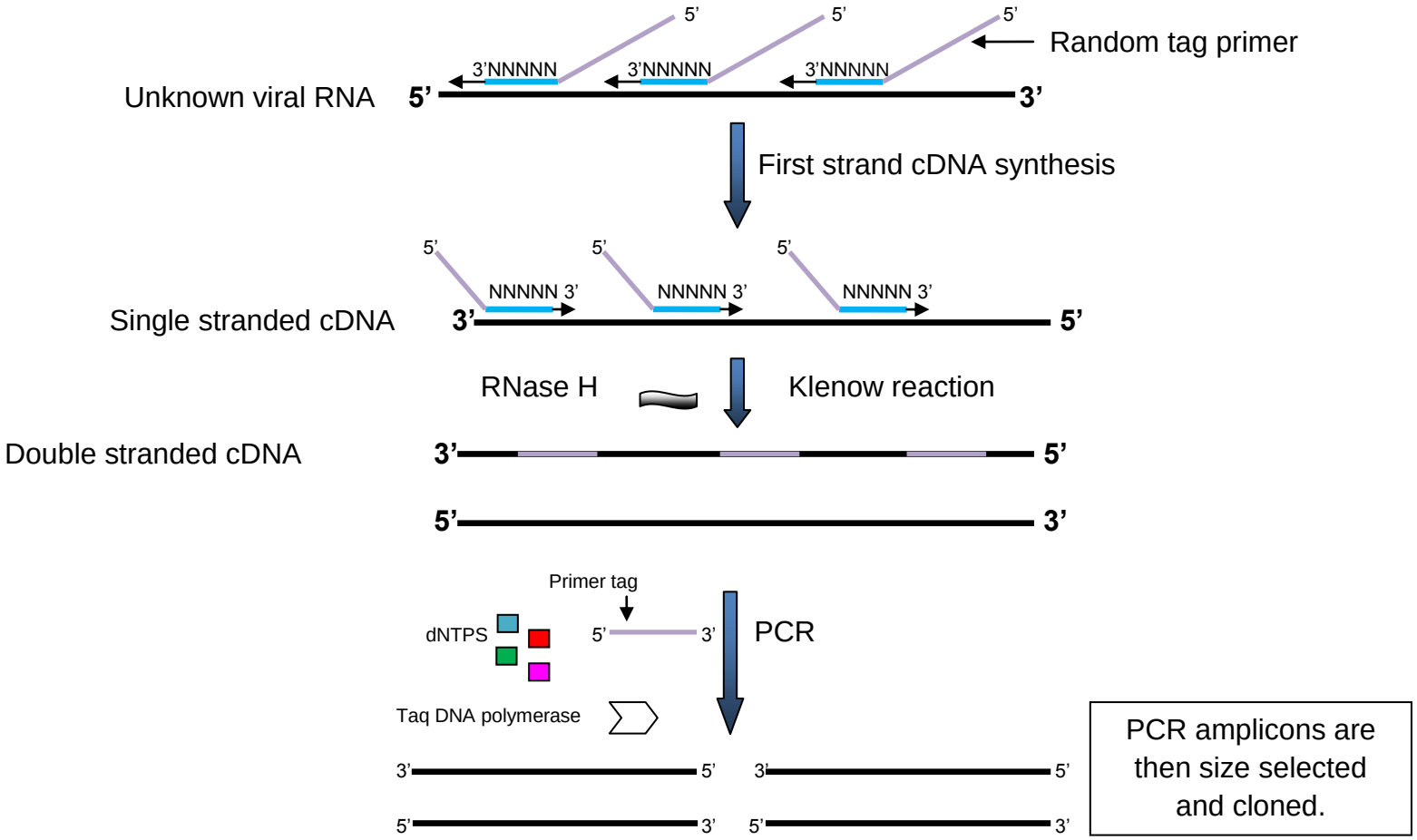

Figure 2: Schematic of SISPA

Unknown viral RNA is converted to single stranded cDNA using primers that have a random tag at the 5’ end and 5 degenerate nucleotides at the 3’ end. Double stranded cDNA is synthesized using Klenow exo-DNA polymerase, in the presence of the random tag primer. Double stranded cDNA is amplified by PCR with the same primer tag as before with Taq DNA polymerase

RNase A is a ribonuclease that specifically cleaves 3’ uracil and cytosine residues. RNase A has been successfully used to confirm RNA characteristics of a viral genome (Valles, Strong et al. 2007). Benzoase is an endonuclease that degrades all forms of DNA and RNA, which are not protected within a viral capsid. Since the genomic status of the unknown viral samples is not known, such as double stranded DNA or single stranded RNA, these enzymes may be used for the removal of host contaminants.As a result, it is necessary to minimized host contamination of viral filtrates to enhance cloning efficiency and specificity.

Ultimately, the development of a universal virus detection assay will allow for the identification of not only arboviruses, but potential viral bioweapons and emerging viruses. Application of the SISPA technique will allow for the identification of potential emerging infectious disease which will, in turn, safeguard public health.

Objectives

Currently, the screening panel utilized by the Florida Department of Health, Bureau of Laboratories (BOL-Tampa) for identification of arboviruses enzootic to Florida does not identify some environmental isolates *de novo.* Therefore, a rapid method to identify unknown viral isolates is needed. The Sequence Independent Single Primer Amplification (SISPA) method may be used to determine the genetic identity of uncharacterized viruses and can be applied to samples obtained from clinical and environmental sources. This will allow for the establishment of a standard protocol criterion to identify previously unidentified arboviruses.

My hypothesis is that standard screening panels utilized in public health laboratories and research facilities for environmental isolates are unable to detect all viruses of public health importance. Therefore, a rapid laboratory method for diagnosis and identification would be of value.

This study has three specific aims:

1. To optimize and utilize the SISPA method to determine the genetic identity of previously unknown viral isolates.
2. To characterize phylogenetic relationship and nucleotide sequence homology for viruses identified by SISPA to previously reported viruses.

3. To determine prevalence of infection for virus(es) identified by SISPA technique in different mosquito species in Florida.

Materials and Methods

Viruses

Unidentified viral isolates were obtained during arbovirus surveillance studies in Florida, either from the Florida Department of Health program located at the Bureau Of Laboratories- Tampa (BOL) during 2005 – 2009, or collected during an ongoing project at the University of South Florida College of Public Health (USF) studying the ecology of encephalitis viruses in Florida. Additional positive control viruses were obtained from the BOL for SISPA validation [Table 1].

Mosquito Trapping and Sorting

Adult mosquitoes were trapped by dry ice baited CDC light traps from surveillance sites located in Hillsborough and Walton County Florida and stored at -80 until processing. During 2008, 41,751 mosquitoes were collected at two locations in Hillsborough County (a peri-urban location and a rural location). During 2009, 14 sites in Walton County submitted a total of 2,660 mosquitoes. Mosquitoes were collected from April to December of 2008 in Hillsborough County and June to August of 2009 in Walton County. Mosquitoes were sorted by site, species, sex and date collected, then placed in pools of up to 50

Table 1: Unidentified viral isolates and control strains.

Strain #	Host Species	Collection Date	Location	Source
M08-343	*Cs. melanura*	7/16/2008	Escambia County, Fl	BOL-Tampa
M03-1427	*Cx. Nigripalpus*	5/21/2003	Palm Beach County, Fl	BOL-Tampa
M03-1434	*Cx. Nigripalpus*	6/04/2003	Palm Beach County, Fl	BOL-Tampa
M06-231	*Cx. Salinarius*	6/30/2006	Escambia County, Fl	BOL-Tampa
M06-280	*Cx. Nigripalpus*	6/30/2006	Pinellas County, Fl	BOL-Tampa
SLEV [beAN 156204]	*Gallus gallus*	1969	Brazil	BOL-Tampa
WNV [Egypt 101]	Human	1952	Egypt	BOL-Tampa
Infirmatus virus	*Ae. Infirmatus*	7/8/2008	Hillsborough County, Fl	USF

SISPA was validated using three control strains (St. Louis encephalitis virus [SLEV] beAN 156204, West Nile virus [WNV] Egypt 101 and Infirmatus virus) as positive controls. M08-343, M03-1427, M03-1434, M06-231, M06-280, SLEV beAN 156204 and WNV (Egypt 101) were obtained from the reference collection at BOL-Tampa. Infirmatus virus was collected by the University of South Florida (USF).

individuals per tube. Mosquito species that were collected with greater than 500 individual mosquitoes from one of the surveillance sites in Hillsborough County (Tampa Bay Downs) were screened to determine virus prevalence of Flanders virus and Infirmatus virus and to determine mosquito species of interest [Table 2]. Mosquito traps at the peri-urban location, Tampa Bay Downs, collected a total of 11,375 mosquitoes [Figure 3]. *Aedes vexans, Aedes infirmatus,* and *Culex nigripalpus* were the three most abundant species collected from the Tampa Bay Downs surveillance site. Mosquito trapping at the rural location, Eureka Springs, collected a total of 30,376 mosquitoes. *Culex nigripalpus, Culex erraticus,* and *Aedes infirmatus* were the three most abundant species collected from the Eureka Springs surveillance site [Figure 4].

During 2009, 2,660 mosquitoes were submitted to USF as part of an ongoing arbovirus surveillance study from 14 sites located in Walton County. *Culiseta melanura, Culex nigripalpus, and Aedes infirmatus* were the three most abundant species collected from the Walton County surveillance sites [Figure 5].

Table 2: Mosquito abundance at surveillance sites and percentage of mosquitoes screened.

Surveillance Site	County	Mosquitoes collected	Percentage of mosquitoes screened
Tampa Bay Downs	Hillsborough	11,375	86%
Eureka Springs	Hillsborough	30,376	21%
Walton	Walton	2,660	12%

During 2008, 41,751 mosquitoes were collected at two locations in Hillsborough County, Fl (a peri-urban location and a rural location) and in 2009, 14 sites in Walton County, Fl submitted a total of 2,660 mosquitoes. Mosquitoes were collected from April to December of 2008 in Hillsborough County and June to August of 2009 in Walton County using dry ice baited CDC light traps.

Mosquito Processing

Mosquito pools removed from the -80 freezer and thawed on ice. Mosquito pools were homogenized by the addition of a 4.5 mm copper clad steel bead (BB-caliber airgun shot, Copperhead brand) and 1 ml of BFD [Appendix A] to a 2ml microcentrifuge tube containing up to 50 mosquitoes using a Tissue Lyser (Qiagen) at 25 Hz for 4 minutes and subsequently centrifuged at 4°C for 4 minutes at 10,000 rpm (9,341 rcf) (Eppendorf Centrifuge 5810 R). Samples were kept on ice throughout processing. The homogenate was subsequently filtered through a Cellulose acetate syringe filter, 0.2 µM pore size (Nalgene Cat. No. 0974061A), that had been pretreated with inactivated Fetal Bovine Serum (FBS: Hyclone Cat. No. SH3007003) to remove cellular debris. A 1.0-ml aliquot of each sample was inoculated into a 25cm^2 tissue culture flask (Nalgene Nunc

International, Cat. No. 156340) of African green monkey kidney (ATCC, Cat. No. CCL-81, passage 140) [Vero cells] using a sterile 1 ml pipet, which were concurrently being maintained. Vero cells were seeded into a 25cm^2 tissue culture flasks 10 ml outgrowth media and incubated at 37°C until confluent (approximately 4 days). The remaining mosquito pool homogenate was stored at -80. The flasks were then rocked at 37°C every 15 minutes for 1 hour and fed with 10 ml of liquid maintenance media for Vero Cells [Appendix A]. Cultures were incubated at 37°C in a Thermo Scientific Forma Series II Water jacketed 5% CO_2 incubator and cell monolayers were examined daily for fourteen days under a microscope for evidence of cytopathic effect (CPE). Cultures which exhibited positive CPE were frozen at -80°C, rapidly thawed at 37ºC.

Nucleic acid was isolated using the QIAamp Viral RNA Mini Kit (Qiagen) using in the automated QIAcube (Qiagen) according to manufacturer's protocol. Pools were screened following SISPA identification of unknown viral isolates using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA polymerase (Invitrogen, Karlsruhe, Germany).

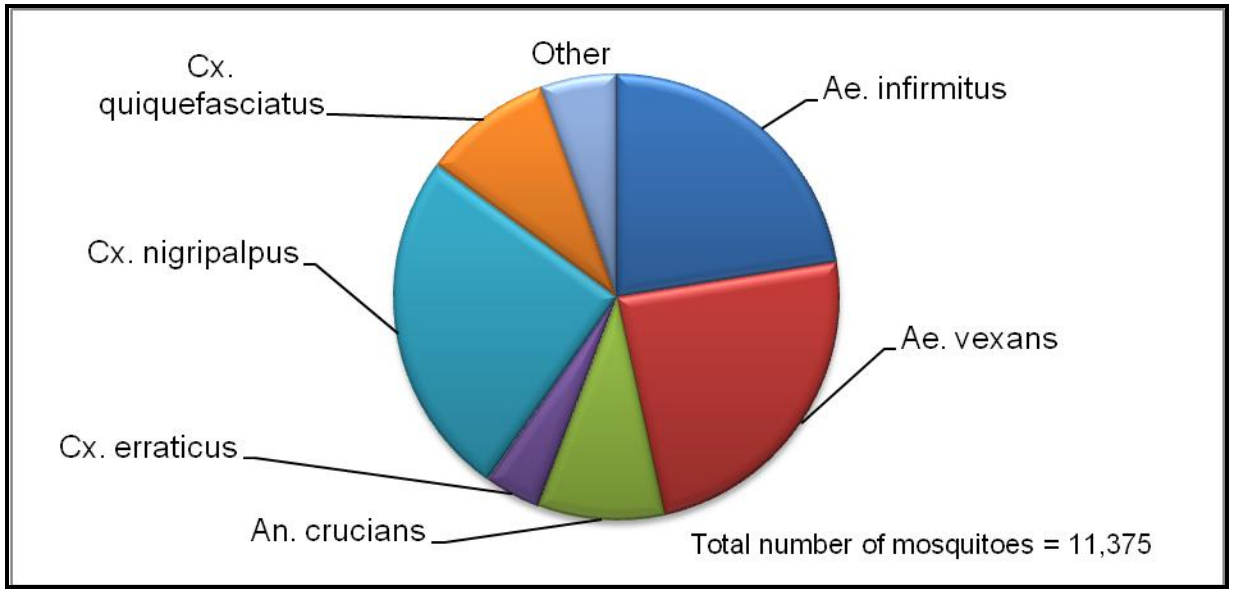

Figure 3: Number of mosquitoes collected from Tampa Bay Downs (2008)

A total of 11,375 mosquitoes were collected from the Tampa Bay Downs, peri-urban location, surveillance site in western Hillsborough County. *Aedes vexans, Aedes infirmatus,* and *Culex nigripalpus* were the three most abundant species.

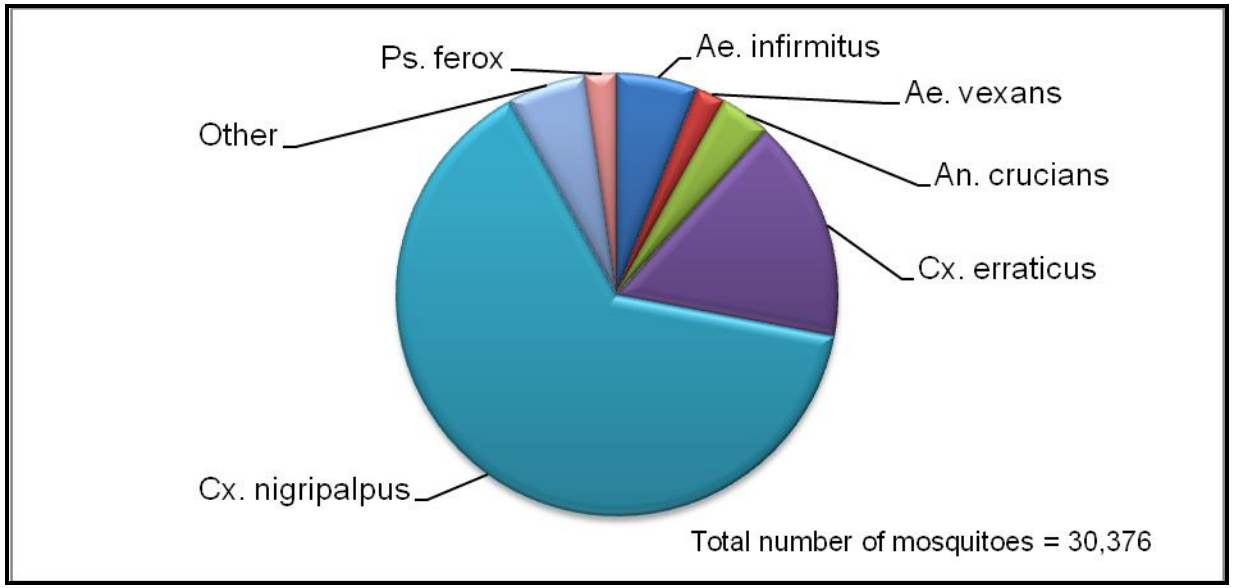

Figure 4: Number of mosquitoes collected from Eureka Springs (2008)

A total of 30,376 mosquitoes were collected from the Eureka Springs, rural location, surveillance site in central Hillsborough County. *Culex nigripalpus, Culex erraticus,* and *Aedes infirmatus* were the three most abundant species.

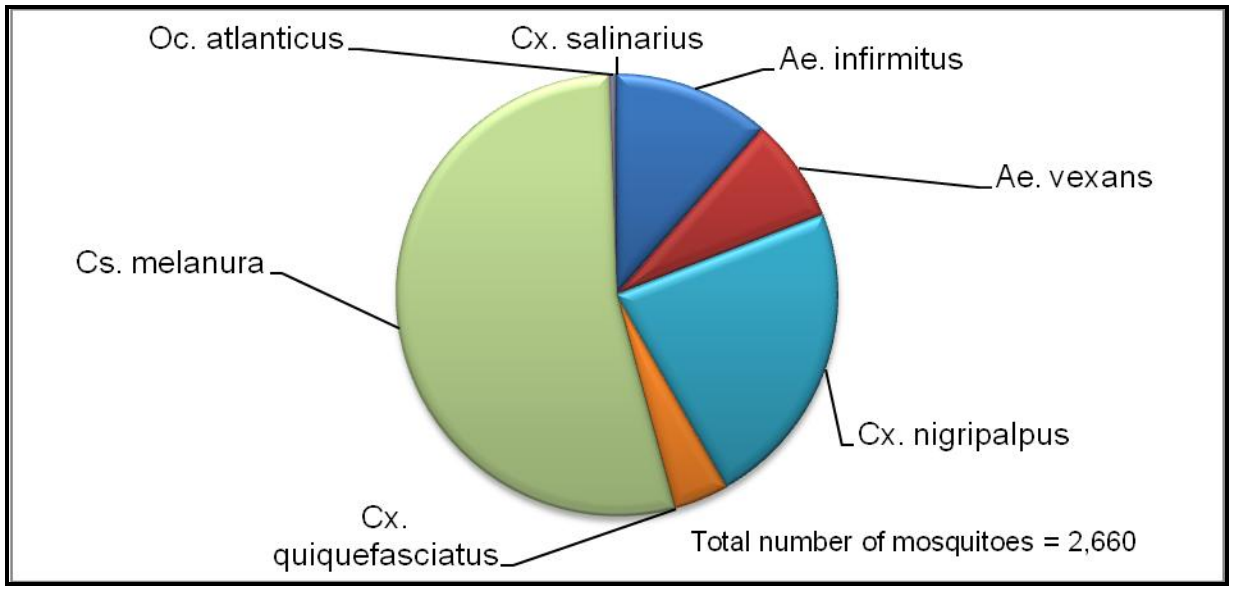

Figure 5: Number of mosquitoes collected from Walton County (2009)

A total of 2,660 mosquitoes were submitted from 14 surveillance site in Walton County. *Culiseta melanura, Culex nigripalpus, and Aedes infirmatus* were the three most abundant species.

RT-PCR Screening Panel

A total of six primer sets were used to test viral isolates that were not identified by real-time RT-PCR as WNV, EEEV or SLEV viruses. This reverse transciptase- polymerase chain reaction (RT-PCR) screening panel is used by the BOL-Tampa for the detection of additional enzootic arboviruses with known circulation in Florida [members of the *Alphaviridae* (Powers, Brault et al. 2001), *Bunyaviridae* (Kuno, Mitchell et al. 1996) and *Flaviviridae* (Lanciotti, Calisher et al. 1992; Kuno 1998; Lanciotti, Kerst et al. 2000)] [Appendix B]. RT-PCR reactions were performed as described in these studies.

Viral isolates that tested negative as a result of the screening panel were prepared for SISPA.

SISPA Sample Preparation

Culture supernatants of positive control viruses (1 ml) were centrifuged for 30 seconds at 10,000 rpm (9,341 rcf) (Eppendorf Centrifuge 5810 R) and then filtered through a Cellulose acetate syringe filter, 0.2 µM pore size (Nalgene Cat. No. 0974061A), that had been pretreated with inactivated Fetal Bovine Serum (FBS: Hyclone Cat. No. SH3007003), in order to enrich virus particles and remove cellular debris [Figure 6]. The filtered supernatant was then treated with benzonase (1U/ µl) (Novagen Cat. No. 70664-3) at 37ºC for 1 hour to remove additional cellular contaminants and immediately followed by the addition of the Trizol LS reagent (Invitrogen Cat. No. 10296-028).

Viral Nucleic Acid Isolation and Amplification

Viral nucleic acid was isolated using TRIzol LS reagent (Invitrogen. No. 10296-028) as per manufacturer's instructions. Sample nucleic acid concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). Samples with an A260/280 ratio of less than 1.6 were processed. Samples with an A260/280 ratio of greater than 1.6 were discarded due to host/culture contamination.

Viral cDNA synthesis was performed in multiple stages [Figure 2]. Reverse transcription consisted of two stages. First, primer annealing was achieved by incubating ~800ng of the viral RNA, dNTPs, and 20μm of a primer consisting of twenty known nucleotides followed by a span of five degenerate nucleotides (N) from a previously described technique (Djikeng, Halpin et al. 2008) at 95°C for 5 min followed by a quick chill on ice. Superscript III Reverse Transcriptase (Invitrogen, No. 18080-093), RNaseOUT RNase Inhibitor, 0.1 M DTT and 5X First-Strand Buffer was added to the reaction mix. Samples were incubated in a MyCycler Thermal Cycler System (BioRad Cat. No. 170-9703) at 25°C for 5 minutes, 50°C for 30 minutes and 70°C for 15 minutes followed by a 4°C hold. RNAse H (Thermo Scientific Cat. No. AB-1280A) was applied to the sample for 20 minutes 37°C. RNase H is an endonuclease that degrades the RNA portion of DNA-RNA hybrids by hydrolyzing the phosphodiester bonds of RNA.

The reverse transcript product was diluted and additional primer was added. The product was incubated at 95°C for 5 minutes followed by a quick chill on ice. cDNA synthesis was performed using 3'-5' exo- Klenow fragment of DNA polymerase (New England Biolabs, Ipswich, MA, Cat. No. M0212L), Klenow buffer and 10 mM dNTPs. Amplification was performed at 37°C for 1 hour and 75°C for 10 minutes followed by a 4°C hold.

Ten microliters of the cDNA reaction was used as a template for PCR. PCR amplicons were produced by incubating the cDNA product, 10X PCR buffer, 10mM dNTPs, Taq DNA polymerase and 20μm of a primer consisting of the known nucleotides present in the primer used for cDNA synthesis at 72°C for 5 minutes, 36 cycles of 94°C for 3 minutes, 94°C for 30 seconds, 40°C for 1 minute and 72°C for 30 seconds followed by 72°C for 5 minutes and a 4°C hold in a MyCycler Thermal Cycler System. Products were analyzed on a 2% agarose gel and the cDNA cleaned using the QIAquick PCR Purification Kit (Qiagen Cat. No. 28104).

Cloning

PCR purification products were then cloned into the pCR 4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen Cat. No. K4575-40) and OneShot TOP10 Chemically Competent *E. coli* cells according to manufacturer's protocol. Briefly, 4 µl of fresh PCR product, 1µl of kit salt solution and 1µl of TOPO vector were gently mixed to prepare the TOPO Cloning reaction and incubated for 5 minutes at room temperature. Two microliters of the TOPO

Cloning reaction was then added to a vial of OneShot Chemically Competent *E. coli* and gently mixed. The reaction was then incubated on ice for 5 minutes and heat-shocked for 30 seconds at 42ºC without shaking. The reaction tube was then immediately transferred to ice and 250 µl of S.O.C. Medium (2% Tryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5% KCl, 10Mm $MgCl_2$, 10Mm $MgSO_4$ and 20mM glucose) was added to each tube. Each tube was then shaken

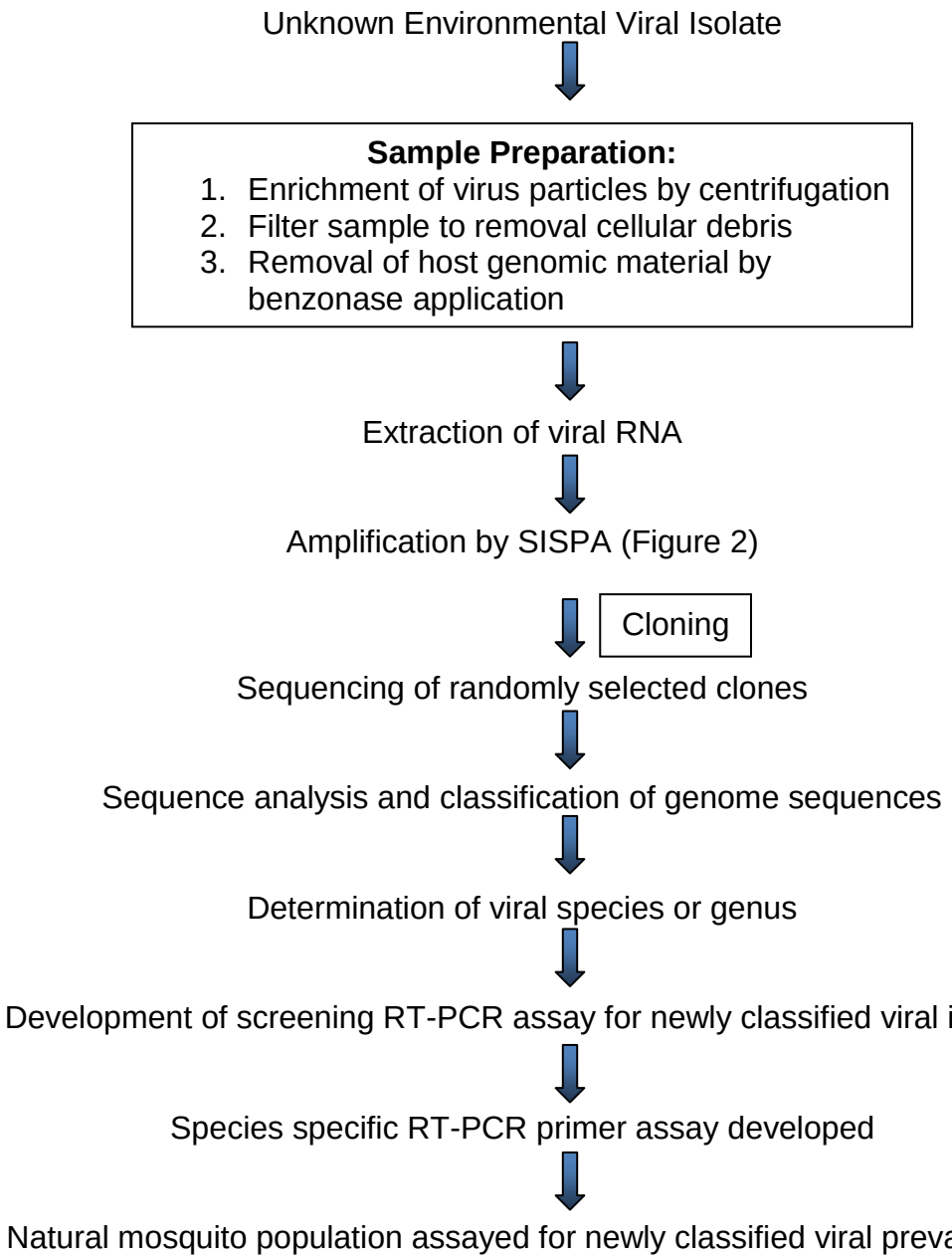

Figure 6: An overview of SISPA application

This figure outlines the overall methodology of this study. Unidentified environmental viral isolates are initially prepared in order to aid in the purification of viral nucleic acids. Viral RNA is then extracted and amplification is achieved by the SISPA methodology. Amplicons are size-selected and cloned. Sequences were analyzed by GenBank query and samples were classified by virus species or genus. An RT-PCR assay was developed to for the newly classified virus in order to screen a natural mosquito population. A species specific confirmation RT-PCR assay was then developed to confirm identification.

horizontally (200 rpm) at 37°C for 1 hour in a Forma Orbital Shaker (Thermo Scientific). The clones were spread on imMedia Amp Agar plates (Invitrogen Cat. No. Q601-20) and incubated at 37°C overnight. Several colonies from each dilution were selected and cultured in 5ml of Luria-Bertani broth (1.0% Tryptone, 0.5% Yeast Extract, 1.0% Sodium Chloride, pH 7.0) (Fisher Scientific Cat. No. BP1421-100; BP1422-100; S640-10) with 50 µg/ml ampicillin (Fisher Scientific Cat. No. BP902-25) overnight. Plasmid DNA was isolated using a Quick Plasmid Miniprep Kit (Invitrogen Cat. No. K2100-11).

Sequence Analysis

Plasmid DNA was shipped to a commercial laboratory (GeneWiz, New Jersey) for traditional DNA sequencing at room temperature. Sequences were evaluated for quality score and contiguous read length. After a manual review of trace files, sequences with quality scores between 25-39 and contiguous read length over 500 were submitted to GenBank and a query search was preformed. Sequences with lower quality scores and contiguous read lengths were discarded.

The basic local alignment search tool (BLAST) was used to identify or classify virus subtypes by percent homology to the GenBank database [http://www.ncbi.nlm.nih.gov/genbank/] utilizing the “other” database and optimizing through discontiguous megablast. Sequences with homology to the cloning vector were discarded and sequences with homology to arboviruses were further analyzed.

Sequences producing significant alignments within GenBank were downloaded and used to construct an alignment with sequences derived from SISPA amplicons. Sequence alignment using the ClustalW 1.6 method was performed in MEGA 4.0 (Tamura, Dudley et al. 2007). If the viral isolate was less than 85% homologous to a known species the classification was determined and related viral species were aligned to determine relatedness.

Phylogenetic analysis was computed in MEGA 4.0 (Tamura, Dudley et al. 2007) for viruses classified to infer the evolutionary relationships of virus strains. The evolutionary history was inferred using the Neighbor-joining method, with 1000 bootstrap replicates. Phylogenetic trees were evaluated for accuracy of branch points and phyletic clusters.

Viral Culture Confirmation

Once a virus was identified by SISPA, a confirmation RT-PCR assay were designed. A gene specific RT-PCR primer set was designed for the classified virus, Flanders virus, in Primer3 (Rozen 2000) and a previously described primer set was employed to target Infirmatus virus. The primer set used to screen for Infirmatus virus was designed to target previously determined and newly derived S segment sequences of human pathogens of the *Orthobunyavirus*, *Phlebovirus*, and *Nairovirus* genera of the family *Bunyaviridae* (Lambert and Lanciotti 2009). Once a mosquito pool isolate was amplified by the *Bunyaviradae* primer set, a confirmation primer set was used that targeted the G_C glycoprotein of the genus

Orthobunyavirus [Appendix C]. These RT-PCR assays were utilized to screen mosquito pools collected during 2008-2009 from Hillsborough and Walton County, Florida. This allowed for the estimation of the prevalence of these newly identified viruses of interest. Viral isolates were amplified for subsequent nucleotide sequencing using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA polymerase (Invitrogen Cat. No. 12574-018) per manufacturer's protocol.

Amplicons derived from mosquito pool screening were visualized using the automated QIAexcel (Qiagen),which allows for the analysis of DNA and RNA fragments. Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen) according to manufacturer's protocol. Identified samples were then submitted for traditional DNA sequencing to GeneWiz. Phylogenetic analysis was computed using Clustal W1.6 method for viruses classified in MEGA 4.0 to infer the evolutionary relationships of virus strains. The evolutionary history was inferred using the Neighbor-joining method, with 1000 bootstrap replicates. Phylogenetic trees were evaluated for accuracy of branch points and phyletic clusters. The overall mean was determined using the Jukes-Cantor computation to determine the suitability of the data for a Neighbor-joining tree. If the average pairwise Jukes-Cantor distance is >1.0 the data is not suitable for a Neighbor-joining tree (Tamura, Dudley et al. 2007). PoolScreen was used to calculate

prevalence (Katholi, Toé et al. 1995). It is a probability-based program that calculates infection rates and associated confidence intervals that account for the potential presence of multiple positive insects.

Results

Since the 1960's, the BOL-Tampa has collected and maintained an extensive reference collection of clinical and environmental virus isolates from cultured specimens. As a result, several new arboviruses have been discovered along with a number of unknown environmental viral isolates. Unidentified viral isolates were obtained from historical arbovirus surveillance studies at the BOL-Tampa Florida or collected from April to December of 2008 in Hillsborough County and June to August of 2009 in Walton County by the University of South Florida. The unknown viral isolates had not been identified by the BOL-Tampa screening panel. Therefore, a new methodology was needed to classify these viruses. A modified SISPA technique was successfully used in this study to classify unknown viral isolates. Once an unknown viral isolate was classified, the natural mosquito population was assayed to estimated viral prevalence at surveillance sites in Florida.

SISPA Validation

SLEV and WNV were first amplified by the standard SISPA technique to validate the method for correct identification or classification of characterized arboviruses [Table 1]. The SISPA technique resulted in the amplification of multiple gene fragments, as depicted by a "smear" pattern following sample electrophoresis (1% agarose gel) and EtBr staining [Figure 7]. Samples were

size selected and subsequently cloned. Selected colonies were sequenced and submitted to GenBank for query. BLASTn analysis of these sequences correctly identified both control strains [Table 4]. For example, several clones of SLEV (strain beAN 156204) had high sequence homology to published SLEV strains in GenBank, including 98% identity to the prototype beAN 156204 strain. In addition, the success of this random amplification technique was further validated by the identification of sequences with homology to different regions of the SLEV genome [Table 3].

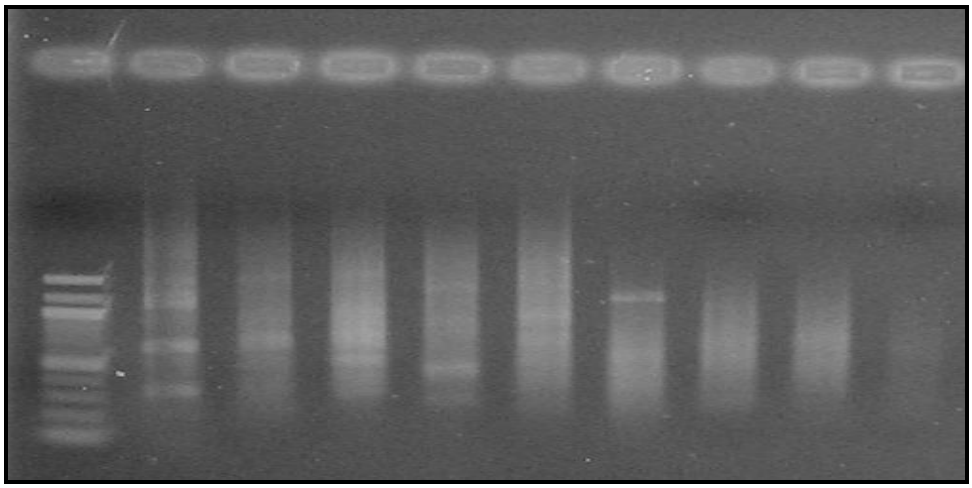

Figure 7: Evaluation of SISPA Method.

The SISPA method successfully amplified sequences derived from a panel of known viruses (West Nile virus [Egypt 101], and St. Louis encephalitis virus [SLEV strain beAN 156204]). Amplicons can be visualized by smeared banding patterns on a 1% gel after electrophoresis. Lane 1 is a 100 base pair (bp) ladder (New England BioLabs Cat. No. N0467L) which ranges from 100-1,517 bp.

Table 3: Alignment of SLEV beAN 156204 fragments obtained from SISPA method identified to genomic segments.

Sequences found	Number of clones sequenced	Homology
SLEV Envelope protein	1	93%
SLEV Polyprotein	2	98%

A total of 10 clones were obtained from SISPA application to the control virus SLEV strain beAN156204. Three clones showed a high homology to genomic segments of SLEV.

However, the preliminary validation results for the control arboviruses indicated that only 30% of clones had SLEV specific amplicons inserted into the plasmid [Table 4]. The remaining clones were found to have contaminating non-SLEV sequences inserted into the cloning vector. Similarly, 60% of the West Nile virus clones were WNV specific and 40% of clones had contaminating non-WNV sequences inserted into the plasmid. The contaminants were identified as artifacts of the culture system used to amplify the virus (Vero cell specific templates).

Table 4: SISPA Validation using control viral strains

Virus Name	Number of clones	Number matching viral strain	Number matching vector/other	% of clones matching viral strain
SLEV [beAN 156204]	10	3	7	30%
WNV [Egypt 101]	10	6	4	60%

Standard SISPA methodology was applied to control virus strands, St. Louis encephalitis virus [SLEV beAN 156204] and West Nile virus [WNV Egypt 101] The results showed a low cloning efficiency and optimization was preformed.

SISPA Optimization

Due to its low cloning efficiency of virus specific amplicons, the standard SISPA technique was further optimized [Table 4]. The method was modified to include steps to remove host nucleic acid contamination. First, culture supernatant of the unknown viral isolates was centrifuged and filtered to enrich virus particles and remove cellular debris. In addition, previous studies have shown the success of nuclease application, such as DNase I RNase A and benzonase, application to improve the cloning efficiency of virus-specific PCR products (Allander, Emerson et al. 2001; Clem, Sims et al. 2007; Valles, Strong et al. 2007). Since the physical properties of the unidentified viral isolates were not known, DNase I, RNase A and benzonase were compared for reaction efficiency. Benzonase was found to remove a greater amount of host

contaminants without damaging the viral genomic material, whereas DNase I and RNase A resulted in lower viral genomic yields following extraction (data not shown). These steps increased the proportion of clones derived from the control viral nucleic acids [Table 5].

Table 5: Optimization of SISPA using control viral strains

Virus Name	Number of clones sequenced	Number matching viral strain	Number matching vector/other	% of clones matching viral strain
SLEV [beAN 156204]	10	5	5	50%
WNV [Egypt 101]	10	8	2	80%

SISPA methodology was optimized through sample preparation steps of centrifugation, filtration and benzonase application. These steps were applied to control virus strands. The results showed an increase in cloning efficiency.

Identification of Viral Isolates

Flanders Virus

The SISPA method successfully amplified sequences derived from an unknown viral isolate, M08-343. Sample SISPA amplicons were purified using a QIAquick PCR Purification Kit, subsequently cloned and five clones were submitted for sequencing. Two of the unknown M08-343 clone sequences (approximately 604 bp) had a 93% identity to the M gene of Flanders virus (AF523197.1) and the others were a result of host nucleic acid contamination.

An M gene specific primer set for Flanders virus was developed and confirmed that the identity of the M08-343 sequences derived from SISPA were Flanders virus [Figure 8]. This Flanders virus RT-PCR assay was then used to rescreen the unidentified viral isolates obtained from the BOL-Tampa archive and the University of South Florida [Appendix C]. A total of five previously unknown mosquito pool isolates were successfully identified as Flanders virus using these M gene specific primers [Table 6]. Flanders virus was detected in three pools of mosquito species (*Culiseta melanura, Culex nigripalpus* and *Culex salinarius*) submitted to the BOL-Tampa.

A total of 9,623 mosquitoes (416 pools) collected from Hillsborough and Walton County in 2008 and 2009 were screened using the M gene primer set for Flanders virus. Flanders virus was not detected.

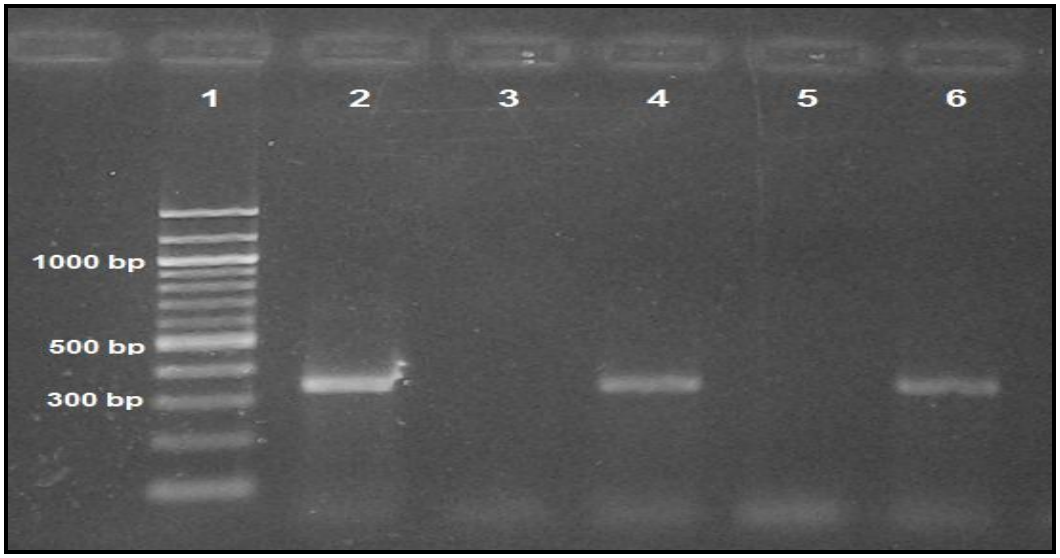

Figure 8: Flanders Virus RT-PCR Assay

A Flanders virus RT-PCR assay was developed following sequence analysis of SISPA clones. Five previously unknown isolates were identified as Flanders virus with this assay and confirmed by DNA sequencing. Lane 1: 100 base pair (bp) ladder (New England BioLabs Cat. No. N0467L) which ranges from 100-1,517 bp; Lane 2: M06-231; Lane 3: M08-319; Lane 4: M08-280; Lane 5: SLEV 12- TRVL 35928; Lane 6: M08-343; Lane 7: H-68; Lane 8: FL-06 S649; Lane 9: Negative Control.

Table 6: Flanders virus positive mosquito pools, BOL-Tampa archive

Strain #	Host Species	# Mosquitoes per pool	Collection Date	County	Source	Identity
M03-1427	*Cx. nigripalpus*	50	5/21/2003	Palm Beach	BOL-Tampa	Flanders virus
M03-1434	*Cx. nigripalpus*	50	6/04/2003	Palm Beach	BOL-Tampa	Flanders virus
M06-231	*Cx. salinarius*	12	6/30/2006	Escambia	BOL-Tampa	Flanders virus
M06-280	*Cx. nigripalpus*	17	6/30/2006	Pinellas	BOL-Tampa	Flanders virus
M08-343	*Cs. melanura*	20	7/16/2008	Escambia	BOL-Tampa	Flanders virus

Phylogenetic analysis of Flanders virus isolates

A phylogenetic analysis of approximately 332bp of the M gene from the newly determined Flanders virus strains was preformed with published M gene sequences downloaded from GenBank of members of the genus Rhabdoviradae [Figure 9]. The neighbor-joining tree was constructed using a pair-wise deletion and the Maximum Composite Liklihood substitution model. The prototype of Flanders virus, strain 61-7484, was used for sequence comparison (Whitney 1964).

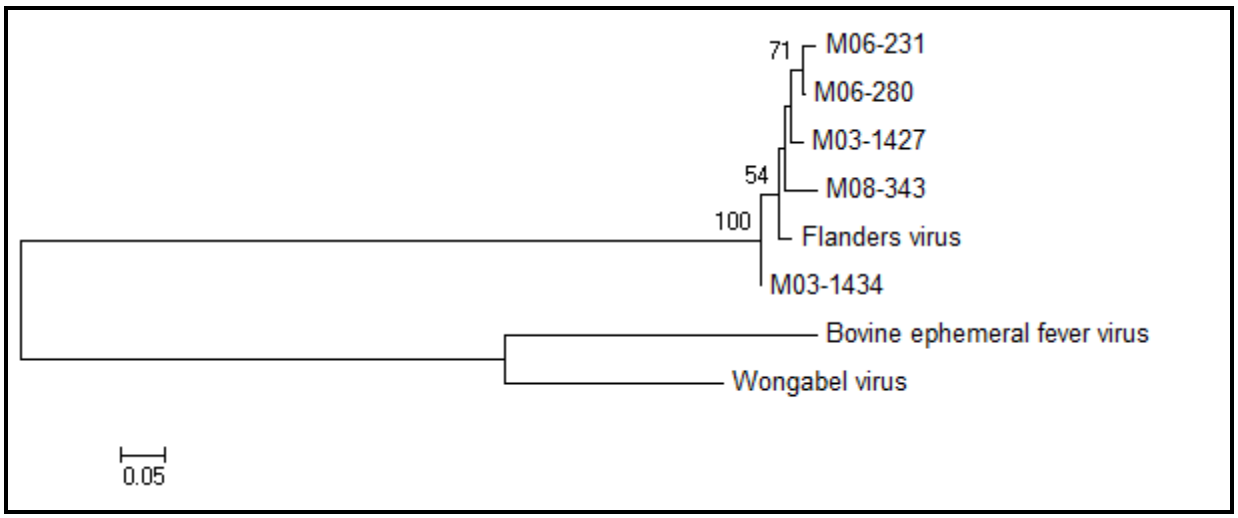

Figure 9: Phylogenetic tree of Flanders virus isolates, M gene.

Previously published sequence data of members of *Rhabdoviridae* in GenBank (AF523197.1; EF612701.1; AF234533.1) were used to make a multiple sequence alignment and neighbor-joining phylogenetic tree (1000x bootstrap replicates, consensus tree) along with the previously unknown environmental sample, M06-231, M06-280, M03-1427, M08-343, M03-1434, of approximately 332 bp. The previously unknown environmental isolates grouped closely with the published Flanders virus strain.

Table 7: Estimates of Evolutionary Divergence between Sequences of Members of Rhabdoviradae and Flanders virus isolates.

Strain	BEFV	WONV	FLAN	M03-1434	M06-231	M03-1427	M06-280
BEFV	--	--	--	--	--	--	--
WONV	63%	--	--	--	--	--	--
FLAN	42%	41%	--	--	--	--	--
M03-1434	41%	42%	97%	--	--	--	--
M06-231	40%	40%	96%	98%	--	--	--
M03-1427	40%	40%	96%	97%	96%	--	--
M06-280	40%	40%	95%	98%	98%	96%	--
M08-343	67%	40%	93%	95%	94%	94%	93%

Members of *Rhabdoviridae* (BEFV: Bovine emphermal fever virus, WONV: Wongabel virus, FLAN: Flanders virus) were compared to Flanders viral isolates and percent identity between sequences is shown. All results are based on the pairwise analysis of 8 sequences. 329 positions in the final dataset were included in the dataset and all positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

Infirmatus Virus

As part of an arbovirus ecology study conducted by USF in 2009, the SISPA method was used to characterize an unidentified virus cultured from a pool of *Ae. infirmatus* mosquitoes. This virus was designated as Infirmatus virus. Nucleotide sequence data and phylogenetic analysis indicate that Infirmatus virus is a newly described member of the California serogroup of *orthobunyavirus* (Ottendorfer, unpublished data).

A previously described RT-PCR assay for the detection of members of *Bunyaviradae* was used to screen the natural mosquito population (Lambert & Lanciotti, 2009). After postive identifciation through this assay to a member of Buynaviradae, a species specific primer set for Infirmatus virus was used to determine classification. A confirmation primer set was used that targeted the G_C glycoprotein of the genus *Orthobunyavirus* to confirm the identity of viral isolates [Appendix C]. A total of 462 pools (10,557 mosquitoes) were screened for Infirmatus virus and 14 pools were found to be positive [Table 8]. Infirmatus virus was isolated from the surveillance site in Hillsborough County, Tampa Bay Downs and Eureka Springs. Mosquitoes from other locations tested negative. Infirmatus virus was identified in *Anopheles crucians, Aedes infirmatus, Culex nigripalpus* and *Culex quinquefasciatus* mosquito pools in April, May, June and September 2008. The infection prevalence was determined, utilizing PoolScreen, to be the highest in *Culex quinquefasciatus* (3.4×10^{-3}, 95 % CI 4.75×10^{-4} to 6.87×10^{-3}) [Table 9].

Table 8: Infirmatus virus positive mosquito pools isolates, Field Surveillance (2008)

Strain #	Host Species	# Mosquitoes per pool	Collection Date	Collection Site	Source	Identity
H-198	*An. crucians*	35	4/2/2008	Tampa Bay Downs	USF	Infirmatus virus
H-277	*Cx. quinquefasciatus*	50	9/27/2008	Tampa Bay Downs	USF	Infirmatus virus
H-371	*Ae. infirmatus*	2	4/22/2008	Tampa Bay Downs	USF	Infirmatus virus
H-372	*Cx. quinquefasciatus*	4	4/22/2008	Tampa Bay Downs	USF	Infirmatus virus
H-472	*Cx. nigripalpus*	8	5/12/2008	Tampa Bay Downs	USF	Infirmatus virus
H-474	*Ae. infirmatus*	8	5/12/2008	Tampa Bay Downs	USF	Infirmatus virus
H-734	*Cx. nigripalpus*	50	4/17/2008	Tampa Bay Downs	USF	Infirmatus virus
H-735	*Cx. nigripalpus*	50	4/17/2008	Tampa Bay Downs	USF	Infirmatus virus
H-736	*Cx. nigripalpus*	50	4/17/2008	Tampa Bay Downs	USF	Infirmatus virus
H-743	*Cx. nigripalpus*	50	4/17/2008	Tampa Bay Downs	USF	Infirmatus virus
H-744	*Cx. nigripalpus*	50	4/17/2008	Tampa Bay Downs	USF	Infirmatus virus
H-746	*Cx. nigripalpus*	50	4/17/2008	Tampa Bay Downs	USF	Infirmatus virus
H-747	*Cx. nigripalpus*	26	4/17/2008	Tampa Bay Downs	USF	Infirmatus virus
S-710	*Cx. quinquefasciatus*	4	6/4/2008	Eureka Springs	USF	Infirmatus virus

Table 9: Infirmatus virus prevalence at the Tampa Bay Downs surveillance site (2008)

Host Species	Point Estimate	95% CI	
		Lower Limit	Upper Limit
Cx. quinquefaciatus	3.4×10^{-3}	4.75×10^{-4}	6.87×10^{-3}
Cx. nigripalpis	2.97×10^{-3}	1.22×10^{-3}	5.29×10^{-3}
Ae. infirmatus	7.9×10^{-4}	5.8×10^{-5}	2.2×10^{-3}
An. crucians	1.01×10^{-3}	1.6×10^{-6}	3.93×10^{-3}

Species collected in excess of 500 individual mosquitoes were screened for Infirmatus virus. The infection prevalence was determined to be the highest in *Culex quinquefasciatus*.

Phylogenetic analysis of Infirmatus virus isolates

A phylogenetic analysis on approximately 392bp of the M segment from the newly determined Infirmatus virus strains identified in the natural mosquito population of Hillsborough County was performed with additional M gene sequences downloaded from GenBank of members of the *Bunyaviradae* family (Figure 10).The M gene sequence for the prototype Infirmatus virus, isolated from a pool of *Ae. infirmatus* collected in July 2008, was used for comparison (Ottendorfer, unpublished data). Based on phylogenetic analysis, Trivittatus virus appears to be the closest relative to Infirmatus virus (Ottendorfer, unpublished data). This finding is supported by BLASTn analysis of Infirmatus positive mosquito pools with 77- 78 % max identity to the published Trivittatus virus (AF123491.1).

Table 10: Estimates of Evolutionary Divergence between Sequences of Members of Bunyaviradae and Infirmatus virus isolates.

Strain	JCV	KEY	CEV	SSH	SDN	JS	LAC	TVT	Infirmatus	H-198
JCV	--	--	--	--	--	--	--	--	--	--
KEY	78%	--	--	--	--	--	--	--	--	--
CEV	72%	73%	--	--	--	--	--	--	--	--
SSH	74%	75%	72%	--	--	--	--	--	--	--
SDN	77%	76%	71%	74%	--	--	--	--	--	--
JS	97%	77%	71%	76%	76%	--	--	--	--	--
LAC	79%	75%	76%	80%	77%	78%	--	--	--	--
TVT	72%	71%	74%	70%	72%	72%	72%	--	--	--
Infirmatus	72%	72%	71%	73%	73%	73%	72%	78%	--	--
H-198	72%	72%	71%	73%	73%	73%	72%	78%	100%	--
S-710	72%	72%	71%	73%	73%	73%	72%	78%	100%	99%

Members of *Bunyaviradae* (JCV: Jamestown Canyon virus, KEY: Keystone virus, CEV: California encephelitis virus, SSH: Snowshoe hare virus, SDN: Serra do Navio virus, JS: Jerry Slough virus, LAC: LaCrosse virus, TVT: Trivittatus virus) were compared to the prototype Infirmatus virus and Infirmatus positive mosquito pools. Percent identity between sequences is shown. All results are based on the pairwise analysis of 11 sequences.391 positions in the final dataset were included in the dataset and all positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

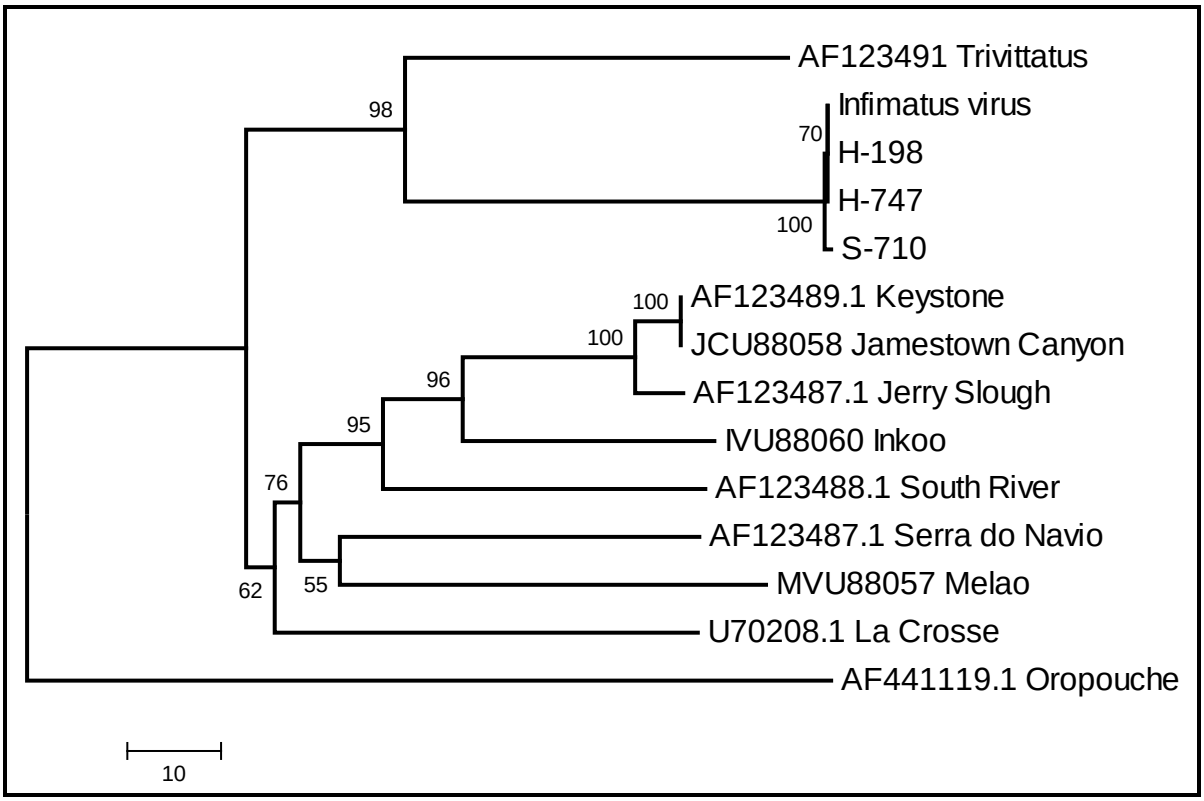

Figure 10: Phylogenetic tree of Infirmatus virus isolates, M segment.

Previously published sequence data of members of *Bunyaviradae* in GenBank (AF123491; AF123489.1; JCU88058; AF123487.1; IVU88060; AF123488.1; AF123487.1; MVU88057; U70208.1; AF441119.1) were used to make a multiple sequence alignment and neighbor-joining phylogenetic tree (1000x bootstrap replicates, consensus tree) along with the previously unknown environmental sample , H-198, H-747, S-710. The previously unknown environmental isolates grouped closely with the published Trivittatus virus strain and the prototype Infirmatus virus.

Discussion

Advances in molecular biology have allowed for the identification of previously unknown viral isolates that could not be typed through common serological methods (hemagglutination inhibition and complement fixation assays) or nucleic acid tests. Several viruses have been identified using SISPA methodology in previous studies such as an Astrovirus, a Rotavirus, Hepatitis G and several Parvoviruses (Matsui, Kim et al. 1991; Lambden, Cooke et al. 1992; Linnen, Wages et al. 1996; Allander, Emerson et al. 2001; Jones, Kapoor et al. 2005). Difficulties in identifying isolates was overcome by the utilization of SISPA, which can identify viral nucleic acids from both DNA (Woodchuck hepatitis virus, enterobacteriophage M13, Hepatitis B virus) and RNA (enterobacteriophage MS2, bovine leukemia retrovirus, hepatitis C virus) templates (Reyes and Kim 1991; Allander, Emerson et al. 2001; Djikeng, Halpin et al. 2008). Earlier applications of SISPA have successfully amplified viruses purified from a number of sources, including bacterial growth media, plasma, serum, fecal material, and allantoic fluid (Reyes and Kim 1991; Allander, Emerson et al. 2001; Djikeng, Halpin et al. 2008; Victoria, Kapoor et al. 2008). Arbovirus surveillance conducted in Florida by the BOL-Tampa and USF has isolated several unidentified viruses. The SISPA methodology was selected as a rapid, flexible technique for the characterization of these agents.

Initially, control viruses (West Nile and St. Louis encephalitis viruses) were amplified by the standard SISPA technique for method validation (Reyes and Kim 1991). However, the random amplification of both host and viral genomic material in the sample resulted in low cloning efficiency for the control viruses. As a result, this technique was optimized to limit host genomic contamination of the samples. The control viruses showed an increase in cloning efficiency following centrifugation, filtration and benzonase application. These sample preparation techniques for SISPA were simple, timely and did not require the expensive equipment typically used for virus purification. Thus, the optimized SISPA technique may be adaptable for use in a public health laboratory.

Flanders Virus

The SISPA method successfully amplified sequences derived from an unidentified virus, strain M08-343, isolated from a pool of *Culiseta melanura* mosquitoes. A query of GenBank database with these sequences identified this isolate as Flanders virus. Flanders virus has been previously described and is an unassigned member of the *Rhabdoviradae* family of the order Mononegavirales in the Hart Park serogroup. Flanders virus has been isolated from many different insects and vertebrates, including *Culiseta melanura,* house sparrows, red-winged blackbirds and an oven bird (Whitney 1964; Kokernot, Hayes et al. 1969; Rose 2001). It has been shown that close variants of Flanders virus are distributed throughout the United States (Boyd 1972). Three genera of Rhabdoviruses are known to infect mammals: *Vesiculovirus*, *Lyssavirus*, and

Ephemerovirus (van Regenmortel 2000). Only members of the genus *Lyssavirus* (i.e. Rabies) and *Vesiculovirus* (i.e. Chandipura virus, which was recently identified in an encephalitis outbreak in children, Andhra Pradesh, India) are known to cause disease in humans (Rao, Basu et al. 2004).

Flanders virus was originally isolated in New York from mosquitoes and birds in 1961 (Whitney 1964). Flanders virus is not believed to be a human pathogen but is of importance due to its temporal relationship with pathogenic arboviruses. For example, Flanders virus has been shown to circulate earlier than SLEV in the mosquito breeding season in the central Ohio-Mississippi Basin (Kokernot, Hayes et al. 1969). In addition, a recent report suggests that this may also be true for West Nile virus and that Flanders virus may be useful as an early indicator of *flavivirus* amplification in the southeastern USA (Moncayo, unpublished data). Furthermore, Flanders virus transmission appeared to decline in the late summer months and is not supported by the hot and dry weather preferred by SLEV (Kokernot, Hayes et al. 1969).

Recent Flanders virus circulation has been detected in Florida based on the newly identified viruses isolated at the BOL-Tampa from 2003 to 2008. These five Flanders virus isolates were derived from different regions in Florida. Two of the isolates (M08-343 and M06-231) were derived from mosquito pools collected in the panhandle (Escambia County). M08-343 was collected in July of 2008 and interestingly, Escambia County reported two locally acquired human cases of West Nile virus in September 2008, as well as sentinel chicken WNV

seroconversions in September and October 2008 (Collins 2008). This relationship was also seen in two of the isolates (M03-1427 and M03-1434) that were derived from pools collected the south Florida (Palm Beach County) in May and June of 2003. Several sentinel chickens tested positive for WNV in July of 2003 and several dead birds (Eurasian Collared Doves, Purple Gallinules, mockingbirds, blue jays, Chinese geese and cockatoos) were collected during the same time frame (Collins 2003). This circumstantial information also suggests that Flanders virus may serve as an early indicator for later West Nile virus transmission in Florida.

To assess the prevalence of Flanders virus in the natural mosquito populations in Florida, 9,623 mosquitoes (total of 416 pools), comprising of ten different mosquito species, were screened for Flanders virus from the Tampa Bay area in 2008 (344 pools) and the panhandle (Walton County) in 2009 (72 pools). Flanders virus was not detected in these pools, which may be due to the fact that arboviral infections in mosquito populations are low, and observations of zero infection in mosquito samples are common (Gu and Novak 2004). Furthermore, Flanders virus transmission may have limited spatial distribution since it was not detected in the central region despite the identification of strain M08-343 in the panhandle (Escambia County) of Florida in 2008 (Collins 2008).

Flanders virus may also have a temporal transmission pattern as it was not detected in the following season (2009) from 72 pools collected in the panhandle of Florida (Walton County, Fl). As a result, Flanders virus likely has

specific temporal and spatial distributions in Florida. It is recommended that surveillance studies for Flanders virus analyze a larger mosquito population from several geographic locations within Florida and further analyze the potential temporal association of Flanders virus with *Flaviviruses*.

The Rhabdoviridae M gene organizes the assembly of the virion by interacting with the ribonucleocapsid and mediates the budding of virions from the infected cell (Jayakar, Jeetendra et al. 2004). Proteins derived from the M gene generally share little similarity between members of Rhabdoviradae (Gubala, Proll et al. 2008). However, the M gene has been determined to be a key component in the assembly of virus-like particles and may not be subject to immunological pressures that could cause strain divergence (Jayakar, Jeetendra et al. 2004). Thus, this region was targeted due to the differences between members of Rhabdoviradae for strain identification and its relative conservation between strains as a major structural component. Flanders virus strains isolated from Florida during 2003 through 2008 by the BOL-Tampa had high homology (range 93 – 97%) to the prototype strain originally isolated in New York in 1961. The M gene was also highly conserved between these isolates collected from different locations in Florida (range 93 - 98%). This is supported by phylogenetic analysis that found minimal divergence of the Florida isolates from the prototype New York strain. A future analysis of the glycoprotein gene is recommended to study the divergence between Flanders virus isolates collected in different locations and time periods. Study of the glycoprotein gene is recommended due

to previous studies that proposed that as the external protein it is more likely to undergo genetic variability (Benmansour, Basurco et al. 1997).

Orthobunyaviruses

In a concurrent study at USF, the SISPA method was used to characterize a previously unknown viral isolate that was designated as Infirmatus virus. Based on nucleotide sequence identity, Infirmatus virus is considered to be a newly described Orthobunyavirus in the California serogroup. Orthobunyaviruses are members of the diverse Bunyaviradae family, which contains important human and veterinary pathogens and is found throughout the world with the exception of Australia (Elliott 1990; Nichol 2001). The Orthobunyavirus genome consists of three segments of negative-sense single-stranded RNA designated as Large, Medium and Small (Nichol 2001). Several members of the group, such as Rift Valley fever and Crimean-Congo hemorrhagic fever, are considered emerging infectious diseases (Elliott 2009). This may be due to the ability of RNA to rapidly evolve through mutation or genome segment reassortment or recombination.

The segmented genome of orthobunyaviruses allows for the possibility of antigenic shift (Lambert and Lanciotti 2009). The three genome segments of the different genera within the family Bunyaviradae have the same complementary nucleotides at the 3' and 5' termini which may allow for reassortment between viral species (Schmaljohn 2001). Due to this ability, it is believed that members of *Bunyaviradae* will continue to be agents of public health importance. Reassorted bunyaviruses have been shown to cause severe disease, such as febrile illness

and hemorrhagic fever (Bowen, Trappier et al. 2001; Gerrard, Li et al. 2004; Briese, Bird et al. 2006). Although the pathogenicity of Infirmatus virus is not known, it has the potential for reassortment and emergence as a public health threat based on its segmented genome.

Although the SISPA method was instrumental for the characterization of Infirmatus virus, it is not a practical tool for high throughput screening of either clinical or environmental samples for detection of the virus. As a result, a traditional RT-PCR assay was utilized to detect Infirmatus virus using a previously described primer set targeting the S segment of the *Orthobunyavirus*, *Phlebovirus*, and *Nairovirus* genera of the family *Bunyaviridae* (Lambert and Lanciotti 2009). The S segment encodes for the nucleoprotein, N, and another nonstructural protein, NSs, and has been shown to be highly conserved (Lambert and Lanciotti 2009). However, BLASTn sequence analysis of the 210 bp product of the RT-PCR assay targeting the S segment failed to clearly distinguish members of the California serogroup isolated in Florida and led to misidentification of viral species. This issue was not found in the prior study (Lambert and Lanciotti 2009).

Once a putative mosquito pool was detected, a confirmation RT-PCR assay was preformed with a primer set targeting the G_C glycoprotein of the genus *Orthobunyavirus* (Appendix C). This assay had been shown to detect Infirmatus virus (Ottendorfer, unpublished data). A total of 14 mosquito pools out of 462 mosquito pools tested were confirmed positive for Infirmatus virus. These

mosquitoes were collected in Hillsborough County at two surveillance sites (Tampa Bay Downs and Eureka Springs). Infirmatus virus was not detected in the 72 pools tested from the panhandle (Walton County) of Florida. Infirmatus virus may not circulate at the other surveillance sites due to ecological and host constraints.

Natural mosquito populations in Florida were assayed to determine the infection prevalence of Infirmatus virus in 10,557 mosquitoes (total of 462 pools) comprised of 10 different species from the Tampa Bay area in 2008 (390 pools) and the panhandle (Walton County) in 2009 (72 pools). Infection prevalence was determined to be the highest in *Culex quinquefasciatus. Culex quinquefasciatus* was not collected from the Eureka Springs site in Hillsborough County and were collected in relatively few numbers at the Walton County surveillance site. Arbovirus transmission cycles have a relatively complex relationship between the arbovirus, the arthropod, and the vertebrate. Arbovirus infection at the surveillance sites was found to be relatively low. Previous analysis of blood meals derived from *Culex quinquefasciatus* have shown that they feed approximately equally on mammals and birds and suggests that they are opportunistic feeders (Niebylski, Savage et al. 1994; Zinser, Ramberg et al. 2004). Concurrent studies at USF have characterized blood meal data collected at the same surveillance sites used in this study. Results have shown that the reservoir is cottontail rabbits (Hassan, unpublished data). It is currently not known if the cottontail rabbits are able to perpetuate the cycle.

Phylogenetic analysis of the M segment from the newly determined Infirmatus virus strains and members of the family *Bunyaviradae* with published M gene sequences in GenBank showed that these Infirmatus virus strains grouped closely with the prototype virus. Infirmatus virus appears to be related to Trivittatus virus (Ottendorfer, unpublished data). Trivittatus virus is commonly vectored by *Ae. infirmatus* in the southeast United States and has a widespread distribution in the eastern United States. Trivittatus virus has been shown to cause mild neurologic disease in humans (Romero and Newland 2003). The detection of multiple species in the California serogroup of Orthobunyaviruses in Florida is important for arbovirus surveillance programs and public health because previous studies have described medically important Orthobunyaviruses and have shown that several members can cause human infections (Gates 1968; Lambert and Lanciotti 2009).

The application of SISPA will allow for better surveillance and rapid detection of an unknown agent. Due to the lack of available human vaccines, surveillance programs play a critical role in the reduction of human disease caused by arboviruses. Elucidation of unknown viral isolates enhances the surveillance efforts employed by clinical and public health laboratories.

Recognition of nonpathogenic arboviruses, such as Flanders virus, can lead to better public health measures through an increase in surveillance at peak arbovirus transmission months. An example can be seen in the discovery of Highlands J virus. Highlands J virus, while non-pathogenic to humans, has a

similar distribution and transmission cycle as Eastern Equine Encephalitis virus (EEEV) (Allison and Stallknecht 2009). EEEV is a severe human and equine neuropathogen with apparent case fatality rates of 30% and 90%, respectively (Przelomski, O'Rourke et al. 1988; Deresiewicz, Thaler et al. 1997). Therefore, surveillance efforts that result in Highlands J virus positives are of public health importance since it signifies that transmission of EEEV is possible. Some studies have suggested that surveillance for Flanders virus may also be useful as an early indicator of *flavivirus* amplification of pathogenic arboviruses, such as WNV and SLEV.

In conclusion, the optimized SISPA method was successfully used for the genetic characterization of two unidentified viruses isolated in Florida. This technique may be useful for the rapid identification of viral agents and may have broad applications in biodefense, agricultural and clinical settings for the detection of emerging infectious diseases. Future studies are recommended to assess the risk of human infection and the role of various mosquito species in transmission for these viruses classified by SISPA.

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Appendices

Appendix A

Media Components

Abbreviations:

EMEM ..... Minimal Eagle Medium, Earle's salts

FCS ..... Fetal Calf Serum

HMEM ..... Minimal Eagle Medium, Hank's salts

L_{15} ..... Lebowitz Media

NCS ..... Newborn Calf Serum

Hepes ..... 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid

Outgrowth Media to Passage Vero Cells

Reagent	ml	Vendor	Catalog Number
1X HMEM	45	Sigma	M-1018
1X L_{15}	45	Sigma	L-4386
NCS (inactivated)	10	HyClone	SH30118.03
Penicillin (200,000 U/ml)	0.1	Sigma	P-7794
Streptomycin (200 mg/ml)	0.1	Sigma	S-9137
Amphotericin B (2.5 mg/ml)	0.1	Sigma	A-9258
Kanamycin (50 mg/ml)	0.1	Sigma	K-1377

Appendix A (Continued)

Liquid Maintenance Media to Maintain Vero Cells After Inoculation

Reagent	ml	Vendor	Catalog Number
1X EMEM	100	Sigma	M-1018
NCS (inactivated)	2	HyClone	SH30118.03
Penicillin (200,000 U/ml)	0.1	HyClone	P-7794
Streptomycin (200 mg/ml)	0.1	Sigma	S-9137
Amphotericin B (2.5 mg/ml)	0.1	Sigma	A-9258
Kanamycin (50 mg/ml)	0.1	Sigma	K-1377
HEPES (1 M)	1	Sigma	H-4034

Biology Field Diluent (BFD)

Reagent	ml	Vendor	Catalog Number
1X HMEM	90	Sigma	M-1018
FCS (inactivated)	10	HyClone	SH30070.03
Penicillin (200,000 U/ml)	0.1	HyClone	P7794
Streptomycin (200 mg/ml)	0.1	Sigma	S9137
Amphotericin B (2.5 mg/ml)	0.1	Sigma	A9258
Kanamycin (50 mg/ml)	0.1	Sigma	K1377

Appendix B

BOL-Tampa environmental isolate RT-PCR screening panel for endemic Arboviruses

Target genus/ genomic target	Forward Primer		Reverse Primer		Appox. amplicon size (bp)	Source
	Name	Sequence (5' to 3')	Name	Sequence (5' to 3')		
California serogroup	CAL-A1	ATGACTGAGTTGGAGTTTCATGATGTCGC	CAL-A2	TGTTCCTGTTGCCAGGAAAAT	250	CDC
Alphavirus	α10247A	TACCCNTTYATGTGGG	T_{25}V-Mlu	TTACGAATTCACGCG-T_{25}	1.0 - 1.5 kb	(Powers et al., 2001)
SLEV	SLE-C1	GTAGCCGACGGTCAATCTCTGTGC	SLE-C2	ACTCGGTAGCCTCCATCTTCATCA	392	CDC
Dengue group	D1	TCAATATGCTGAAACGCGCGAGAAACCG	D2	TCAATATGCTGAAACGCGCGAGAAACCG	511	CDC

Appendix B (Continued)

BOL-Tampa environmental isolate Real Time RT-PCR screening panel for endemic Arboviruses

Target genus/ genomic target	Forward Primer		Reverse Primer		Probe		Source
	Name	Sequence (5' to 3')	Name	Sequence (5' to 3')	Name	Sequence (5' to 3')	
WNV	WN-A1	CAGACCACGCTACGGCG	WN-A2	CTAGGGCCGCGTGGG	WN-A BHQ	CTGCGGAGAGTGCAGTCTGCGAT	CDC
SLEV	SLEA-P2	GAAAACTGGGTTCTGCGCA	SLE-A-P1	GGTGCTGCCTAGCATCCATCC	SLE-A BHQ	TGGATATGCCCTAGTTGCGCTGGC	CDC
EEEV	EE9391	ACACCGCACCCTGATTTTACA	EE9459c	CTTCCAAGTGACCTGGTCGTC	EEE 9414	TGCACCCGGACCATCCGACCT	CDC

Appendix C

RT-PCR Assays used for identity confirmation

	Forward Primer		Reverse Primer	
Genomic Target	**Name**	**Sequence (5' to 3')**	**Name**	**Sequence (5' to 3')**
Flanders virus Medium gene	Flanders FWD	CTTTGAATCCTGGTCGTGGT	Flanders REV	TTACGCTCGACACACCATGT
Orthobunyavirus N ORF	Cal/BWA FWD	GCAAATGGATTTGATCCTGATGCAG	Cal/BWA REV	TTGTTCCTGTTTGCTGGAAAATGAT
Orthobunyavirus G_C glycoprotein	M3 FWD	GTGGTTGCATACATAAAATCT	M3 REV	TAGGCAGGCTGTAACTCTCA

Appendix C (Continued)

Master Mix Components for RT-PCR

SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase Master Mix components for use with amplification of the S segment of members of the family *Bunyaviridae* primer set (Lambert and Lanciotti 2009) and amplification of the GC glycoproten of Infirmatus virus.

Component [Final Concentration]	Volume	Stock Concentration
RNase/DNase free water	5.5 µl	N/A
2X Reaction Mix	12.5 µl	Proprietary
20 um Forward primer	0.5 µl	100 µM
20 um Reverse primer	0.5 µl	100 µM
SuperScript III Platinum Taq	1.0 µl	Proprietary
Template	5.0 µl	N/A
Total	25 µl	

SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase Master Mix components for use with Flanders M gene specific primers

Component [Final Concentration]	Volume	Stock Concentration
RNase/DNase free water	4.5 µl	N/A
2X Reaction Mix	12.5 µl	Proprietary
20 um Forward primer	1.0 µl	100 µM
20 um Reverse primer	1.0 µl	100 µM
SuperScript III Platinum Taq	1.0 µl	Proprietary
Template	5.0 µl	N/A
Total	25 µl	

Appendix C (Continued)

Thermal Cycler Parameters

Thermacycling parameters for amplification of the S segment of members of the family *Bunyaviridae* primer set (Lambert and Lanciotti 2009) and amplification of the GC glycoproten of Infirmatus virus.

Reverse Transcription (1 cycle)	50° 30 min 95° 5 min
PCR (45 cycles)	94° 20 sec 55° 30 sec 68° 2 min
Final Extension (1 cycle)	72° 20 min 4° ∞

Thermacycling parameters for amplification using Flanders M gene specific primers

Reverse Transcription (1 cycle)	50° 30 min 95° 5 min
PCR (35 cycles)	95° 5 min 55° 30 sec 72° 30 sec
Final Extension (1 cycle)	72° 7 min 4° ∞

About the Author

Jessie L. Dyer received a Bachelor of Science degree in Molecular Biology from the Florida Institute of Technology in 2007. She has co-authored several peer-reviewed journal articles on arboviruses. During her time as the University of South Florida, she received the University of South Florida Student Research Award 2008 to support her graduate research project, served as the President of the Infectious Disease Association and was actively involved in several student organizations.