DETECTION AND PHYLOGENETIC ANALYSIS OF TAURA SYNDROME VIRUS OF SHRIMP FROM ARCHIVED DAVIDSON'S FIXED PARAFFIN EMBEDDED SHRIMP TISSUE

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Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

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ABSTRACT

Taura syndrome (TS) is an OIE-listed (World Organization for Animal Health) disease of marine shrimp that is caused by Taura syndrome virus (TSV). TSV has caused more than US $2 billion losses since the initial discovery of the disease in 1992. This work focuses on the detection and genome reconstruction of TSV from Davidson's fixed paraffin embedded (DFPE) shrimp tissues. The data generated for this thesis demonstrate the utility of archived DFPE shrimp tissues as biological samples for detection and genetic studies in TSV.

In Chapter 1, the status of TS is summarized. The review gives an overview of the diseases since it was first identified in Pacific white shrimp (*Penaeus vannamei*) in Ecuador in 1992. The review covers the identification of the etiologic agent, clinical signs and geographic distribution of the diseases, development of histopathological and molecular diagnostic tools. Efforts made to control the disease using biosecurity in shrimp farming, and development of genetically resistant lines of shrimp are also reviewed.

Chapter 2 describes the detection of TSV from archived DFPE shrimp tissues. Twenty-nine DFPE *P. vannamei* shrimp tissue blocks from 2005 representing samples originating in 4 geographical locations were selected for this study. Sample screening indicated lesions of acute stages pathognomonic of TSV infection. Total RNA was isolated from DFPE blocks using three different commercial kits to compare the quality and quantity of extracted nucleic acids. The results showed that RNA isolated from Qiagen RNeasy FFPE Kit provided highest quality of RNA based on 260/280 and 260/230 ratios. Conventional and real-time RT-PCR results show that TSV nucleic acids can be detected in archived DFPE tissue samples. Sanger sequencing of representative amplicons confirmed the identity of TSV. Finally, a phylogenetic analysis based on the nucleotide sequence of the capsid proten (VP1) gene shows the genetic relationship among

different geographical isolates and homologous sequences of TSV isolates deposited in the GenBank database

The feasibility of virus detection and genome reconstruction from archived DFPE tissues opens opportunities for the discovery of novel pathogens and will help enhance our understanding of the evolution of shrimp viruses. This study is the first of its kind in the field of shrimp pathology in using archived DFPE-derived tissue samples for pathogen detection. These results suggest that archived DFPE tissue samples can be utilized for the discovery of novel pathogens and evolutionary analyses. In addition, the findings have direct implications for disease management in shrimp.

CHAPTER 1: Literature Review

Origin and Identification of Taura Syndrome Virus

Taura Syndrome (TS), caused by Taura Syndrome Virus (TSV), first emerged in Ecuador from an unknown source in 1991, and by 1993 the disease was recognized as a major disease affecting penaeid shrimp (Jimenez 1992; Hasson et al., 1995; Lightner et al., 1995). Early reports of TS-like symptoms indicate that TS might have been present as early as 1990 (Lightner et al., 1997). Taura Syndrome spread rapidly throughout Latin America, and by 1996 the disease had spread to all shrimp-farming regions of the Americas (Lightner et al., 1997; Hasson et al., 1999). Cumulative mortality rates due to TS ranged from 75%-95% among infected Pacific white shrimp (*Penaeus vannamei*) populations (Hasson et al., 1995). TSV is estimated to have caused up to 2 billion USD in damage to shrimp farming worldwide (Dhar et al., 2004; Lightner et al., 1995; Lightner et al., 2012).

During the initial outbreaks of TS in 1992 in Ecuador, shrimp mortality rates climbed and TSV became a major epidemic; this caused local scientists to search unsuccessfully for a bacterial or viral agent responsible for the epidemic (Colburn 1997). Finally, different forms of pollution and chemical imports were examined for causality, and it was noted that there were substantial increases in banana fungicides that correlated with the timing of the increases in shrimp mortality rates (Colburn 1997). In 1992, a fungal disease of banana, black leaf wilt disease caused by *Sigatoka negra*, had spread from north of the country, and caused an outbreak of black leaf wilt disease among banana plantations in Ecuador (Colburn 1997). Banana growers in the region responded with aerial spraying of the banana fields with fungal pesticides such as Tilt, Calixin, and Benlate (Lightner et al., 1994; Colburn 1997). Fungicides were applied more heavily during the rainy seasons, and the rivers from the banana growing region drained into the Gulf of

Guayaquil (Colburn 1997). It was already established that shrimp mortality had increased during the rainy season, so it was hypothesized that the banana fungicides were the causative agents of TS (Lightner 1994; Colburn 1997). Popular press coverage reported by Rosenberry (1994) and Wigglesworth (1994), along with research completed by Intriago et al. (1997), among others, perpetuated the belief that a toxic etiological agent caused TS disease (Hasson et al. 1995; Lightner et al. 1995).

In 1994, Ciba-Geigy, the producers of the Tilt fungicide, requested to have a special workshop on Taura Syndrome that was hosted by The University of Arizona (Colburn 1997). Expert scientists and leading industry representatives attended the workshop to gather and review all available information and make recommendations for scientific studies to explore the etiology, disease control and prevention, and disease management methods among other topics (Colburn 1997). It was recommended that studies be performed to evaluate whether fungicides or an infectious agent was the etiological cause of TS (Colburn 1997).

The methodology for experimental infection and induction of TS disease was demonstrated by Brock et al. 1995 by feeding minced TSV-infected carcasses to Specific Pathogen Free (SPF) shrimp. In 1994, the viral etiology of TS was confirmed at the University of Arizona through a series of infectivity studies which fulfilled River's postulates (Hasson et al., 1995; reviewed by Dhar et al., 2004). Hasson and colleagues demonstrated that viral particles can be isolated and purified from experimentally infected shrimp, and the observation of virions corroborated the findings of Brock et al. 1995 (Hasson et al., 1994). Although TS disease was scientifically shown to because by TSV, Ecuadorian shrimp farmers maintained that shrimp losses were caused by a toxic agent- a claim that was supported by litigation for 16 years after viral etiology was determined (OIE, 2019). TSV, the causative agent of TS, is currently recognized by the OIE as a

notifiable agent due to the destructive impact the epidemic had on shrimp farming areas in the Americas and Asia (Dhar et al., 2004, OIE 2019).

Geographic distribution and lineages of Taura Syndrome Virus

Taura Syndrome Virus originated in Ecuador in 1992, but quickly spread throughout the Americas (Lightner et al., 1997; Hasson et al., 1999; Lightner, 2011). Between 1996 and 2003, TSV outbreaks were confirmed in the US (in Texas), China, Taiwan, and Thailand, and South America (Lightner, 2011; USDA, 2004). Taura Syndrome Virus has now been isolated on five continents, and a genetic analysis using TSV capsid protein sequence by Wertheim et al. 2009 found support for four distinct lineages of TSV: Mexico, Southeast Asia, Belize/Nicaragua, and Venezuela/Aruba. Phylogenetic analysis conducted by Wertheim et al. 2009 suggested that all TSV lineages originated from the Americas, with the most basal lineage leading to a cluster of strains from Ecuador, Columbia, and the US. This corroborates the hypothesized origin of TSV in Ecuador (Wertheim et al., 2009). Disbursement of Texas, Hawaii, and Eritrea strains among the Mexico isolates suggest that the Mexico TSV lineage was the progenitor of these outbreaks (Wertheim et al., 2009). The Southeast Asia lineage cluster was hypothesized to originate from Honduras, and contained isolates from China, Taiwan, Thailand, and Indonesian dispersed amongst each other (Wertheim et al., 2009). The Belize/Nicaragua lineage cluster contained the Saudi Arabia strains, suggesting they share a recent common ancestor (Wertheim et al., 2009). The Venezuela/Aruba lineage comprises the fourth genetically different cluster, but also contained isolates from Ecuador, which was consistent with findings from Cote et al., (2008) (Wertheim et al., 2009). According to the phylogeny completed by Wertheim et al., (2009), there were at least five separate introductions of TSV into the United States. This analysis revealed that US strains

were not closely related and stemmed from different lineages in most cases (Wertheim et al., 2009). This is not unexpected considering most shrimp diseases spread within and between countries through the movement of diseased broodstock and post-larvae.

Later phylogenetic analysis shows that isolates have clustered into lineages that correspond with their geographical locations. These lineages include isolates from the Americas including Ecuador, Columbia, Honduras and USA isolates; Southeast Asia which contains Thailand, China, Taiwan, and Myanmar isolates; Mexico, Belize, Venezuela, Columbia, and Saudi Arabia (Dhar et al., 2004; Cote et al., 2008; Tang et al., 2012; Aranguren et al., 2013). The increasing diversity of TSV strains can be explained by a high mutation rate, 2.37×10^{-3} substitution/site/year, in the CP2 region of the viral genome. This mutation rate is similar to other rapidly evolving RNA viruses such as human immunodeficiency virus type 1 (Wertheim et al., 2009).

Disease Biology

Properties of Taura Syndrome Virus & Genome organization

Taura Syndrome virions are non-enveloped, 31-32 nm in diameter, with a buoyant density of 1.337 g/ml and display icosahedral symmetry (Hasson et al., 1995, Dhar et al., 2004; Lightner, 2011). Taura Syndrome Virus was first classified as a member of the *Picornaviridae* family due to morphology, site of replication, similarities of the capsid proteins, possession of a liner positive-sense single stranded RNA genome, genome length, and genome organization (Hasson et al., 1995; Bonami et al., 1997; Robles-Sikisaka et al., 2001). Upon analyzing the capsid protein gene sequence of 3.3 kb clones and northern blot results, Robles-Sikisaka and colleagues made two important observations (Robles-Sikisaka et al., 2001). First, unlike the picronavirus genome that contains single open reading frame (ORF), the TSV genome contains more than one ORF, and

second, the capsid protein gene of TSV is not expressed as a sub-genomic RNA unlike many positive-sense RNA containing viruses (Robles-Sikisaka et al., 2001). Later, Mari et al., (2002) determined the complete genome sequence of TSV and called for this virus to be reclassified as a member of *Dicistroviridae* family based on the presence of two citrons in TVS genome. TSV is currently classified as a member of the *Dicistroviridae* family, genus *Aparavirus* (ICTV, 2012). The TSV genome is genome approximately 10205 nucleotides in length, excluding the 3' poly-A tail (Mari et al., 2002; Lightner et al., 2011). A schematic diagram of the genome organization can be seen in Figure 1.1 derived from Mari et al., (2002).

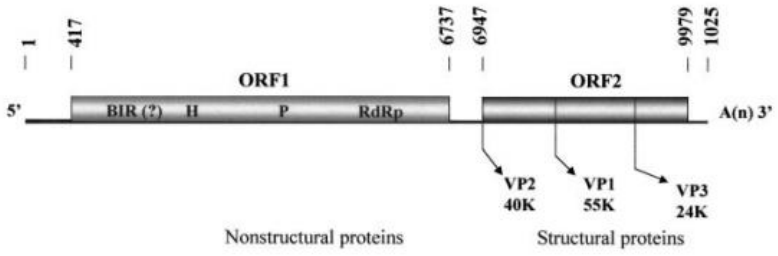

Figure 1.1 Schematic diagram of the genome organization of TSV (Mari et al., 2002).

The genome of TSV contains two ORFs, as seen in Figure 1.1, which are separated by a 207-nucleotide intergenic region. The ORF1 is flanked by a 377 nucleotide 5' untranslated region (UTR), and ORF2 is flanked by a 226 nucleotide 3' UTR followed by the 3' poly-A tail. These two ORFs represent 92% of the TSV genome. There are two AUG codons at the 5'-end of TSV genome. The second in-frame AUG codon at nucleotide position 417 is a candidate for the translation initiation site for ORF1 which extends to nucleotide position 6736. The ORF1 encodes a 234 kDa nonstructural polyprotein (Mari et al., 2002). The TSV genome also contains a short stretch of amino acid (aa) sequence at the 5' end of ORF1 between nucleotide positions 166 and

230 that show similarities to inhibition of apoptosis proteins (IAP) found in other DNA viruses, mammals, yeast, and insects (Dhar et al., 2004). The amino acid products of TSV ORF1 contain sequence motifs that correspond to conserved motifs of helicase, protease, and RNA-dependent RNA polymerase commonly found in other members of the picorna-like viruses (Mari et al., 2002). The AUG codon at nucleotide position 6947 is the translation initiation site for ORF2 which extends to nucleotide position 9982. The ORF2 encodes a 112 kDa polyprotein. The amino acid products of ORF2 were compared to databases and revealed significant similarities to structural polyproteins of insect RNA viruses. The 5' termini for sequences which code for VP2, VP1 and VP3 capsid proteins are mapped at 6913, 7937, 9413, respectively. The molecular mass of VP2, VP1, and VP3 were calculated to be 36.4, 54.6, and 21.1 kDa, respectively (Mari et al., 2002).

Disease cycle and clinical signs

Taura Syndrome disease has three clinically and histologically distinct phases: acute phase, transition phase and chronic phase (Brock et al., 1997; Hasson et al., 1999; Dhar et al., 2004; Lightner, 2011). The acute phase begins rapidly within 24 hours of exposure, lasts between 7-10 days, and has the highest mortality rate (Brock et al., 1997; Hasson et al., 1999; Dhar et al., 2004). The acute phase targets the cuticular epithelium of the foregut, appendages, hindgut, and body cuticles (Brock et al., 1995; Hasson et al., 1995; Lightner et al., 1995; Dhar et al., 2004). During this phase, the infected cuticular epithelium cells may detach from the underlying stroma and form a spherical shape, which is followed by cell lysis (Hasson, 1998). These spherical bodies contain remnants of necrotic cells that are abundant in the acute phase lesions (Brock et al., 1995; Hasson et al., 1995; Hasson et al., 1999; Lightner, 1995; Dhar et al., 2004; Lightner, 2011).

Clinical signs of Taura Syndrome have been documented in most stages of the shrimp life cycle in *P. vannamei* (Lightner et al., 1996; Lightner, 2011). Clinical signs of the acute phase include anorexia, flaccid bodies, cuticle softening, erratic swimming, opaque musculature, and lethargy (Dhar et al., 2004; Lightner, 2011). Moribund shrimp in the acute phase often present a pink or red hue that is often concentrated in the tail fin, resulting from the expansion of chromatophores (Brock et al., 1995; Hasson et al., 1995; Lightner et al., 1995; Dhar et al., 2004). Examples of these signs can be seen in Figure 1.2, derived from Dhar et al., (2004). During the acute phase, histological lesions consisting of necrotic epithelial cells with pyknotic and karyorrhectic nuclei are present. Sized cytoplasmic inclusion bodies and cytoplasmic eosinophilia can be observed (Brock et al., 1995; Hasson et al., 1995; Hasson et al., 1999; Lightner, 1995; Dhar et al., 2004). These characteristics have been appropriately termed "buckshot" or "peppered" lesions due to their appearance (Brock et al., 1995; Hasson et al., 1995; Lightner et al., 1995).

Shrimp that survive the acute phase enter a transitional phase, characterized by declining mortality rates and lasting several days (Hasson et al., 1999; Dhar et al., 2004). During this phase, resolving acute phase epithelial lesions are still visible; there is an accumulation of visible melanized lesions on the tail and thorax due to the accumulation of hemocytes at necrosis sites, leading to irregular black spots, as seen in Figure 1.2 (Hasson et al., 1995; Hasson et al., 1999; Lightner et al., 1996; Dhar et al., 2004; Lightner, 2011). During the transitional phase, sections of the lymphoid organ arterioles may appear normal, and spheroid development begins in the lymphoid organ (Hasson et al., 1995; Hasson et al., 1999; Dhar et al., 2004; Lightner, 2011). Other shrimp diseases, such as yellow head disease, may display similar spheroids. However, the presence of spherical bodies and pyknotic nuclei (giving the "peppered" or "buckshot-riddled appearance") in the absence of necrosis of the parenchymal cells of the lymphoid organ tubules

are indicative of a TSV infection (Lightner, 1996; OIE, 2006). Death may still occur at this phase due to the consequences of cuticular epithelium destruction or infections by opportunistic bacteria (Lightner, 1996; Brock et al., 1997). Clinical signs marking the transition phase include anorexia and lethargy, largely due to the expenditure of resources on wound repair and disease recovery. The transition phase is resolved when the infected shrimp resumes the normal molting cycle and sheds the melanized exoskeleton (Hasson et al., 1995; Hasson et al., 1999; Dhar et al., 2004; Lightner, 2011).

The final phase of infection, the chronic phase, is marked by the cessation of gross signs of infection and mortalities (Hasson et al., 1999; Lightner et al., 1999). A chronic TSV infection will last for a minimum of eight months, and infected shrimp may carry the virus for the duration of their lifetime (Hasson et al., 1998; Hasson et al., 1999). Histopathology of chronic phase infection is characterized by the presence of prominent spheroids in the hypertrophied lymphoid organ that consists of phagocytic granular hemocytes (Hasson et al., 1999; Anggraeni et al., 2000; Lightner, 2011). Lymphoid organ spheroids (LOS) were initially thought to be a unique cellular change upon TSV infection. However, LOS were later found to be associated with other RNA virus infections, such as infectious myonecrosis virus (IMNV), and yellowhead virus (YHV) (Lightner et al., 2006; OIE, 2006). It is possible that LOS formation could be a general host response to RNA virus infection in shrimp (Hasson et al., 1999; Anggraeni et al., 2000). During the chronic phase, shrimp resume normal feeding and swimming behaviors (Dhar et al., 2004; Lightner, 2011). Although shrimp that survive through the chronic phase may appear normal, there is evidence to suggest that they are less tolerant to salinity stress and that they may pass the virus vertically to offspring (Hasson et al., 1999, OIE, 2006).

Figure 1.2. *Penaeus vannamei* juveniles infected with TSV. (A) Signs of acute phase of infection. Juvenile shrimp in the upper right corner appears healthy and translucent while the remaining five acutely infected shrimp display a darker coloration overall ranging from lavender to red. Darker coloration is due to chromatophore expansion. (B) Signs of transition phase of infection. Three shrimp display multifocal melanized lesions in the cephalothorax and tail, indicative of the transition phase of a TSV infection. Lesions indicate foci of cuticular epithelium that were destroyed during the acute phase and are in the ongoing process of lesion resolution (Dhar et al., 2004).

Susceptible species

TSV infects a number of farmed shrimp species, including *P. vannamei*, the most common penaeid species farmed worldwide currently (Hasson et al., 1995; Lightner et al., 1999; Tu et al., 1999; Anderson et al., 2018). Mortality rates resulting from TSV infections in *P. vannamei* populations range from 50-90% (Bonami et al., 1997; Brock et al., 1997). Other American penaeid species can be infected, including *P. stylirostris*, *P. schmitti*, *P. setiferus*, *P. duorarum* and *P. aztecus*. TSV causes severe disease in post-larvae, juvenile, and adult stages of *P. vannamei*, post-larvae and juvenile stages of *P. setiferus*, juvenile stage of *P. schmitti,* and in post-larvae and juvenile *P. stylirostris* (Overstreet et al., 1997; Lightner et al., 1996; Brock, 1997; Robles-Sikisaka et al., 2002; Dhar et al., 2004). *P. monodon* was demonstrated to have signs of disease and *P. chinensis* also displayed mortality from TSV infectivity studies (Srisuvan et al., 2005; Overstreet et al., 1997).

Disease transmission

In a pond or a tank, disease transmission most often occurs through cannibalism of dead or infected shrimp. This leads to exponential and rapid spread of TSV to the rest of the shrimp population (Brock, 1995; Hasson, 1995; Dhar et al., 2004). TSV may remain pathogenic in decaying shrimp carcasses for up to 3 weeks postmortem, which allows the carcasses to serve as a source for outbreak if consumed by healthy susceptible shrimp (Dhar et al., 2004). Water borne transmission can also occur for up to 48 hours following experimentally induced TSV epizootics (Dhar et al., 2004). *P. vannamei* that survives infection continue to harbor infectious TSV in the lymphoid organ and hemolymph (Hasson, 1998; Hasson et al., 1999). New outbreaks may result

from the cannibalization of chronically infected shrimp by uninfected shrimp (Hasson, 1998; Hassan et al., 1999).

Aquatic insects such as *Trichocorixa reticulata*, known as water boatmen, can serve as a vector of transmission from pond to pond, or between farms (Hasson et al., 1995; Lightner et al., 1995; Garza et al., 1997). Water boatmen prey on TSV-infected post larval shrimp and then transmit infectious virus from pond to pond through fecal matter or by being consumed by healthy shrimp (Hasson, 1995; Lightner et al., 1997). *In situ* hybridization assays of water boatmen show TSV-positive gut contents, but no indication of viral replication, indicating that they can transport the virus intestinally without being actively infected (Lightner, 1996; Hassan, 1998). Sea birds such as the seagull *Larus atricilla* can also transmit TSV between ponds and farms (Garza et al., 1997). These sea gulls consume the TSV-infected shrimp, and then transmit the virus to other ponds or farms through defecation. Shrimp in the subsequent ponds ingest the TSV-infected feces while scavenging for food. (Garza et al., 1997). Vanpatten et al., (2004) demonstrated that TSV particles remained infectious for up to one day following passage through both chickens and seagulls, which demonstrates the importance of aquatic birds as mechanical vectors for TSV.

TSV can survive long-term freezing as well, which enables the virus to spread easily from country to country. TSV can disseminate into the new population if a TSV-infected shrimp is used for bait, through improper disposal procedures at shrimp processing plants, careless introduction of TSV-infected tissues into local waterways, or disposal of TSV-infected tissues at landfills where seagulls could ingest and subsequently spread TSV particles (Hasson et al., 1995; Lightner et al., 1995; Lightner, 1997).

Current diagnostic techniques

There is a wide variety of diagnostics techniques available for the detection of TSV, including gross and clinical sign observation, histological (H&E) methods, immunological methods, conventional reverse transcriptase polymerase chain reaction (RT-PCR), and real-time RT-PCR. During the acute phase of infection with TSV, shrimp exhibit an overall pale reddish color with a distinctly red tail due to chromatophore expansion. A 10x lens/ magnifying glass can be used to examine the appendages for signs of epithelial necrosis. Shrimp will typically display an empty gut and soft shells at this point. Gross signs of infection during the transition phase, including gross multifocal melanized lesions, may only provide a tentative TS diagnosis. Shrimp in the chronic phase display no disease symptoms so gross clinical signs are not used for diagnosis (Brock et al., 1995; Hasson et al., 1995; Lightner et al., 1995; Lightner, 1996; OIE, 2009).

Shrimp tissues can be prepared for histological analysis through Hematoxylin and Eosin (H&E) staining. Pathognomonic acute-phase lesions present as multifocal necrotic areas in the cuticular epithelium that display increased cytoplasmic eosinophilia and pyknotic/karyorrhectic nuclei, as seen in Figure 1.2. Remnants of necrotic cells in these lesions present as spherical bodies and range in staining from pale basophilic to eosinophilic and are responsible for the “buckshot-riddled” appearance. These lesions are sufficient in making a TSV diagnosis (Lightner, 2011). During the transition phase, masses of hemocytes become melanized and give rise to irregular black spots; in H&E sections, these lesions show cuticle erosion and exposed surface hemocytes (Lightner, 1996; Lightner, 2011). Routine H&E histology of tissues in the chronic phase of infection may only show numerous prominent lymphoid organ spheroids which lack the central vessel typically seen in normal lymphoid organ tubules (Anggraeni et al., 1998; Hasseon et al., 1999; Lightner, 2011).

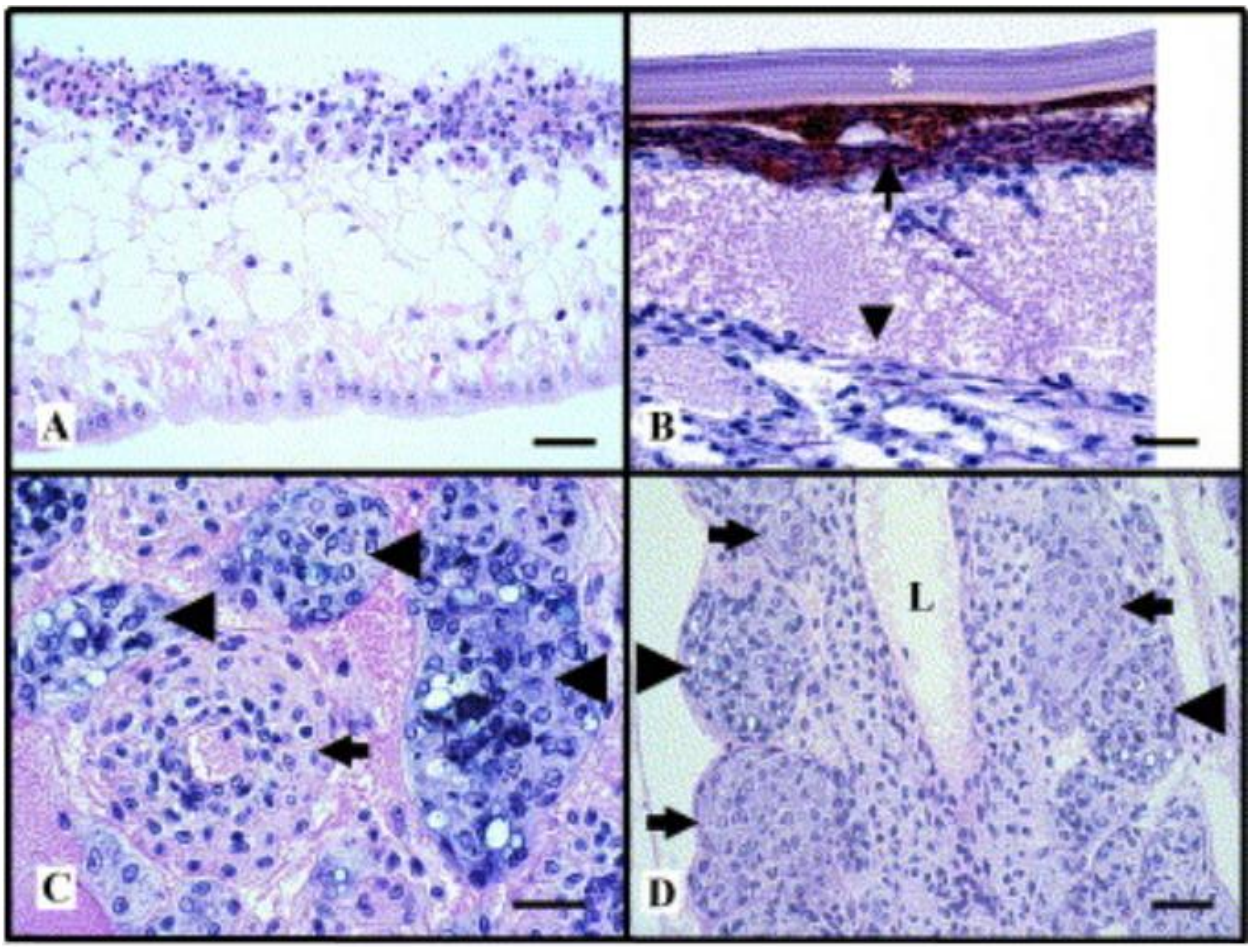

Figure 1.3. Routine histology of the acute, transition, and chronic phases of TSV infections in *Penaeus vannamei*. (A) Head appendage with pathognomonic severe acute phase "peppered" or "buckshot riddled" lesions in the cuticular epithelium and subcutis. Normal uninfected epithelium is present at the bottom for comparison. (B) Transition phase TSV lesion in the cuticular epithelium with a layer of melanized (brown) hemocytic infiltrates (small arrowhead) that has replaced epithelium destroyed by TSV. Fibrous tissue and edema (large arrowhead) indicate ongoing wound repair. (C) Lymphoid organ in a chronically infected shrimp showing three basophilic spheroids (large arrowhead) with a normal arteriole cross-section (small arrowhead). (D) Arrows indicate spheroids bordering the sub gastric artery wall, with the lumen (L) in the center. Scale bar = 30 μm (Dhar et al., 2004).

In situ hybridization methods for detecting TSV utilizes two TSV-specific, digoxigenin-labeled cDNA probes, and provides a greater sensitivity of detection than H&E. TSV lesions provide blue-black areas in the cytoplasm when reacting with cDNA probes (Mari et al., 1998; OIE, 2009).

Antibody-based detection methods were developed for the detection of TSV from tissue homogenate, hemolymph, or DFPE samples (OIE, 2009). In the dot-blot immunoassay method, the antigen is dotted to the surface of the multiscreen filtration plate (MAHA-N45), reacted with monoclonal antibody (mAb) or mouse polyclonal antibodies, and a secondary Alkaline-phosphatase-labelled goat anti-mouse IgG antibody is used for detection (OIE, 2009). ELISA-based detection utilizes a TSV-specific mAb to detect the capsid protein of TSV. This method is advantageous because it is economical and is a rapid four hour assay, however, some mAbs routinely used in ELISA failed to detect some strains, such as the Mexico strain of TSV seen in *P. stylirostris* (Poulos et al., 1999, Robles-Sikisaka et al., 2002).

Both conventional and real-time RT-PCR have been developed for the detection and quantification of TSV. These methods are rapid, less labor intensive, highly specific, and robust. In addition, non-lethal tissue sampling method was developed for PCR-based assay using hemolymph as sample (Nunan et al., 1998; Tang et al., 2004). RT-PCR amplifies a 231 base pair sequence from a conserved intergenic region between ORF1 and ORF2 in TSV genome (Nunan et al., 1998). Real-time RT-PCR-based methods using SYBR Green dye or the TaqMan probe were developed for a rapid detection and quantification of TSV (Dhar et al., 2002; Tang et al., 2003). The SYBR Green-based method measures the accumulation of target amplicon during the exponential phase of the reaction, and the increase in fluorescence is recorded as the cycle threshold (Ct) value (Dhar et al., 2002). This method uses 112F/162R or 004F/081R primers to

amplify a 50 or 78 base pair sequence of the TSV genome, respectively (Dhar et al., 2002). The TaqMan method, described by Tang et al., (2003), uses a probe that is labelled with fluorescent dyes FAM on the 5' end and TAMRA on the 3' end, and amplifies a 72 base pair sequence of the TSV genome. This method uses 1004F/1075R primers.

Disease management

Biosecurity

Biosecurity practices are important for mitigating the impacts of shrimp diseases and ensuring long-term sustainability of the aquaculture industry. Biosecurity approaches include the use of Specific Pathogen Free (SPF) shrimp and on-farm practices. These protocols allow shrimp breeders to focus on selection pressures on survival rather than focusing solely on disease resistance (Moss et al., 2012).

SPF shrimp can help control the disease status of captive broodstock (Lotz, 1997; Lightner et al., 2009). SPF shrimp are free of at least one specific pathogen; this pathogen must be reliably diagnosable, physically excluded from a facility, and pose a significant threat to the aquaculture industry (Lightner et al., 2009). There are currently SPF shrimp available for TSV, White spot syndrome virus, Yellow head virus, and infectious myonecrosis virus (Moss et al., 2012). *P. vannamei* is the only species of farmed shrimp that is available commercially as SPF, which has led to a 1,490% increase in *P. vannamei* production over 9 years (Moss et al., 2012).

On-farm biosecurity measures are designed to mitigate crop loss from disease and may be more practical and cost-effective than the use of SPF shrimp and selective breeding programs (Moss et al., 2012). Traditional farming practices included shrimp that were cultured in coastal ponds where flow-through water was utilized to maintain water quality standards (Hopkins et al.,

1993; Moss et al., 2012). However, this water source was a significant way for pathogens to enter shrimp farms, which ultimately spread from both pond to pond and farm to farm (Lotz, 1997). Eventually, researchers determined that high rates of water exchange were not necessary to maintain high shrimp biomass; these results led to the development of The Belize system, a commercial shrimp farm that relied on no water exchange, heavy aeration, and the use of in situ microbes for both biofiltration and as a supplemental food source for shrimp (Hopkins et al., 1991; Browdy et al., 1993; McIntosh, 1999; Moss et al., 2012). There are shrimp farms that use recirculation aquaculture systems (RAS) that are stocked at high densities and experience very limited water exchange (Taw et al., 2011, Moss et al., 2012). These systems may also include plastic-lined ponds, physical barriers for other sea life like crabs, and bird scare lines (Taw et al., 2011). The use of RAS technologies along with SPF shrimp have greatly increased production potential and may help lead to increased profitability for shrimp farmers (Taw et al., 2011; Moss et al., 2012; Taw, 2017).

Breeding for disease resistance

Since the emergence of TSV in the early nineties, a tremendous effort has been dedicated to selectively breeding resistant lines to control Taura Syndrome disease (Lotz et al., 1995; Moss and Moss, 2009; Cock et al., 2009). These breeding programs were designed to enhance survival to TSV exposure and have resulted in families of *P. vannamei* shrimp that can survive TSV infection (Moss et al., 2010). Due to the availability of these shrimp for commercial breeding purposes, the threat of TSV has been largely eliminated in shrimp farming regions worldwide (Moss et al., 2012).

Significant improvements have been seen in developing TSV resistant line of *P. vannamei* shrimp despite low heritability estimates (Argue et al., 2002; Cock et al., 2009). Selected lines of *P. vannamei* showed up to an 18.4% survival increase after a single generation of selection (Argue et al., 2002) and other families showed between 24-37% mean survival rates after TSV exposure over a three-year period (White et al., 2002). White et al., (2002) demonstrated a 65-100% increase in survival in some families of *P. vannamei* during the same three-year period. Researchers have been selecting for TSV resistance genes for more than fifteen years now, and multiple families are now developed that typically show a 100% survival rate after TSV exposure (Moss et al., 2012). Shrimp breeding programs have distributed TSV-resistant shrimp lines of *P. vannamei* and *P. stylirostris* to commercial hatcheries worldwide to the invaluable benefit of the shrimp industry (Moss et al., 2012).

TSV virulence varies between strains and, although the reason remains unknown, this variation plays an important role in developing TSV-resistant shrimp lines (Tang and Lightner, 2005; Srisuvan et al., 2006). The molecular mechanisms that underlie TSV resistance in shrimp remain unknown as well (Sookruksawong et al., 2013). Veloso et al., (2011) reported that during infection with TSV there was an upregulation of immune gene expression in the hepatopancreas, but the molecular pathways were not identified. Sookruksawong et al., (2013) identified 697 unigenes that were expressed and associated with TSV-resistance. Other studies have demonstrated that growth and energy-related genes seem to be down-regulated in TSV resistant strains (Argue et al., 2002; Veloso et al., 2011)

Significance

The aquaculture industry is an important source of both food and income for millions of people around the world (FAO, 2016). This industry has grown from supplying a mere 7% of marine food for human consumption in 1974 (FAO, 2016) to supplying an estimated 50% of seafood for human consumption by 2017 (National Marine Fisheries Service, 2018). Global aquaculture production was estimated to have supplied approximately 23.4 million individuals with employment in 2005, and current global seafood production by aquaculture is estimated to be 74.81 million tons (FAO, 2016; Technavio Report, 2018).

The aquaculture industry is currently valued at USD 180.2 billion and expected to grow to USD 224.2 billion by 2022 (Technavio Report, 2018). In 2016, the United States aquaculture industry was valued at USD 1.5 billion, which accounts for 21 percent of the United States' total seafood production (National Marine Fisheries Service, 2018). The United States consumption of fish and/or shellfish is 16 pounds per capita, and in 2017 USD 102.2 billion was spent on fishery products by consumers in the United States (National Marine Fisheries Service, 2018). Shrimp aquaculture in the Western Hemisphere alone produced more than 200,000 tons of shrimp and was valued at USD 2 billion (Lightner, 2011).

Infectious diseases caused by viruses, bacteria and fungi remain an ongoing obstacle for the sustainable growth of the shrimp aquaculture industry worldwide. Therefore, the detection of diseases in the aquaculture industry is critical for controlling, predicting, and preventing potential outbreaks and large economic losses. There is limited work that has been done with the detection of viral pathogens from archived DFPE shrimp tissues, and it is critical to explore the potential of archives histological tissue samples for pathogen detection, discovery, and evolutionary studies.

Use of formalin fixed tissue samples for studying human diseases

In recent years, optimization of nucleic acid extraction methods has enabled formalin fixed paraffin embedded (FFPE) tissues to be used for pathogen discovery and Next Generation Sequencing (NGS). These tissues are processed following a standard protocol that utilizes formalin, and then embedded in paraffin blocks. In cancer, the large collection of diseased and normal tissue stored at hospitals has provided an excellent source for molecular genetic studies, as seen in a study by Wilkins et al., (2018). Cimino et al., (2014) detected two distinct Herpesvirus sequences through NGS that were derived from gliomas preserved in FFPE tissues. Bodewes et al., (2015) utilized FFPE tissues for the discovery of a novel rotavirus. FFPE tissues were used to reconstruct the 1918 Spanish influenza, and sequences were compared to the modern H1N1 strain. It was observed that the 1918 H1N1 strain was able to replicate in the absence of trypsin, and that the expression of the 1918 virus genes conferred the unique high-virulence phenotype observed with the pandemic virus (Tumpey et al., 2005). Schweiger et al., (2009) analyzed normal and tumor breast tissues from archived FFPE tissues using Illumina sequencing to detect genomic variations in samples.

Davidson's fixed paraffin embedded tissues

Routine screening of animals for a panel of diseases including TS is needed to bio-secure a production facility. As a part of the disease surveillance program, live shrimp are collected from a hatchery or a grow-out pond and preserved for shipment. Those who work in shrimp pathology face an additional challenge when working with archival tissue from shrimp because the tissues are not fixed in the traditional 10% buffered formalin. These shrimp tissue samples are fixed in Davidson's fixative (with a pH of approximately 3.5-4) that contains acetic acid to soften the

shrimp exoskeleton for successful future microtome sectioning. Upon receiving the samples in the laboratory, shrimp tissues are processed following a standard tissue processing protocol (Bell and Lightner, 1992) and then embedded in paraffin blocks.

Using Davidson Fixed Paraffin Embedded (DFPE) tissues is a durable, efficient, and cost-effective means of archiving tissues for histopathological analyses and disease screening. The University of Arizona's Aquaculture Pathology Laboratory has a biobank of DFPE samples dating from 1991, from initial TSV outbreaks to 2019. These samples represent a priceless biobank of molecular information and are often the only tissues available for retrospective studies. The use of FFPE and DFPE tissues gives researchers access to unutilized resources for retrospective evolutionary studies.

Rationale

Despite the ease and convenience of DFPE tissue storage, the recovery of nucleic acids from shrimp tissue specimens is particularly challenging. The fixative process leads to the cross linking between DNA/RNA and proteins and contributes to the fragmentation of both DNA and RNA. The use of Davidson's fixative presents an additional obstacle due to the acid hydrolysis that further contributes to nucleic acid degradation. These changes interfere with many classical molecular analyses requiring high quality nucleic acids (Hasson et al., 1997, Andrade et al., 2008). As a result, high quality viral RNA has not yet been extracted from Davidson's Fixed Paraffin Embedded Shrimp Tissues.

The utilization of archived DFPE shrimp tissues will allow researchers to perform important retrospective evolutionary studies, as well as pathogen detection and discovery. Results from this study could indicate that these tissue archives represent an important untapped resource in the aquaculture industry. Using DFPE tissues for future studies has direct implications in disease management, including understanding pathogen-host evolution in shrimp lines, pinpointing mutations relating to pathogen virulence, and could aid in preventing large economic losses in the future.

Hypothesis and objectives

Hypothesis

Archived Davidson's fixed paraffin embedded shrimp tissues can be utilized for pathogen detection and phylogenetic analysis, using TSV as a model.

Objectives

1. **Evaluate total RNA isolation methods using archived TSV-infected DFPE tissues.** To test objective one, three commercially available RNA extraction kits will be utilized. Mean 260/280, 260/230, and concentration values will be compared.
2. **Evaluate the feasibility of detecting TSV using RNA isolated from archived DFPE tissues.** TSV will be detected via RT-PCR. Overlapping primers were designed to cover the VP1 capsid protein gene in ORF2 of TSV.
3. **Determine the genetic relationship of TSV isolates using viral capsid protein VP1-gene sequence.** The VP1-gene is a variable region in the TSV genome and is therefore a good candidate for building a phylogenetic tree from the samples selected for this study.

A schematic representation of the workflow of the detection and phylogenetic analysis of Taura syndrome virus from Davidson's fixed paraffin embedded tissue samples can be seen in Figure 1.4.

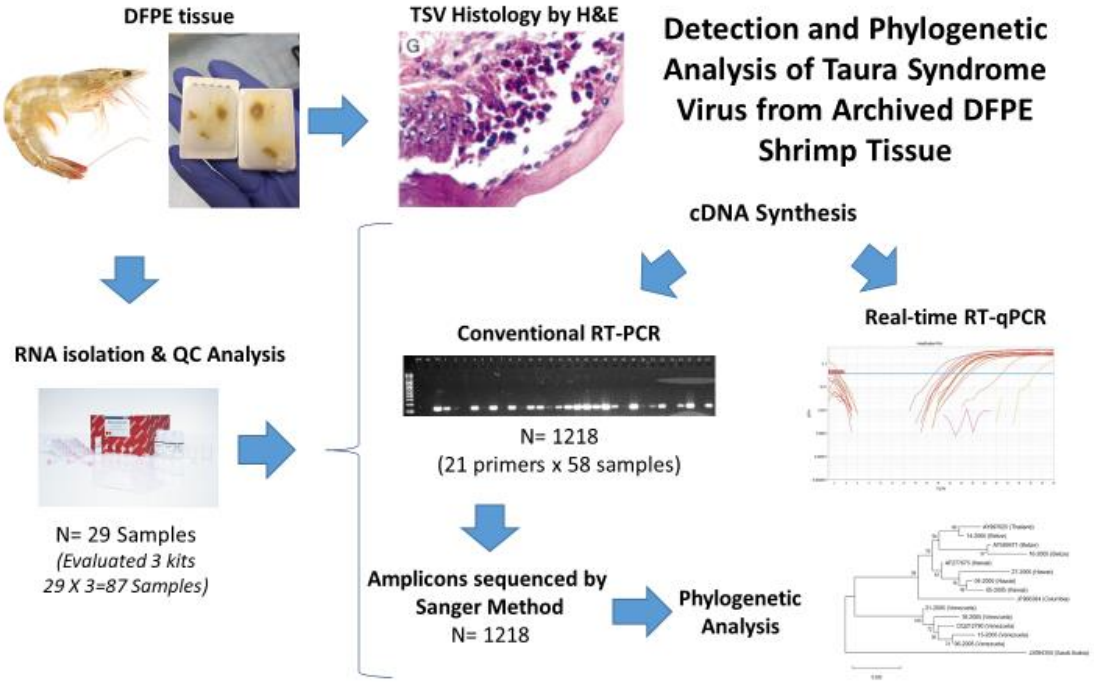

Figure 1.4. A schematic representation of the workflow in detection and phylogenetic analysis of Taura syndrome virus from Davidson's fixed paraffin embedded tissue samples. First, a histological analysis was conducted to select TSV infected DFPE shrimp tissue samples from 2005. Next, a comparison between three commercially available RNA extraction kits was completed to evaluate total RNA quality and quantity. cDNA was synthesized and TSV was readily detected by reverse-transcriptase polymerase chain reaction (RT-PCR) and reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR). Sanger sequencing of the amplicons confirmed the identity of TSV, and a phylogenetic analysis was performed using capsid protein gene, VP1.

CHAPTER 2:

Detection and phylogenetic analyses of Taura syndrome virus from archived Davidson´s fixed paraffin embedded shrimp tissue

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Abstract

Taura syndrome is an OIE-listed disease of marine shrimp that has caused more than US $2 billion losses since 1992. The disease is caused by Taura syndrome virus (TSV), a single-stranded RNA virus with ~10 kb genome. This study demonstrates the utility of using 15-year-old archived Davidson's fixed paraffin embedded (DFPE) shrimp tissues for TSV detection and phylogenetic studies. Total RNA was isolated from known TSV-infected DFPE tissues (N=29) using three commercially available kits and, the purity and ability to detect TSV in the isolated RNA were compared. Qiagen RNeasy FFPE Kit provided the highest quality RNA as determined by the 260/230 and 260/280 ratios and lowest mean Ct value obtained while detecting TSV by real-time RT-PCR. . TSV was successfully detected through conventional RT-PCR. Among the TSV-specific primers screened through RT-PCR, primer pair TSV-20 for the RdRp gene, primer pairs TSV-15 and TSV-16 primers for the VP2 gene, and primer pair TSV-5 for the VP1 gene amplified the highest number of samples, 20, 28, and 25, respectively. In contrast, EF-1α gene was amplified from 28 out of 29 samples. To assess the phylogenetic relation among different strains of TSV, the VP1-gene was amplified in 14 overlapping segments and the amplicons were sequenced. A consensus sequence was generated from smaller fragments were taken for phylogenetic analyses. The results showed that the TSV isolates from this study generally clustered with homologous isolates from the corresponding geographical regions for which nucleotide sequences were retrieved from the GenBank database. This supports the authenticity of the nucleotide sequence generated from DFPE tissues. These results show that archived DFPE shrimp tissues can be amplified for pathogen detection and retrospective genetic analyses.

Key words: Taura syndrome virus, TSV, DFPE Tissue, shrimp.

Introduction

Taura syndrome (TS), caused by Taura syndrome virus (TSV), is a disease of Penaeid shrimp that is estimated to have caused between USD 1.2-2 billion worth of damage in the Americas during its widespread panzootic between 1992 and 1996 (Hasson et al., 1995; Lightner et al., 1995; Tu et al., 1999; Dhar et al., 2004). Taura syndrome is an OIE-listed disease that has been detected on five continents (Wertheim et al. 2009). This pathogen is a non-enveloped single-stranded RNA (~10 kb genome) containing virus classified as a member of the family *Dicistroviridae*, Genus *Aparavirus* (ICTV, 2012). The detection of shrimp pathogens, such as TSV, is critical for controlling, predicting, and preventing potential outbreaks and large economic losses.

In the study of shrimp diseases, Davidson's fixed paraffin embedded (DFPE) tissues are considered prized biological samples for histological analysis. These samples are often the only tissues available for retrospective studies, yet there is no work that has been done with the detection of viral pathogens from archived DFPE shrimp tissues. Despite the ease and convenience of DFPE tissue storage, the recovery of nucleic acids from shrimp tissue specimens is particularly challenging. The fixative process leads to the cross-linking between DNA/RNA and proteins and contributes to the fragmentation of both DNA and RNA (Bodewes et al., 2015). When working with archival DFPE shrimp tissue, there is an additional challenge because shrimp tissues are fixed in Davidson's fixative instead of formalin, the fixative used for FFPE tissues. Davidson's fixative contains acetic acid to soften the shrimp exoskeleton which further contributes to nucleic acid degradation through acid hydrolysis. These changes interfere with many classical molecular analyses requiring high quality nucleic acids. The negative effects of Davidson's fixative on virus detection by *in situ* hybridization using non-radiolabeled probe are discussed while detecting TSV (Hasson et al., 1997), and infectious myonecrosis virus (IMNV) in shrimp (Andrade et al., 2008). These studies highlight the challenges when working with DFPE shrimp tissues.

Formalin fixed paraffin embedded (FFPE) tissues are similar to DFPE tissues but are fixed in formalin instead of Davidson's fixative. Although there are challenges when working with both FFPE and DFPE tissues, researchers have recently extracted nucleic acids from FFPE tissues for pathogen detection while studying human and animal diseases. For example, researchers utilized RNA isolated from FFPE tissues and next generation sequencing (NGS) to discover a novel strain of rotavirus (Bodewes et al., 2015). FFPE tissues were recently used to reconstruct the 1918 Spanish influenza and compare the viral sequences with a recent H1N1 strain to identify the unique high-virulence phenotype observed with the pandemic virus (Tumpey et al., 2005). In human cancer studies, a large collection of diseased and normal tissue stored at hospitals has provided an excellent source for molecular genetic studies (Wilkins et al., 2018). While FFPE tissues are now widely used for disease research in human medicine, similar studies are non-existent in the aquaculture realm including shrimp aquaculture. In shrimp disease research, there is only one published study that utilized DFPE tissue for pathogen detection. In this study, the researchers utilized DFPE tissue derived DNA for the detection of Necrotizing Hepatopancreatitis (NHP) disease caused by a bacterium *Hepatobacter penaei* B (NHP-B) by PCR (Brinez et al., 2003).

However, there are no published reports of utilizing DFPE tissue for viral disease detection in shrimp.

In the present study, the utility of DFPE tissue in pathogen detection was evaluated using TSV as a proof-of-concept (POC) study. Twenty-nine known TSV-infected DFPE tissues representing different geographical locations were utilized to isolate total RNA. TSV was successfully detected through real-time RT-PCR. A series of primers designed to target the non-structural and structural genes provided successful detection of TSV by conventional PCR. Phylogenetic analyses using capsid protein gene (VP1) sequences shows the genetic relatedness of TSV isolates from different geographical region to corresponding isolates for which sequences were available in the GenBank database. The data presented here clearly established the utility of DFPE tissues as biological resource for pathogen detection and evolutionary studies. To our knowledge, this is the first report of a virus detection and genetic analyses of viruses infecting shrimp using archived DFPE tissue.

Materials and Methods

Samples. A histological analysis of archived DFPE tissue blocks of known TSV cases from 2005 was performed. The samples that presented TSV infection with severity level as Grades 3 to 4 were selected for total RNA extraction (n=29). The severity of the TSV infection was graded based on a semi-quantitative scale that ranges from Grade 0 to Grade 4 (Lightner, 1996). Grade 3 TSV infection shows moderate to high signs of disease shown by number and severity of pathogen caused lesions and Grade 4 TSV infection shows high numbers of pathogen caused lesions and tissue destruction. A microtome was used to cut three sections from archived DFPE tissue, 20 microns in thickness, from each sample. For the purpose of this study, ‘case’ and ‘sample’ may be used interchangeably. A summary of samples taken for RNA isolation and PCR amplification is shown in Table 2.1.

Table 2.1. Archived DFPE tissue samples selected for analyses. The cases (n=29) are displayed with their corresponding sample numbers from each of the three extraction kits (Invitrogen PureLink FFPE RNA Isolation Kit, Norgen Biotek FFPE RNA Purification Kit, and Qiagen RNeasy FFPE Kit), place of origin, the year each case was received, shrimp species, and grade of infection (from G1 to G4, G4 representing the highest level of TSV infection).

Case #	Norgen Kit Sample #	Qiagen Kit Sample #	Invitrogen Kit Sample #	Year	Origin	Species	Grade of Infection
1	1	58	73	2005	Belize	*L. vannamei*	G3
2	2	59	74	2005	Venezuela	*L. vannamei*	G3-4
3	3	60	75	2005	Thailand	*P. monodon*	G3-4
4	4	61	76	2005	Hawaii	*L. vannamei*	G2-4
5	5	62	77	2005	Hawaii	*L. vannamei*	G3-4
6	6	63	78	2005	Venezuela	*L. vannamei*	G3
7	7	64	79	2005	Thailand	*P. monodon*	G3
8	8	65	80	2005	Belize	*L. vannamei*	G3
9	9	66	81	2005	Hawaii	*L. vannamei*	G3-4
10	10	67	82	2005	Venezuela	*L. vannamei*	G3-4
11	11	68	83	2005	Belize	*L. vannamei*	G4
12	12	69	84	2005	Thailand	*L. vannamei*	G4
13	13	70	85	2005	Belize	*L. vannamei*	G4
14	14	71	86	2005	Belize	*L. vannamei*	G3-4
15	15	72	87	2005	Venezuela	*L. vannamei*	G3-4
16	44	30	16	2005	Belize	*L. vannamei*	G4
17	45	31	17	2005	Thailand	*L. vannamei*	G4
18	46	32	18	2005	Venezuela	*L. vannamei*	G3-4
19	47	33	19	2005	Belize	*L. vannamei*	G4
20	48	34	20	2005	Thailand	*L. vannamei*	G4
21	49	35	21	2005	Venezuela	*L. vannamei*	G3
22	50	36	22	2005	Hawaii	*L. vannamei*	G3-4
23	51	37	23	2005	Hawaii	*L. vannamei*	G3-4
24	52	38	24	2005	Venezuela	*L. vannamei*	G2-4
25	53	39	25	2005	Hawaii	*L. vannamei*	G3
26	54	43	26	2005	Thailand	*L. vannamei*	G3
27	55	42	27	2005	Hawaii	*L. vannamei*	G3
28	56	40	28	2005	Thailand	*P. monodon*	G3-4
29	57	41	29	2005	Thailand	*P. monodon*	G3-4

RNA extraction and quality assessment. Total RNA extraction was performed using three commercially available kits, Invitrogen PureLink FFPE RNA Isolation Kit (Invitrogen), Norgen Biotek FFPE RNA Purification Kit (Norgen Biotek), Qiagen RNeasy FFPE Kit (Qiagen) in accordance with the manufacturer's recommendations. To assess total RNA quantity and quality, 1.0 µl of RNA were utilized in triplicate to obtain a mean value for RNA concentration, 260/280, and 260/230 ratios using a NanoDrop™ 2000 All reactions were performed in a sterile environment, and the isolated RNA were stored at -20°C until further use.

Detection of TSV through real-time RT-PCR (RT-qPCR). RNA samples were heated to 90±2°C then immediately quenched. Real-time reverse transcription-polymerase chain reaction (RT-qPCR) were performed in accordance with Tang et al., (2004) for the detection of TSV. A reaction mixture was prepared consisting of 2.5 µl of TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems™), 1 µl forward and reverse primers (5 µM each), 0.5 µl TaqMan probe (2 µM), 2 µl RNA and 4 µl of HPLC grade water in a final reaction volume of 10 µl. TSV primers 1004F (5'- TTG GGC ACC CGA CAT T- 3') and 1075R (5'- GGG AGC TTA AACTGG ACA CAC TGT -3') and TSV probe (FAM-CAG CAC TGA CGC ACA ATA TTC GAG CAT C-TAMARA) were used to amplify a 72 bp amplicon. These primers can be seen in Table 2.2. RT-qPCR was performed with an Applied Biosystems Step One Plus® real-time PCR machine. Thermocycling conditions consisted of a reverse transcription step at 48°C for 5 min, a denaturation step at 95°C for 20 s, followed by an amplification step at 40 cycles of 95°C for 1 s and 60°C for 20 s. Each sample was run in triplicate, and a TSV-positive control, a negative control representing RNA isolated from a specific pathogen free (SPF) shrimp, and no template control were used for all RT-qPCR detection.

Statistical analysis. Statistical analysis of mean Ct Values was performed using the Kruskal-Wallis non-parametric test with SPSS v16.0 software. The result of the analysis is presented in Table 2.4.

Detection of eukaryotic translation elongation factor 1 alpha (EF-1α) through real time RT-PCR. Elongation factor 1-α (EF-1α) was selected for use as an internal control gene (Dhar et al., 2010) to determine if inhibitory substances are present in RNA isolated from DFPE tissue. The mRNA expression EF-1α was measured by quantitative RT-PCR using PowerUp™ SYBR® Green Master Mix (Applied Biosystems™). The cDNA synthesis was performed using Tetro cDNA Synthesis Kit (Bioline Meridian BIOSCIENCE) following the manufacturer's protocol. Briefly, two µl of total RNA from each sample (n=29) were combined with a reaction mixture comprised of 1 µl random hexamer, 4 µl 10 µM dNTP, 1 µl 5x RT Buffer, 1 µl RiboSafe RNase Inhibitor, 1 µl Tetro Reverse Transcriptase, and 12 µl DEPC-treated water in a final reaction volume of 20 µl. The cDNA was diluted to 50 ng/µl. The RT-qPCR was performed with an Applied Biosystems Step One Plus® real-time PCR machine. The EF-1α primers 123F (5'-TCGCCGAACTGCTGACCAAGA-3') and 123R (5'- CCGGCTTCCAGTTCCTTACC-3') were used to generate a 55 bp amplicon as described by Dhar et al., (2009). The RT-PCR mixture contained 7 µl of PowerUp™ SYBR® Green Master Mix 1 µl of each of forward and reverse primer (10 µM) and 1µl of cDNA. The Applied Biosystems Step One Plus® program consisted of an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for

10 s, annealing for 30 s at 55°C, and extension for 30 s at 72°C. Following amplification, the melt curve analysis was performed. The reaction temperature was increased to 95 °C for 15s, then decreased to 60 °C for 1 min, and increased to 95 °C at a rate 0.3 °C per second with a continuous fluorescence monitoring. Each sample was run in triplicate in a 96 well plate, and the mean Ct value as used for further analysis as seen in Table 2.4.

TSV primer design. TSV-specific primers for conventional RT-PCR were designed using Geneious Prime 2019.2.1 (Biomatters) and the TSV reference genome (GenBank accession: NC_003005). Each set of forward and reverse primers were designed to amplify between 100-150 bp overlapping genome segments. Fourteen sets of primers were designed to amplify the VP1 gene in ORF2 from 7901-9176 nt (Reference TSV genome: NC_003005) to use for phylogenetic analysis. To amplify more conserved regions of the TSV genome, three sets of primers were designed to amplify a portion of the VP2 gene in ORF2 6948-7252 nt 9176 (Reference TSV genome: NC_003005), and four sets of primers were also designed to amplify portions of the RNA-dependent RNA polymerase gene in ORF1 from 5194-6610 nt (Reference TSV genome: NC_003005). A summary of these primers is presented in Table 2.2. Each sample was screened with all primers designed, including the OIE-recommended primers.

Table 2.2. A summary of TSV-specific primers. Primers for this study were designed based on the TSV reference genome (NC_003005) to amplify TSV using RNA isolated from DFPE tissue. The OIE-recommended primers to amplify TSV through RT-PCR and RT-qPCR are listed for reference.

Primer name	Location in TSV genome	Nucleotide position in the genome of TSV Reference Strain	Sequence (5' – 3')	Amplicon size (bp)
TSV 1-F	VP1	7901	GGCGTAGTGAGTAATGTAGCT	137
TSV 1-R	VP1		AGAGACAGGGGTACGCCATA	
TSV 2-F	VP1	7955	ACGAAAGTCAACGCATATGAGA	123
TSV 2-R	VP1		AGGCACTGCAATTGTGGGAT	
TSV 3-F	VP1	8057	GATCCCACAATTGCAGTGCC	125
TSV 3-R	VP1		AGAGACAGGGGTACGCCATA	
TSV 4-F	VP1	8155	TGACACTCCTGATGCGCATG	122
TSV 4-R	VP1		CTAGACTAACTGGGGCAGCG	
TSV 5-F	VP1	8257	CGCTGCCCCAGTTAGTCTAG	122
TSV 5-R	VP1		AGGGGAGATATTGCACCAGC	
TSV 6-F	VP1	8359	GCTGGTGCAATATCTCCCCT	150
TSV 6-R	VP1		GGATCGTACACTCGCATCCA	
TSV 7-F	VP1	8414	TCACAGATCATCGACATCTCACA	124
TSV 7-R	VP1		CACAATCTGCCGTGTACCCA	
TSV 8-F	VP1	8518	TGGGTACACGGCAGATTGTG	145

TSV 8-R	VP1		AAGCGTACCTGGTTCAGCAA	
TSV 9-F	VP1	8643	TTGCTGAACCAGGTACGCTT	149
TSV 9-R	VP1		TTCCCCCAAAGACACCTTCG	
TSV 10-F	VP1	8721	CAGTAACGCGTGCTCCAGTA	147
TSV 10-R	VP1		GCAGTCCGGCATAAGCTAGT	
TSV 11-F	VP1	8834	GGTGGAAGGCACAGACTAGC	121
TSV 11-R	VP1		CAAGAGTTGGAGCGCTGGTA	
TSV 12-F	VP1	8935	TACCAGCGCTCCAACTCTTG	150
TSV 12-R	VP1		TCACCAATCGCTGCCATACT	
TSV 13-F	VP1	9031	TGGTATTTCCGAGGAGACGT	137
TSV 13-R	VP1		TCACTGGAGCTTTGGACTCA	
TSV 14-F	VP1	9071	GCAGCGATTGGTGAAGCTAC	127
TSV 14-R	VP1		TGACCACGGTATAGTTACCTGG	
TSV 15-F	VP2	6948	TGCCTGCTAACCCAGTTGAA	55
TSV 15-R	VP2		AGTCCTCCACTGGTTGTTGT	
TSV 16-F	VP2	7117	AGTCCAGGACCAAGCTCTCA	119
TSV 16-R	VP2		CTGTTGCAAGCTGTTCCTGC	
TSV 17-F	VP2	7233	CAGAATTCAATCAGCCACAC	131
TSV 17-R	VP2		TACTCGTACAGTAACCTCGT	
TSV 18-F	RdRp	5194	CAATGGCCATTGGTTCCGTT	111
TSV 18-R	RdRp		TATACAAGGTAGCGGGGGCT	
TSV 19-F	RdRp	5576	GTGGTTGGGCTCTGAGGAAT	117
TSV 19-R	RdRp		GCCGCAAAAATACCCAAGCT	
TSV 20-F	RdRp	6099	AACCATTCTCAGCCTTCCGG	105
TSV 20-R	RdRp		CCCGTTTTCTCGCTGAGCTA	
TSV 21-F	RdRp	6529	AAACAACGCGCATTGCTTCT	101
TSV 21-R	RdRp		GTACCCTGCGTTCCTACACG	
TSV 9195F*	Intergenic region/ORF 2	9195	TCAATGAGAGCTTGGTCC	231
TSV 9992R*	Intergenic region/ORF 2		AAGTAGACAGCCGCGCTT	
TSV 1004F**	ORF1	1004	-TTGGGCACCCGACATT	72
TSV 1075R**	ORF1		GGGAGCTTAAACTGGACACACTGT	

*OIE-recommended primers (OIE, 2014)

**Primers used for RT-qPCR

Amplification of TSV by RT-PCR and amplicon sequencing. Total RNA isolated from Norgen Biotek FFPE RNA Purification Kit and Qiagen RNeasy FFPE Kit were selected to carry out RT-PCR due to the superior performance of these two kits in respect to RNA quality (see Result section, below). Complementary DNA (cDNA) was synthesized, and utilized for RT-PCR amplification of TSV VP1, VP2, and RdRp genes. A total of 1218 samples were tested form TSV detection via RT-PCR for this study. Amplicons were generated by 21 sets of primers used to amplify 29 samples each, from Norgen Biotek FFPE RNA Purification Kit and Qiagen RNeasy FFPE Kit (21 primers x 29 samples x 2 extraction kits = 1218 total amplicons). For RT-PCR amplification, 1 µl of each DNA sample was combined with 1 µl forward and reverse primers (5 µM each), and 12.5 µl of DreamTaq™ Hot Start Green PCR Master Mix (ThermoFisher) in a 25 µl reaction volume. Thermocycling conditions consisted of 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, then 72°C for 5 minutes. A TSV-positive control, a negative control representing RNA isolated from a specific pathogen free (SPF) shrimp, and no template control were used for all RT-PCR amplification. A housekeeping gene, EF-1α was used as an internal control. The amplicons were electrophoresed in a 1.5% agarose gel for 60 minutes at 80 volts and were visualized on a GelDoc™ XR+ (Bio-Rad). RT-PCR amplicons were sequenced using the Sanger sequencing method (University of Arizona Genetics Core) and Geneious Prime 2019.2.1 was used to trim and align sequences to the TSV reference genome (NC_003005). Nucleotide and amino acid sequence identity was determined using the BLAST of NCBI.

Isolate selection for phylogenetic analysis. An additional analysis of the amplification pattern of all isolates was performed. We selected 12 representative isolates (4, 5, 6, 8, 10, 14, 15, 16, 17 ,18, 21 and 27) because each of them produced amplification using primers TSV1, TSV12, TSV13 and TSV14. A pictograph that shows the amplification patter of these isolates is shown in Figure 2.1.

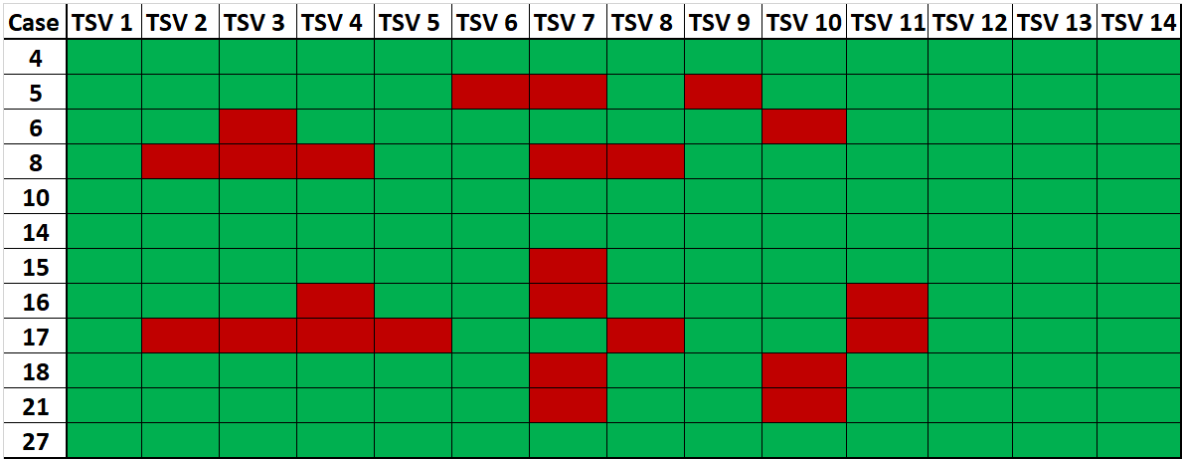

Figure 2.1. **A pictograph of RT-PCR amplification of the TSV VP1 gene for 12 representative isolates.** Each isolate included in the pictograph amplified using primer pairs TSV1, TSV12, TSV13 and TSV14. The primers pairs (TSV-1 to TSV-14) used to amplify the TSV VP1 gene are shown on the top row. The case numbers are indicated in the far-left column. Green boxes indicate amplification and red boxes indicate no amplification for the corresponding primers.

Phylogenetic analysis. Because not every case yielded the same amplification results, only samples that amplified the selected four primer pairs (out of 14) were chosen for analysis. 12 out of 29 representative samples from different geographical regions (04-2005, 05-2005, and 27-2005) from Hawaii; (06-2005, 10-2005, 15-2005, 18-2005, and 21-2005) from Venezuela; (08-2005, 14-2005, and 16-2005) from Belize; and (17-2005) from Thailand were used to generate concatenated consensus sequences of the VP1 gene. The concatenated consensus sequences of the VP1 gene were compared with the homologous concatenated gene sequence of 29 TSV isolates from varying geographical locations that are available in GenBank. Isolates selected from GenBank are summarized in Table 2.3. A multiple alignment was performed using the Geneious Prime program and the CLUSTALw Plugin using the 12 samples from this study and the 29 isolates acquired from GenBank. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 150 positions in the final dataset (Figure 2.6). Phylogenetic analysis was conducted using MEGA X 10.0.5. A neighbor-joining phylogenetic tree (Tamura et al., 2004) was constructed using the multiple alignment, and a bootstrap consensus was inferred from 1000 replicates. The results are shown in Figure 2.7.

Table 2.3. GenBank accessions utilized for a phylogenetic analysis of the TSV VP1 gene.

GenBank Accession Number	Country of Origin	Year of Isolation	Host Species
FJ876481	Belize	2001	*P. vannamei*
FJ876490	Belize	2004	*P. vannamei*
AY590471	Belize	2005	*P. vannamei*
FJ876507	Nicaragua	2005	*P. vannamei*
FJ876516	Nicaragua	2006	*P. vannamei*
FJ876520	Taiwan	2007	*P. vannamei*
FJ876487	Indonesia	2003	*P. vannamei*
FJ876509	China	2005	*P. vannamei*
AY997025	Thailand	2005	*P. vannamei*
GQ359322	Texas, USA	2010	*P. vannamei*
GQ502201	Texas, USA	2004	*P. vannamei*
FJ876492	Texas, USA	2009	*P. vannamei*
FJ876469	Hawaii	1995	*P. vannamei*
AF277675	Hawaii	1994	*P. vannamei*
FJ876468	Hawaii	1995	*P. vannamei*
FJ876466	Ecuador	1994	*P. stylirostris*
FJ876461	Ecuador	1993	*P. vannamei*
FJ876513	Ecuador	2006	*P. vannamei*
FJ876512	Ecuador	2006	*P. vannamei*
FJ7876493	Mexico	2004	*P. vannamei*
AF510515	Mexico	2002	*P. vannamei*
DQ000302	Eritrea	2006	*P. monodon*
FJ876495	Eritrea	2004	*P. monodon*
JN194143	Colombia	2006	*P. vannamei*
JN194141	Colombia	2006	*P. vannamei*
FJ876508	Aruba	2005	*P. vannamei*
FJ876511	Aruba	2006	*P. vannamei*
FJ876503	Venezuela	2005	*P. vannamei*
DQ212790	Venezuela	2005	*P. vannamei*
FJ876502	Venezuela	2005	*P. vannamei*
JQ356858	Saudi Arabia	2010	*P. indicus*
JX094350	Saudi Arabia	2011	*P. indicus*

Results

RNA Quantity and Quality Assessment. Data obtained from the NanoDrop™ 2000 show that the Invitrogen PureLink FFPE RNA Isolation Kit yielded the highest mean concentration value of 355.30 ng/µl for total RNA, while Qiagen RNeasy FFPE Kit yielded the lowest mean concentration value of 249.20. The Qiagen RNeasy FFPE Kit yielded the best mean 260/280 (1.91) and 260/230 (2.00) ratio values, when compared to the two other extraction kits. A 260/280 ratio value of 2.0 is generally accepted as "pure" for RNA, and a 260/230 ratio value of 2.0-2.2 is generally accepted as "pure" for nucleic acids. Lower ratio values may indicate a higher presence of contaminants such as phenols or proteins. A summary of this data is provided in Figure 2.2 and Table 2.4.

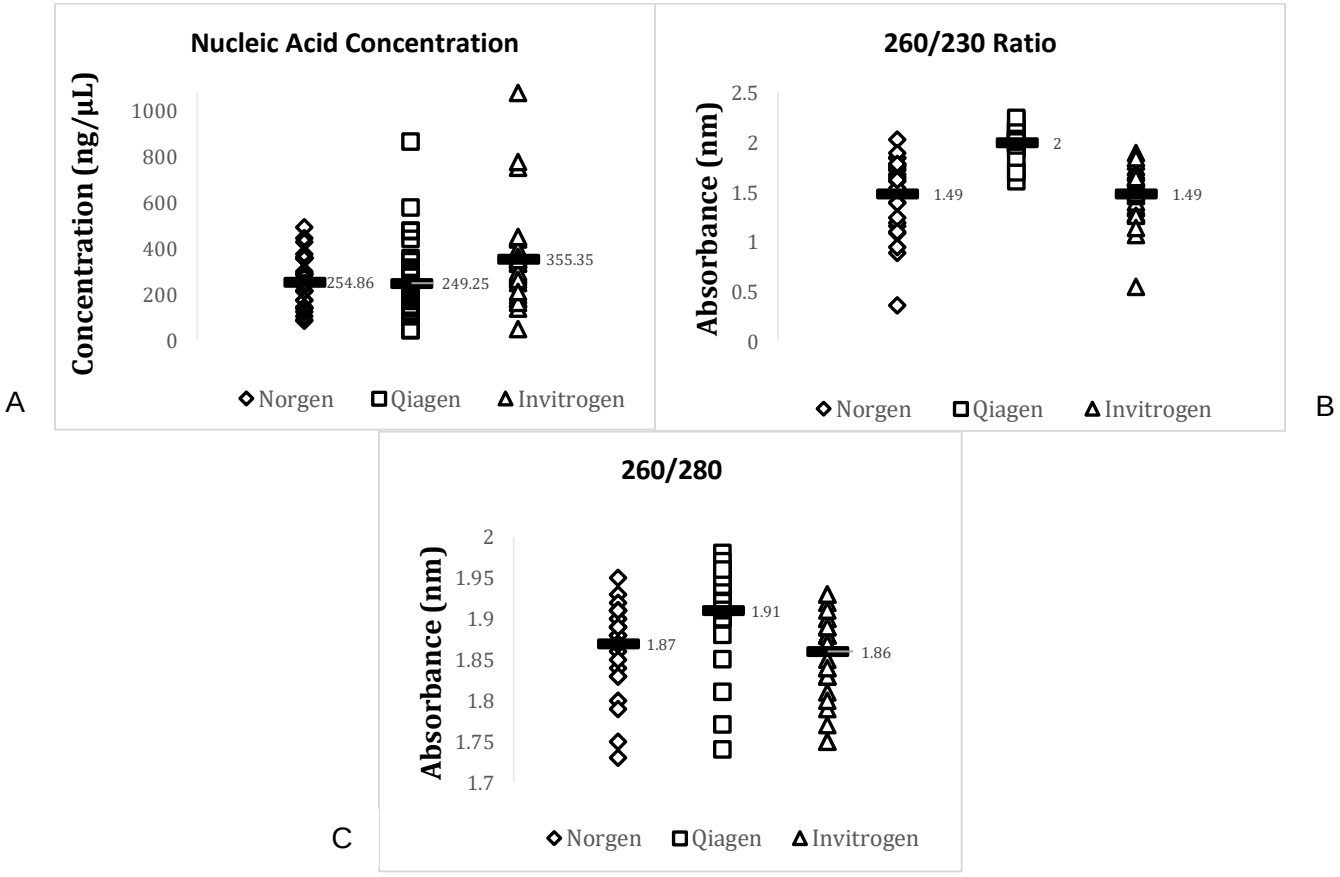

Figure 2.2. Quantity and quality assessment of total RNA isolated from archived TSV-infected DFPE shrimp tissues. Each test was run in triplicate and a mean value for each data set was obtained. Figure 2.2A shows a comparison of the mean nucleic acid concentrations for 29 samples from each extraction kit. Figure 2.2B shows a comparison of the mean 260/230 ratio values for 29 samples from each extraction kit. Figure 2.2C shows a comparison of the mean 260/280 ratio values for 29 samples from each extraction kit. The horizontal line in each group represents a geometric mean of the data in the corresponding vertical data set.

Detection of TSV using RT-qPCR. A 72 bp cDNA amplicon in TSV ORF1 was amplified by TaqMan real-time RT-PCR following an OIE-recommended protocol. RT-qPCR results show that both Qiagen RNeasy FFPE Kit and Norgen Biotek FFPE RNA Purification Kit yielded RNA that provided significantly lower Ct values than RNA isolated using an Invitrogen PureLink FFPE RNA Isolation Kit. While all 29 samples were successfully amplified using RNA isolated using Qiagen RNeasy FFPE Kit and Norgen Biotek FFPE RNA Purification Kit, two out of 29 samples did not provide TSV amplification when RNA isolated using the Invitrogen PureLink FFPE RNA Isolation Kit was used. A summary of this data is provided in Figure 2.3 and Table 2.4.

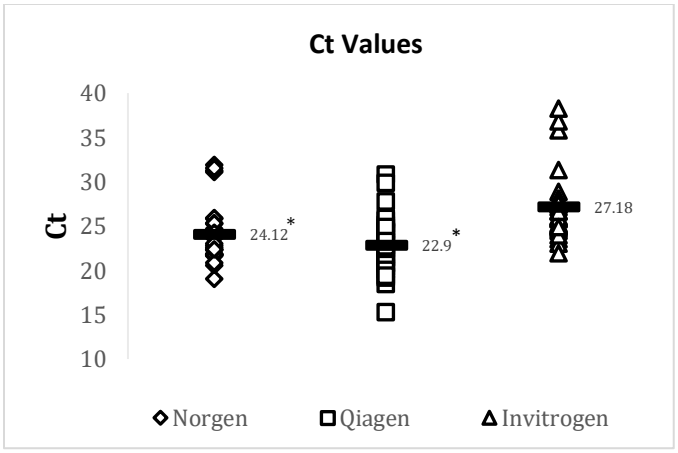

Figure 2.3. Analysis of the Ct values of TSV amplicons generated by real-time RT-PCR. Three commercially available extraction kits were utilized for total RNA extraction, and total RNA isolated from archived TSV-infected DFPE shrimp tissues was used to generate a 72 bp amplicon of the TSV genome. The horizontal line in each group represents a geometric mean of the Ct values of the corresponding vertical data set. Asterisks represent statistical significance ($p<0.05$).

Table 2.4. A comparison of quantity, quality, and Ct values of total RNA isolated with three RNA extraction kits. RNA Concentrations (ng/µl), 260/280 and 260/230 values, and Ct values for TSV and EF-1α gene obtained by real-time RT-PCR are summarized. The mean value and standard deviation in each category are shown. An asterisk (*) indicates statistically significant difference ($p<0.05$).

Extraction Kit	Mean RNA Concentration (ng/µl)	Mean 260/280 ratio	Mean 260/230 ratio	Mean Ct value (TSV)	Mean Ct value (EF-1α)
Norgen Biotek FFPE RNA Purification Kit	254.90 ± 109.5	1.87 ± 0.05	1.49 ± 0.34	24.12 ± 3.4*	28.25 ± 2.3
Qiagen RNeasy FFPE Kit	249.20 ± 179.5	1.91 ± 0.06	2.00 ± 0.14	22.90 ± 3.3*	28.49 ± 1.6
Invitrogen PureLink FFPE RNA Isolation Kit	355.30 ± 207.8	1.86 ± 0.05	1.49 ± 0.27	27.18 ± 3.9	28.68 ± 1.7

Detection of TSV capsid protein genes, VP, VP2, RNA-dependent-RNA polymerase (RdRp), and internal control gene EF-1α by conventional RT-PCR. TSV capsid protein genes VP1 and VP2, TSV RdRp gene, and the internal control gene EF-1α were successfully amplified by conventional RT-PCR using RNA isolated from DFPE tissue and following a Qiagen RNeasy FFPE Kit protocol. However, the number of samples amplified varied depending on target gene and primers used to amplify the corresponding genes. A summary of the percentages of successful amplification of TSV genes using each set of primers is presented in Table 2.5. The internal control gene EF-1α was successfully amplified in all but one case (Sample No. 25), which could be due to low concentration (43.4 ng/µl) and low 260/280 (1.74) and low 260/230 ratios (1.51) for this sample. Subsequently, this sample (Sample No. 25) was excluded from the RT-PCR data analysis. Representative gel images of amplified segments of TSV VP1, VP2 and RdRp genes, and the internal control gene, EF-1α can be seen in Figures 2.4A, 2.4B, 2.4, and 2.4D. Samples 4, 10, 14, and 27 (Figure 2.5) provided successful amplification of all selected regions of the genome. A summary of these results can be seen in Table 2.5. Among three TSV genes targeted for amplification, the VP2 gene in ORF2 was the most successfully amplified gene among the samples tested (28 out of 28 samples amplified by using primer pairs TSV-15 and TSV-16).This was followed by the TSV VP1 gene (25 out of 28 samples amplified using the primer set TSV-5) and RdRp gene (20 out of 28 samples amplified using the primer set TSV-20). Finally, only four out of 28 samples were successfully amplified with the OIE-recommended primers (Table 2.6). Figure 2.5 is a pictograph that represents the entire amplification data obtained from the RT-PCR

amplification of the different segments of the TSV genome. The RT-PCR data revealed that successful amplification occurred when the amplicon size ranges between 100 and 150 bp, and when the amplicon size is increased over 150 bp, the efficiency of amplification is reduced.

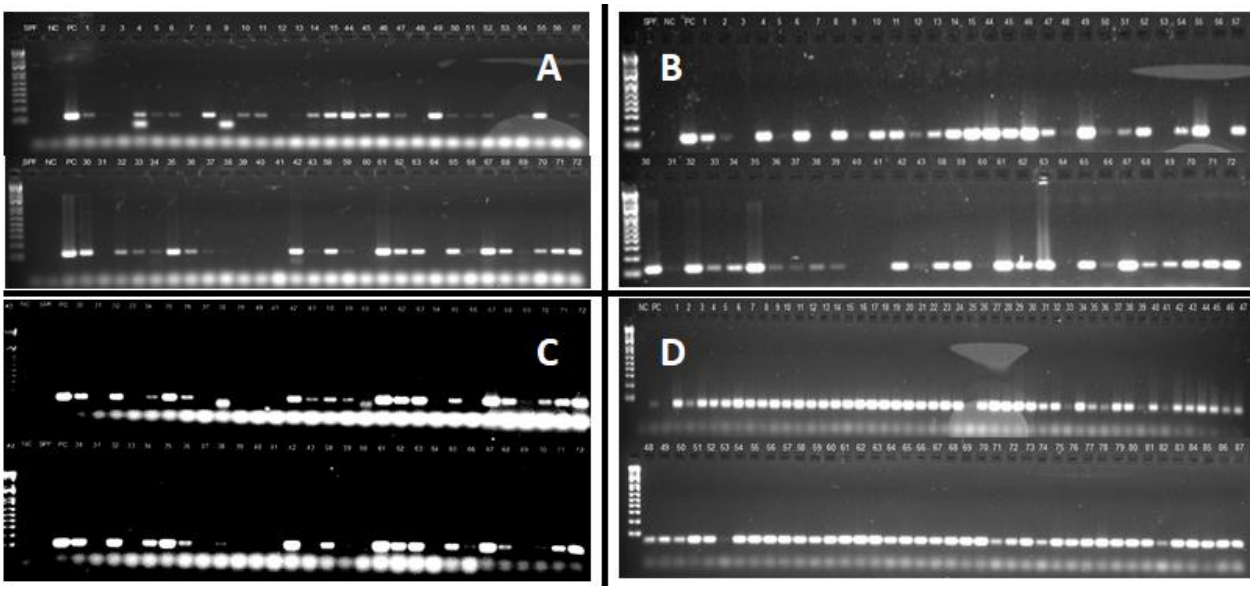

Figure 2.4. Representative agarose gel electrophoresis images for the detection of TSV genes and the internal control gene EF-1α. Pictured above are agarose gel images RT-PCR products from different regions of the TSV genome (A, B, and C) and EF-1α (D). A. TSV VP1 gene (Primer pairs TSV-5, amplicon size 122 bp). , B. TSV VP2 gene (Primer pair TSV-16, amplicon size 119 bp), C. TSV RdRp gene (Primer pair TSV-20, amplicon size 105 bp), and D. Shrimp EF-1α gene. SPF = Specific Pathogen Free negative control, NC = no template control, and PC = TSV positive control.

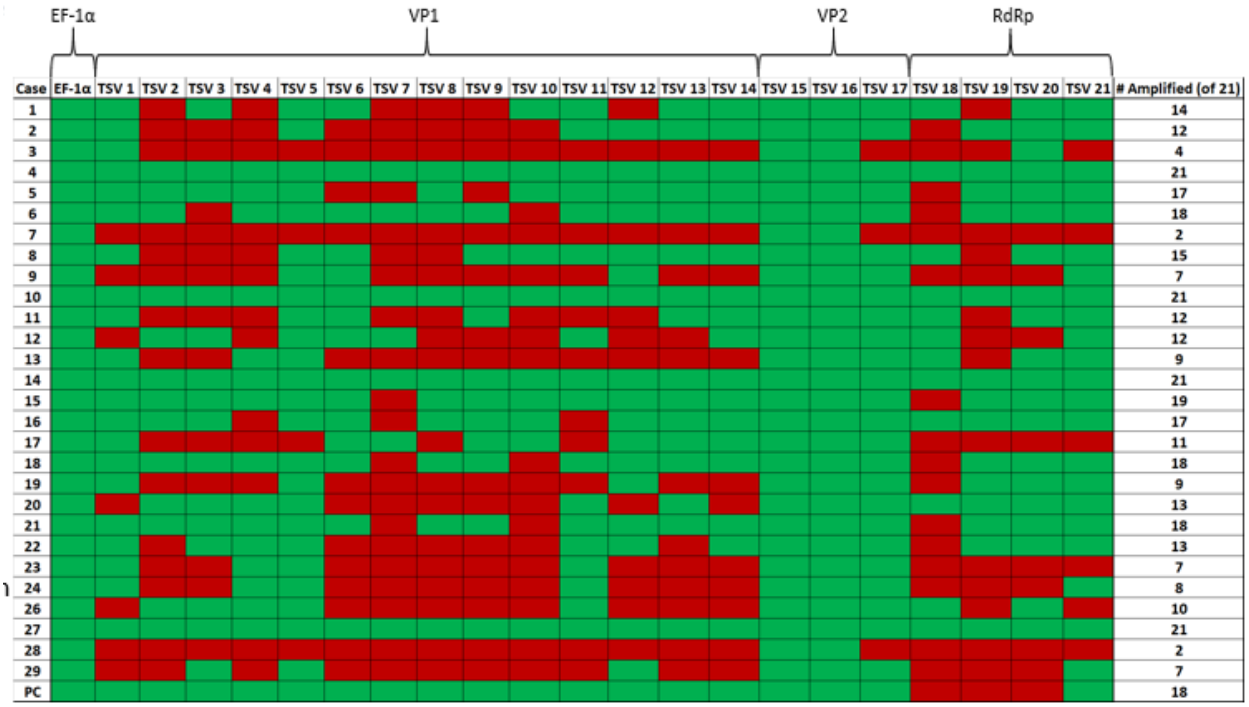

Figure 2.5. A pictograph of RT-PCR amplification of TSV genes VP1, VP2, RdRp and shrimp internal control gene EF-1α. The primer pairs (TSV-1 to TSV-21) used to amplify different TSV genes are shown on top row of the map. The case numbers are indicated in the far-left column (excluding Sample No. 25, as mentioned previously) and total number of primer pairs (out of 21) that were successful in amplifying each sample can be seen in the far-right column. Green boxes indicate amplification and red boxes indicate no amplification for the corresponding primers.

Table 2.5. A comparison of the percent of successful amplifications for different regions of the TSV genome. Each sample number is listed with the corresponding percent of successful amplifications for each region of the TSV genome: VP1 gene (out of 14 total amplicons), VP2 gene (out of three total amplicons), RdRp (out of four total amplicons). Total percentage of all 21 possible amplifications is listed in the far-right column. Sample No. 25 is excluded from data.

Sample #	% VP1 gene	% VP2 gene	% RdRp gene	Total % amplified
1	57	100	75	67
2	43	100	75	57
3	07	67	25	14
4	100	100	100	100
5	79	100	75	81
6	86	100	75	86
7	0	67	0	10
8	64	100	75	57
9	21	100	25	33
10	100	100	100	100
11	43	100	75	57
12	50	100	50	57
13	21	100	75	38
14	100	100	100	100
15	93	100	75	90
16	71	100	100	81
17	57	100	0	52
18	86	100	75	86
19	21	100	75	43
20	43	100	100	62
21	86	100	75	86
22	50	100	75	62
23	29	100	0	33
24	29	100	25	38
26	36	100	50	48
27	100	100	100	100
28	0	67	0	10
29	21	100	25	33

Table 2.6. A comparison of the percent of successful amplifications provided by each TSV-specific primer pair. Each primer pair (EF-1α, TSV-1 through TSV-21, and TSV 9195F/9992R) are listed with the corresponding target gene and percent of samples that were successfully amplified (out of 28). Sample No. 25 is excluded from data.

Primer Pair	Target gene	Total % Amplified
EF-1α	EF-1α	100
TSV-1	VP1	71
TSV-2	VP1	46
TSV-3	VP1	54
TSV-4	VP1	54
TSV-5	VP1	89
TSV-6	VP1	54
TSV-7	VP1	21
TSV-8	VP1	36
TSV-9	VP1	43
TSV-10	VP1	32
TSV-11	VP1	64
TSV-12	VP1	57
TSV-13	VP1	57
TSV-14	VP1	61
TSV-15	VP2	100
TSV-16	VP2	100
TSV-17	VP2	89
TSV-18	RdRp	57
TSV-19	RdRp	39
TSV-20	RdRp	71
TSV-21	RdRp	79
TSV 9195F/9992R	ORF1/2 Intergenic region	11

Phylogenetic analysis. A neighbor-joining phylogenetic tree constructed using the VP1 nucleotide sequence shows that Venezuela isolates 06-2005, 15-2005, 18-2005, and 21-2005 form a well supported South American cluster with GenBnk isolates from Venezuela, Aruba, and Ecuador. GenBank isolates from Taiwan, China, Indonesia, Thailand and Texas, USA form the Asian cluster. Hawaiin isolates 04-2005, 05-2005, and 27-2005 from the present study cluster with GenBank isolates from Hawaii and Ecuador to form the Hawaii/Ecuador cluster. Eritrea isolates forma a separate well supported Eritrea cluster. Thailand isolate 17-2005 clusters with GenBank isolates from Nicaragua and Belize to form the Central American cluster. Interestingly, isolates 14-2005 and 10-2005 form a separate, relatively well supported cluster. The ancestral GenBank isolate from Ecuador from 1993 stands alone. The alignment used to generate the phylogenetic tree can be seen in Figure 2.6.

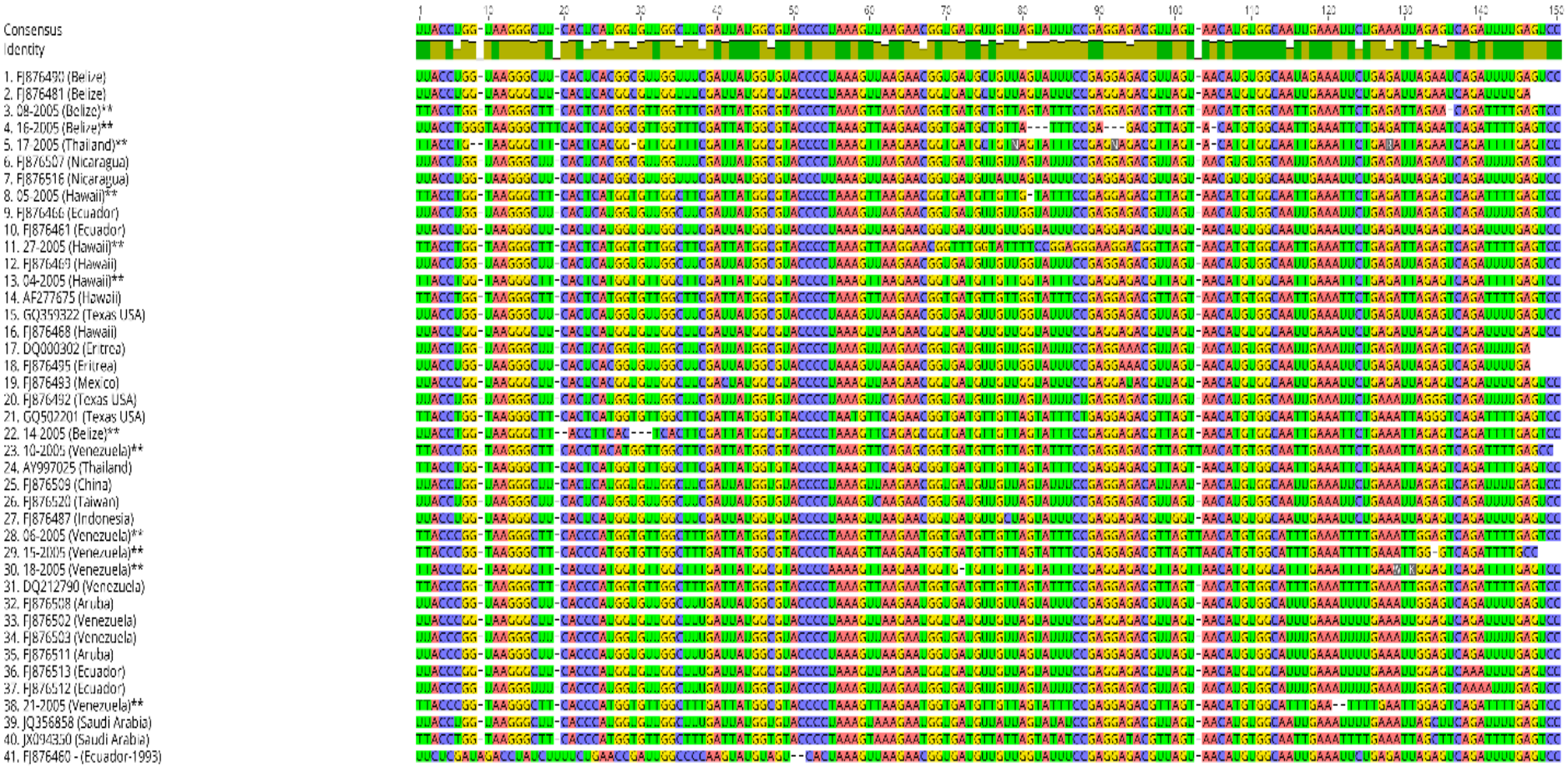

Figure 2.6. Alignment of 41 concatenated TSV sequences. The sequences displayed were obtained from isolates from the present study (Figure 2.1) and GenBank (Table 2.3)

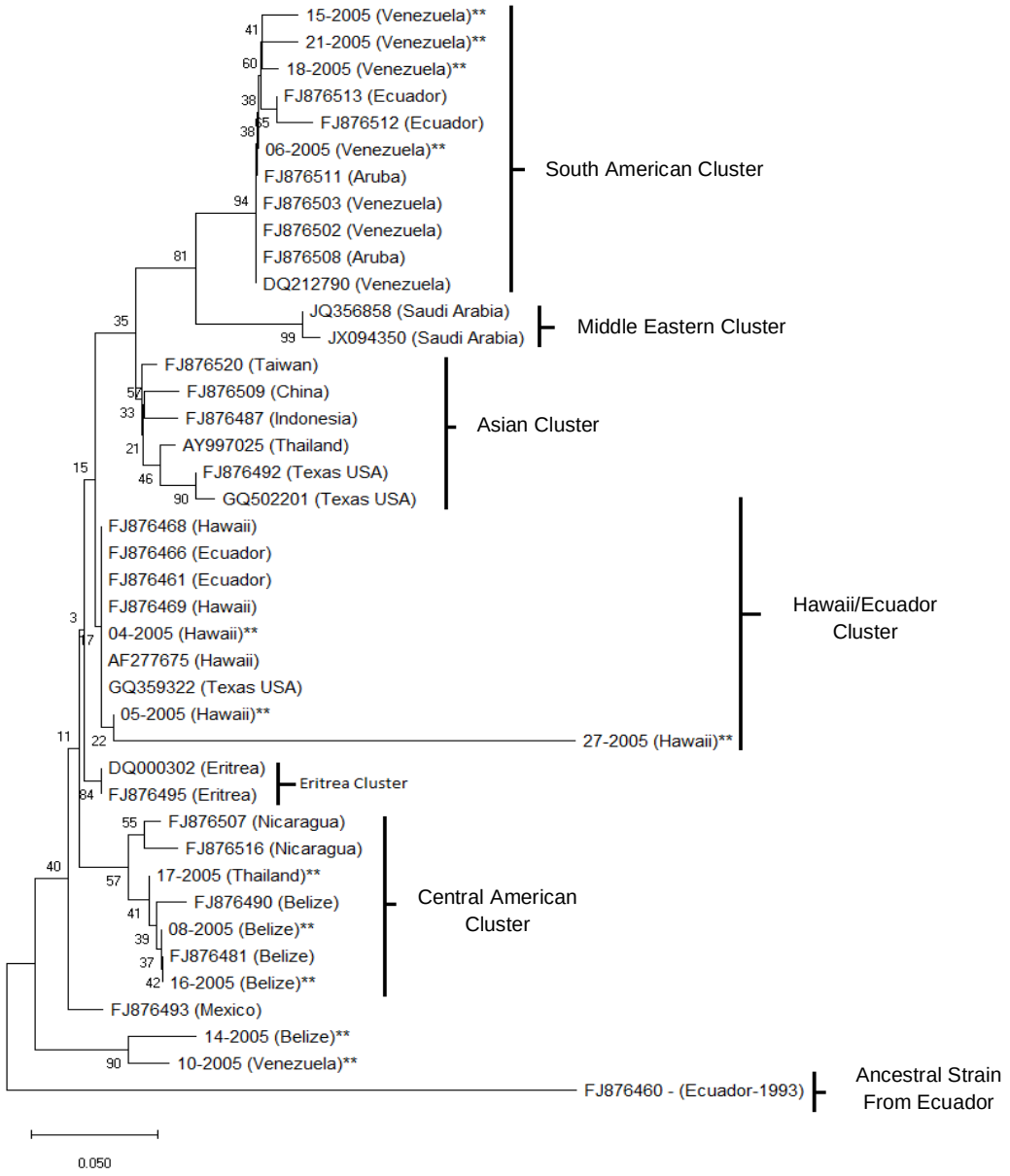

Figure 2.7. Phylogenetic analysis of 41 TSV isolates. The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 0.87629497 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [3] and are in the units of the number of base substitutions per site. This analysis included 12 samples from the present study (denoted by ** on the tree) and 29 GenBank accessions (as seen in Table 2.3).

Discussion

In the aquaculture industry, detection of infectious diseases in a timely manner is critical for predicting, preventing, and controlling potential outbreaks and large economic losses. Davidson's fixed paraffin embedded tissues are often the only samples available for retrospective studies for determining the origin, evolution, and spread of pathogens across countries and continents. While DFPE tissues can be extremely valuable in addressing these questions, retrieving genetic information from DFPE histological samples has presented significant challenges in pathogen discovery and genetic studies in humans and animals, and these sorts of studies have not been attempted in shrimp disease research. This is the first study that demonstrates the utility of using archived DFPE histological blocks for shrimp RNA viral pathogen detection, using TSV as model.

Three different commercially available kits were used to isolate total RNA from DFPE shrimp tissue to detect TSV. Although the Invitrogen PureLink FFPE RNA Isolation Kit yielded the highest mean concentration, Qiagen RNeasy FFPE Kit provided RNA that yielded lowest mean Ct value while detecting TSV by TaqMan real-time RT-PCR.. Total RNA isolated using a Qiagen RNeasy FFPE Kit had the best 260/280 and 260/230 values of 1.91 and 2.0, respectively. These data indicate that the RNA obtained with Qiagen RNeasy FFPE Kit was purer than the RNA obtained with the other FFPE RNA isolation kits.

While the amplicon size for TaqMan real-time RT-PCR was 72 bp, the size range of the amplicons in conventional RT-PCR was between 100-231 bp. The results show that the smaller amplicons (100- 150 bp) were most successfully detected through RT-PCR, and amplicon sizes greater than 150bp, including a 231 bp amplicon that was amplified using an OIE-recommended protocol only amplified four samples (OIE 2019). This is most likely due to chemical modification, cross-linking of RNA and proteins, and RNA fragmentation that is seen when extracting nucleic acids from DFPE tissues.

The RT-PCR results show that amplicons originating from the VP2 gene in the TSV genome were the most successful, while amplicons originating from the VP1 gene were the least successful. This is likely because VP2 gene is a more conserved region than VP1 gene region (Wertheim et al. 2009). According to Wertheim et al. 2009, the TSV nucleotide substitution rate in the VP1 region is approximately 2.37×10^{-3} substitution/site/year. It is also interesting to note that primers designed to amplify the RdRp region of the TSV genome were not as successful as the VP2 gene region in terms of total percent of amplified samples. This could be due to fragmentation or chemical modification in the RdRp region during the fixative process, or to the mutations in this region where the TSV-specific primers were intended to bind. Based on the overall amplification success in 28 DFPE tissue samples detected via RT-PCR, it can be concluded that primers designed based on the VP2 gene with an amplicon size less than 150 bp will be optimal for detecting TSV from archived DFPE tissues.

The VP1-gene was selected for phylogenetic analysis because it is a highly variable region of the genome (Lightner, 2005). The phylogenetic analysis presented here is similar in structure to the last published TSV phylogenetic study conducted by Aranguren et al., (2013). A South American cluster, consisting of isolates from Venezuela, Ecuador, and Aruba, is also consistent with the structure shown by Aranguren et al., (2013). The Hawaii/Ecuador cluster formed by isolates from Hawaii and Ecuador is observed in the present study and in the study by Aranguren et al., (2013). A Central American and Asian cluster can also be observed. GenBank isolates from Texas, USA

cluster with the Asian isolates, which is consistent with the structure observed by Dhar et al., (2010). A separate Middle Eastern cluster, consisting of two Saudi Arabian isolates, is similar to the structure shown by Tang et al., (2012).

It is important to note that two of the isolates in this study are observed in different clusters than what was expected (17-2005 from Thailand and 10-2005 from Venezuela). Dhar et al., (2010) carried out the genetic characterization of a TSV isolate affecting cultured shrimp population in Texas. The Texas isolates were more closely related to the isolates from China and Thailand than to the Hawaii isolate. As mentioned previously in the introduction of the thesis, the movement of infected shrimp is the main cause for the spread of disease in shrimp aquaculture (Lightner et al., 2012). We hypothesize that while the 17-2005 isolate from Thailand lies within the Central American cluster, and the 10-2005 isolate from Venezuela forms a sub-cluster within the Central American cluster with isolate 14-2005 from Belize, these TSV isolates could have been introduced from a distinct geographical locations like Thailand or Venezuela.

It is fundamental to point out that for the phylogenetic analysis, the concatenated consensus sequences are 150 nt in length. It must be clarified that sequences less than 800 nt in length are not desirable for phylogenetic analysis due to the limited information that these shorter sequences can yield (Dwivedi and Gadagkar, 2009). However, in our study, even though sequence length was not optimal, the overall topology of the phylogenetic tree showed resemblance to previous studies. To obtain higher support for the distinct clusters, new primers could be designed to amplify and sequence the regions not used in this study; new data could be subsequently incorporated in the phylogenetic analysis.

The results from the present study demonstrate that both health assessment and targeted pathogen screening in shrimp can be done using DFPE tissue samples that are routinely used for histological analyses. The feasibility to detect viral pathogens from archived DFPE blocks opens unlimited possibilities for retrospective studies and the discovery of novel pathogens. These applications have direct implications in disease management in shrimp aquaculture, and the potential to be applied to pathogens which affect other aquaculture species.

Conclusion

Shrimp diseases caused by viral pathogens remain a major threat to the long-term sustainability of the shrimp aquaculture industry. Taura Syndrome disease, caused by TSV, has caused up to $2 billion USD globally. Although SPF shrimp and TSV-resistant lines have been introduced to prevent the impact of economic losses caused by TS, the disease still represents a threat to the shrimp aquaculture industry. This is why TS remains as an OIE-listed crustacean disease. The detection and molecular analysis of TSV is essential for the prevention and control of potential outbreaks caused by TS worldwide.

This project describes the development of a method for the isolation of RNA from archived DFPE shrimp tissue for the detection and characterization of a shrimp virus, TSV. Three different commercially available extraction kits were evaluated, and one of these kits provided high-quality RNA which was conducive for the detection of TSV by conventional and real-time PCR. Subsequently, the entire TSV capsid protein gene VP1 was amplified. Phylogenetic analysis using an alignment constructed from the VP1-gene Sanger sequencing results revealed that the isolates selected for this study were similar to the homologous isolates from similar geographical regions for which gene sequences are available in GenBank. These findings supported the claim that high quality RNA can be extracted from archived DFPE tissues and used for pathogen detection and phylogenetic analysis.

The University of Arizona Aquaculture Pathology Laboratory has a collection of archived DFPE tissues that extend decades into the past. Among these DFPE tissues, there are case reports of samples from the late 1980’s from Latin America with reports of unknown disease etiology that align with the clinical and gross signs of TSV infection. Following the protocol presented in this

study, it could now be possible to perform total RNA isolation and detect TSV from these archived DFPE tissues. If TSV can be successfully detected, these samples would predate the reported origin of TSV in Ecuador in 1991.

RNA viruses like TSV exist as quasi-species within the host due to high mutation rates. These quasi-species are generated through mutations that occur during the replication of the virus in infected cells (Andino and Domingo, 2016). Through the utilization of archived DFPE tissues, it is possible to retrospectively detect these quasi-species within the same host or between shrimp hosts from different geographical regions. This could help determine which TSV quasi-species are more abundant or lethal between shrimp species. For example, prior to 1999, the *P. stylirosis* shrimp species was considered to be TSV-resistant (Brock et al., 1995). A new TSV strain emerged in Mexico and caused major economic losses in farmed *P. stylirosis* (Robles-Sikisaka et al., 2002). With the ability to utilize DFPE tissues for nucleic acid isolation, it is now possible to amplify TSV from the affected *P. stylirosis* during this outbreak in order to gain a better understanding of the evolution of the virus over time.

Detection and pathogen characterization from archived DFPE tissue has never been performed for any viral pathogen in shrimp aquaculture. Therefore, this work represents a novel method of detection and genetic analysis of pathogens from archived shrimp tissues. The results obtained from the present study indicates that archived DFPE tissues can be utilized for the detection and genetic characterization of shrimp viruses. These findings can be extended for the detection and characterization of other shrimp viruses and potentially other pathogens of aquaculture species, which is critical for controlling, predicting, and preventing potential outbreaks and large economic losses.

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