Assessment of Cell Cultures to Propagate Crustacean Viruses

Thesis submitted by

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ABSTRACT

Decapod crustaceans (including crabs, shrimp, lobsters, and crayfish) can play host to a wide range of pathogens and parasites. Approximately 60% of current disease-associated losses in shrimp aquaculture may be due to viral pathogens with a further 20% loss due to bacterial pathogens. Global economic losses due to disease within shrimp culture between 2010 and 2017, was counted for > US$ 11billion.

Despite research in crustacean hosts being focused on the use of a combination of traditional (e.g. histopathology, transmission electron microscopy) and modern (molecular) approaches, the underlying mechanisms of host infection are still poorly understood. Furthermore, a better understanding of the viral diseases has been hindered by the lack of permanent *in vitro* models for propagating the viruses of crustacean species.

Due to the lack of continuous cell lines for crustacean organs and tissues, this study attempted to assess alternative cell lines or cell cultures to propagate crustacean viruses. The first investigation involved assessing mosquito cell lines to evaluate the susceptibility and the propagation of Penaeus merguiensis hepandensovirus (PmeHDV). This was conducted by *in vitro* propagation of PmeHDV in *Aedes albopictus* (C6/36) cell lines (Chapter 4). The methodologies used involved cytology assays to visualize cellular changes and PCR to confirm PmeHDV prrsence. PCR of the serial passages of the infected C6/36 cell line indicated no propagation for PmeHDV and the TaqMan quantitative PCR confirmed that the average number of viral copies declined over the serial passages of C6/36. The results indicated that C6/36 cell line allows the initial stage of PmeHDV replication, but the virus was incapable of using C6/36 for patent replication of its' virions.

The second investigation involved by transfecting three putative nuclear location signals (NLSs)-introduced via plasmid DNA (pDNA) of PmeHDV into Vero cells using a lipofection reagent to determine if the three putative NLSs were functioning (Chapter 5). Originally, it was planned to use the C6/36 cell line which, from the literature, should have supported the growth of PmeHDV, but it was proved that C6/36 was unsuitable for PmeHDV' growth. The approaches involved transfection of non-NLSs inserted pDNA and NLSs-inserted pDNA into Vero cell lines. The Vero cells were observed post-transfection using a combination of filter sets and emission/excitation light frequencies. The microscopic analyses suggested that the NLSs are not functioning well but were probably linked to importin-beta-1 as the proteins were blocked at the nuclear membrane of Vero cells not frequently entering the nucleus.

Nevertheless, the results indicating small noticeable differences in the outer nuclei within transfected-Vero cells with the experimental NLSs genes.

Due to unsuccessful propagation of PmeHDV in C6/36 (Chapter 4),coupled with the lack of sensitivity of Vero cell lines to identify the location of NLSs of PmeHDV (Chapter 5), an alternate cell line and virus were examined. Therefore, the third investigation experimentally exposed mouse macrophage interferon reporter cells a.k.a. RAW-Blue™ Interferon Stimulated Gene (ISG) to two recently described RNA viruses include *Chequa iflavirus* and athtab bunyavirus from red-claw crayfish (*Cherax quadricarinatus*). The experiment (Chapter 6) involved *in vitro* propagation of both viruses into RAW-Blue™ cell lines and confirmation of the virus's infection by histological observation and reverse transciptase (RT)-PCR. Haematoxylin-eosin (HE) staining failed to show the signs of vacuoles (CPE) in cells. Interestingly, RT-PCR results demonstrated that both *Chequa iflavirus* and *Athtab bunyavirus* can infect RAW-Blue™ ISG cells and can maintain their survival in the cells until 7 days post-infection (dpi). Nevertheless, possibly the stronger amplicons during 2 and 4 dpi, showed amplification of *Athtab bunyavirus* due to the virus immediate early (IE) gene expression, RNA-dependent RNA polymerase on which the RT-PCR is was based turned on mRNA of the viral genome, but mRNA of RNA polymerase expression shuts down after 4 dpi and when other genes are then transcribed.

Overall, research to study the interaction between crustacean viruses and their host cells is widely open as RAW-Blue™ ISG paved the way to the *in vitro* growth of *Chequa iflavirus* and *Athtab bunyavirus* and to the future assessment of their putative NLSs.

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LIST OF ABBREVIATIONS

aa	amino acid
A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
α	alpha
BLAST	basic local alignment search tool
bp	base pair
CPE	cytopathic effect
DAPI	4′, 6-diamidino-2-phenylindole
DENV	dengue virus
DMEM	dulbecco's minimum essential medium
DNA	deoxyribonucleic acid
DNV	densovirus
dpi	day post infection
dpt	day post transfection
ELISA	enzyme-linked immunosorbent assay
Em	emitation
Ex	exitation
F	forward
FITC	fluorescein isothiocyanate
FAO	food and agriculture organization
FBS	foetal bovine serum
FP	fluorescence protein
H & E	haematoxylin and eosin
HPV	hepatopancreatic parvovirus
ICTV	international committee on taxonomy of viruses
IFN	interferon
Imp	importin
IRF	interferon regulatory factor
ISG	interferon stimulated gene
IU	international unit
kDa	kilodalton
kg	kilogram
LC_{50}	Lethal Concentration 50

M	Molar
MEM	minimum essential medium eagle's
mg	milligram (10^{-3} g)
mM	milliMolar
min	minimum or minute
mRNA	messenger ribonucleic acid
ng	nanogram (10^{-9} g)
NLS	nuclear location signal
nm	nanometer
NPC	nuclear pore complex
NS1	non-structural protein1
NS2	non-structural proten2
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
pDNA	plasmid DNA
PmeHDV	penaeus merguiensis hepandensovirus
PmoHDV	penaeus monodon hepandensovirus
qPCR	quantitative PCR
R	reverse
RNA	ribonucleic acid
rpm	revolution per minute
RPMI	roswell park memorial institute-1640
RT-PCR	reverse transcriptase PCR
RT-qPCR	reverse transcriptase quantitative-PCR
Sf9	spodoptera frugiperda pupal ovarian tissue
SV-40T-antigen	simian virus 40 tumour antigen
TC	tissue culture
USD	United State dollar
VP	viral protein
wpi	week post infection
WST-1	water soluble tetrazolium salt
µg	microgram (10^{-6} g)
µl	microlitre

CHAPTER 1
Introduction

The global production of crustaceans including cultured prawn for 2018, according to FAO statistic in 2017, was expected around 8.63 million tonnes (Shinn *et al*., 2018). However, the success of prawn aquaculture is limited by various of diseases. The economic loss from Thailand's prawn production data between 2010–2017, was counted around US$ 11.458 billion. Prawn disease-related losses in Vietnam in 2015 were assumed >US$ 26 million, due to acute hepatopancreatic necrosis disease (AHNPD), while the costs of white spot syndrome virus (WSSV) in the same year were >US$ 11 million (Shinn *et al*., 2018).

Of all viral diseases, parvoviral diseases are emerging as as major threat to penaeid culture due to their ability to cause slow growth and mass mortality of prawns (reviewed by Safeena *et al*., 2012). To date, only two major prawn parvoviruses, namely hepatopancreatic parvovirus (HPV) and infectious hypodermal and haematopoeitic necrosis virus (IHHNV) have been investigated in detail (Bergoin & Tijssen, 2010; Safeena *et al*., 2012).

One viruses of interest at JCU is Penaeus merguiensis densovirus (PmeDNV) or recently named as P. merguiensis hepandensovirus (PmeHDV), which is a variant of Decapod hepandensovirus1 in the genus *Hepandensovirus*, subfamily *Densovirinae*, family *Parvoviridae* (Bergoin & Tijssen, 2010; Cotmore *et al*., 2013). The word hepandensovirus reflects the original name of hepatopancreatic parvovirus (HPV) while the word Decapod refers to its hosts (Cotmore *et al*., 2013). In Australia, PmeHDV can be isolated from *P. merguiensis* (Owens, 1997) and from *P. esculentus* (Paynter, 1985). The research to discover crustacean viruses from Queensland was continued by JCU team. Sakuna *et al*. (2017a) and Sakuna *et al*. (2018) discovered *Chequa iflavirus* and *Athtab bunyavirus* from cultured redclaw crayfish (*Cherax quadricarinatus*).

A growing number of reports on crustacean viral diseases began emerging in the 1980s. However, the underlying mechanisms of host infection are still poorly understood. Better understanding of the viral diseases has been hindered by the lack of cellular models for propagating the viruses (Roekring *et al*., 2006; Claydon *et al*., 2010). To assist this, studies to understand the crustacean viral diseases have been conducted.

Attempts to use alternative cell cultures or cell lines, such as fish cells (Loh *et al*., 1990); mammalian cells (Audelo-del-Valle *et al*., 2003; Luo *et al*., 2004; Hayakijkosol & Owens, 2011); insect cells (Sudhakaran *et al.*, 2007; Hayakijkosol & Owens, 2013; Madan *et al*., 2013; Hanapi *et al*., 2017) to propagate or to study prawn viruses have been reported.

However, the success of previous approaches is still open to question. Of all attempts, the successful propagation of Taura Syndrome Virus (TSV) in a mammalian cell line (Vero) was reported by Audelo-del-Valle *et al*., (2003), whereas the only successful propagation of hepandensovirus (HDV) in insect cell line (C6/36) was reported by Madan *et al*. (2013). Trying different cell lines or adapting different crustacean viruses may be necessary to successfully replicate the viruses in cell lines and understand the mechanisms involved during the virus infection and replication.

Viruses within *Parvoviridae* are considered intranuclear for all or most of their replication and assembly. Transportation and viral synthesis are absolutely required during the replication. Specific proteins called importins (Imp) types α/β or β alone mediate the transportation of the viral proteins into the nuclei. Upon the binding of nuclear location signals (NLSs) to the Imp, viral proteins are transported in or out of the nuclei (Jans *et al*., 2000).

NLSs themselves are most often stretches of sequences of the basic amino acids lysine (K) and arginine (R) and can be preceded by helix-breaking neutral amino acids, proline (P), glutamine (Q) or glycine (G) and less commonly by the negatively charged aspartic acid (D) and glutamic acid (E) (Cokol *et al*., 2000). NLSs are recognised by importin types α/β or β (Lam *et al*., 1999; Forwood *et al*., 2001; Lam *et al*., 2002; Forwood & Jans, 2002).

Owens (2013) provided evidence that P. merguiensis hepandensovirus (PmeHDV) (DQ458781) has putative NLSs in the ORFs (Table 1.1).

Table 1.1. Putative NLSs in P. merguiensis hepandensovirus (PmeHDV).

Coding domains, ORF	Putative NLS sequences
NS2, ORF1	^{181}**KR**DQQKT(-)EKKDDEPPKKKTK
NS1, ORF2	^{534}**KK**NPELTQFVLASMQYVHSNYMDKPD**RK**F**K**
VP1, ORF3	^{809}**PKKKKK**Y**R**

Bold letters indicate conserved basic residue; Amino acids (aa) in red could be disruptive (modified from Owens, 2013).

These various studies presented above represent initial steps towards the strategies of using alternative cells to crustacean cells to propagate crustacean viruses and provide useful

information on the putative NLSs of penaeid viruses that needs to be experimentally tested. Based on previous efforts of examining different cell cultures to propagate crustacean viruses and supported by the reports of Madan *et al*. (2013) about the successful propagation of Indian strain of hepandensovirus or P. monodon hepandensovirus (PmoHDV) in C6/36 cell culture, this present study was initially designed to (1) repeat the replication of P. merguiensis hepandensovirus (PmeHDV) in C6/36 cell culture.

In previous studies, cell transfection and fluorescent analyses have been widely used to demonstrate the mechanisms of NLSs during viral infection. Examples include the detection of Periplanata fuliginosa densovirus (PfDNV) NS1 in P. fuliginosa haemocytes, S2, and C6/36 cell cultures (Zhou et al., 2009) and Macrobrachium rosenbergii nodavirus capsid protein (MrNVc) in Sf9 cell culture (Hanapi et al., 2017). Vero cells are also have been reported to highly unlikely to refuse transfection because it lacks signalling proteins (interferons) that are produced and released in response to the presence of several viruses (Desmyter *et al*., 1968; Chen *et al*., 2018). Therefore, this present study was continued to evaluate the functioning putative NLSs of PmeHDV (Owens, 2013) by transfecting of putative PmeHDV NLSs-inserted plasmids into C6/36 if PmeHDV can grow in C6/36 or using mammalian cells (Vero) as alternative cell cultures.

More information on alternative cell cultures to propagate crustacean viruses was collected by examining the susceptibility of a mammalian cell culture (mouse macrophage RAW-Blue™ ISG) to *Chequa iflavirus* and *Athtab bunyavirus*. The mouse RAW-Blue™ ISG have been widely used to identify the presence of virus infection and replication and it was proven to demonstrate high efficiency of virus infection, including murine norovirus infection (Cox *et al.,* 2009; Levenson et al., 2018).

The research presented in this thesis examined these background approaches with the goals of finding potential cell cultures for *in vitro* propagation of crustacean viruses of interest and for experimental examination of putative NLSs of crustacean viruses.

CHAPTER 2
Review of Literature

2.1 INTRODUCTION

2.1.1 Hepandensoviruses: characteristics and classification

Wild caught shrimp post larvae or more commonly now, the culture of larvae spawned by brood stock are the main sources for shrimp aquaculture. Thus, all life stages are involved in the process and are potentially subject to diseases (Behringer, 2012). In response to the situation, efforts on understanding and managing shrimp diseases are being made and for the good reason that the industry is continually affected with huge losses due to pathogens - predominantly viruses (Lightner, 2011). Two invertebrate parvoviruses, within subfamily *Densovirinae* (Bergoin, M. & P. Tijssen. 2000; Fe´die`re, 2000; Yang *et al*., 2012; Cotmore *et al*., 2013), namely infectious hypodermal and haematopoietic necrosis virus (IHHV) and hepatopancreatic parvovirus (HPV) are responsible for some of the disease losses (Lightner, 1996; reviewed by Rai *et al*., 2011 and Safeena *et al*., 2012).

The classification of *Densovirinae* is far from settled. The 6th report by the International Committee on Taxonomy of Viruses (ICTV) described three genera, namely *Densovirus*, *Brevidensovirus*, and *Iteravirus*. An additional genus, *Bidensovirus* is also mentioned in some studies (Tijssen & Bergoin, 1995). Bergoin & Tijssen (2010) have proposed a new genus which is *Hepandensovirus* to group HPVs of prawns which were previously known as prawn densoviruses (DNV). Whereas in the 9th report of ICTV (2011), the *Densovirinae* are divided into four genera, including: *Densovirus*, *Brevidensovirus*, *Iteravirus* and *Pefudendovirus*.

A recent proposal by Cotmore *et al*. (2013) simplified the taxonomy by considering six genera, including *Ambidensovirus, Brevidensovirus, Iteravirus, Pefudensovirus, Penstyldensovirus* and *Hepandensovirus* amongst the *Densovirinae*. The genera *Penstyldensovirus* and *Hepandensovirus* are proposed to accommodate shrimp viruses.

The *Penstyldensovirus*, which was previously listed in genus *Brevidensovirus*, defines a genus of viruses that were formerly known as infectious hypodermal and haematopoietic necrosis virus of Decapoda (Cotmore *et al*., 2013). This genus covers four variants of Decapod penstyldensovirus1*,* namely Penaeus stylirostris penstyldensovirus1 (PstPDV1 (AF273215)); P. monodon penstyldensovirus1 (PmoPDV1 (GQ411199)); P. monodon

penstyldensovirus2 (PmoHDV2 (AY124937))*;* and P. stylirostris penstyldensovirus2 *(*PstPDV2 (GQ475529)) (Cotmore *et al*., 2013).

The *Hepandensovirus* defines a genus of shrimp HPVs (Cotmore *et al*., 2013). This genus covers 7 strains of the species Decapod hepandensovirus1, namely Thai isolate P. monodon hepandensovirus1 (PmoHDV1 (DQ002873)); Tanzanian isolate P. monodon hepandensovirus2 (PmoHDV2 (EU247528)); Madagascan isolate P. monodon hepandensovirus3 (PmoHDV3 (EU588991)); Indian isolate P. monodon hepandensovirus (PmoHDV4 (FJ410797)); Chinese isolate P. chinensis hepandensovirus (PchHDV (AY008257)); Australian isolate P. merguiensis hepandensovirus (PmeHDV (DQ458781)); and South Korean isolate Fenneropenaeus chinensis hepandensovirus (FchHDV (JN082231)) (Cotmore *et al*., 2013).

2.1.2 Structures and genome of hepandensoviruses

Hepandensoviruses are small, isometric, non-enveloped viruses with particle sizes of 20-26 nm that comprise linear, single stranded DNA genome with >6 kb length (Shike *et al*. 2000; Hueffer & Parrish, 2003; Chen *et al*., 2004; Bruemmer *et al*., 2005; Bergoin & Tijssen, 2008; Tattersall, 2008; Safeena *et al*., 2012; Fay & Panté, 2013). The viruses are highly pathogenic to their hosts, causing severe diseases called densonucleosis (Vago *et al*., 1964) and in general, the complete genome of hepandensoviruses are monosense organized and organization of genes from 5’ end of the complimentary strand are in the order of open reading frame1 or ORF1 which encodes non-structural protein2 gene (NS2 gene); ORF2 which encodes NS1 gene; and ORF3 which encodes viral protein gene (VP gene) (reviewed by Safeena *et al*., 2012).

2.1.3 Isolation and potential inhibition of hepandensoviruses

The common technique to isolate and propagate shrimp viruses is through experimental injection of the viruses into the host shrimp. However, several attempts such as using animal models or cell lines have been reported in the latter case to reduce the use of living animals for research purposes. Some insects, such as *Acheta domesticus* (house cricket) and *Tenebrio molitor* (mealworms) have been identified as potential insects for studying P. merguiensis hepandensovirus (PmeHDV) (La Fauce & Owens, 2008). The success of using insect (*Aedes albopictus*) cells or C6/36 cells as an alternative cell to crustacean cells to propagate the Indian strain of P. monodon hepandensovirus4 (PmoHDV4) was also reported by Madan *et al*. (2013).

Like other DNA viruses, hepandensoviruses require living cells to propagate (Li *et al*., 2009). Hepandensoviruses are nuclear replicating viruses and a key element called nuclear pore complex (NPC) of the host cells is believed to be the main entrance mechanism for most nuclear replicating viruses (Cokol *et al*., 2000). The process of targeting and transporting viral genomes from the NPC to the nucleus (Whittaker *et al*., 2000) is assisted by the binding between nuclear location signals (NLSs) and cellular receptors called importins (Imp). Wagstaff *et al*. (2012) suggested that the recognition of nuclear location signals by importins can be interrupted by a broad-spectrum anti-parasite medication called ivermectin. In that study, the drug showed potential antiviral activity towards both HIV-1 and dengue virus, both of which are strongly reliant on importin *α*/*β* nuclear import.

Relevant to the NLSs of shrimp parvoviruses, interestingly, the three open reading frames (ORFs) of Decapod hepandensovirus1 have been suggested by Owens (2013) to carry putative NLSs that need to be confirmed in experimental research. The opportunity for future research using ivermectin as an NLS inhibitor is more promising when an *in vivo* test by Nguyen *et al*. (2014) provided the first evidence that ivermectin potentially blocked a putative parvovirus when the number of hyperthropied nuclei in the group of crayfish (*C. quadricarinatus*) with putative parvoviruses (injected with ivermectin at 7 µg/kg with different frequencies) statistically significantly decreased compared to that of the control group (no ivermectin).

This review will integrate primary knowledge of replication properties among Decapod hepandensovirus1, including the characteristics; the encoded proteins; the terminal sequences; and the putative NLSs based on available references. An overview of ivemectin as a potential NLS drug will also put forward. The study by Wagstaff *et al*. (2012) together with that of Owens (2013) and Nguyen *et al*. (2014) paves the way for the development of inhibition of NLSs of hepandensoviruses.

2.2 Decapod hepandensovirus1 RELATED DISEASES

2.2.1 History of Identifications of the disease symptoms

In the past, hepandensoviruses (aka shrimp HPVs or DNVs) were reported as unusual parvoviruses (Bonami *et al*., 1990; Mari *et al*., 1993). The name of hepatopancreatic parvo-like virus (HPV) derived from the consistency of the cytopathological and morphological performance of the identified virus particles with parvovirus infection in other arthropods and

vertebrates as well as the principal target organs, the hepatopancreas (Lightner & Redman, 1985).

2.2.2 Hosts and geographical distribution

The natural host range of hepandensoviruses encompasses an increasing number of cultured and captured decapods (Lightner & Redman, 1992; Lightner, 1993; Manjanaik *et al*., 2005; Safeena *et al*., 2012). In addition, hepandensovirus-like agent was identified in freshwater prawn (*Macrobrachium rosenbergii*) (Anderson *et al*., 1990; Gangnonngiw *et al*. 2009) and in crayfish (*Cherax quadricarinatus*) (La Fauce & Owens, 2007b) but now this isolate is identified as in the genus *Ambidensovirus* (Bochow *et al*., 2015). HDV was also isolated from the haemolymph of the mud crab (*Scylla serrata*) (Owens *et al*., 2010).

Hepandensoviruses are widely distributed among wild, cultured and reared shrimps worldwide. The geographical distribution includes China, Taiwan, Korea, Philippines, Malaysia, Singapore, Australia, Indonesia, Thailand, Kenya, Israel, Kuwait, and Hawaii, Ecuador, Mexico and Brazil (Lightner & Redman, 1992; Lightner, 1993; Manivannan *et al*., 2002; Flegel, 2006a & b; Safeena *et al*., 2012).

2.2.3 Detection of the diseases

Lightner *et al*. (1993); Flegel *et al*. (1996); and Spann *et al*. (1997) determined that the early larval and postlarval stages (PL) are the stages for the high level of hepandensovirus infection. Mass mortality usually occurs within 4 to 8 weeks. However, hepandensovirus-related mortality is still difficult to record as it sometimes occurs without signs of the disease or it might appear as a co-infection with other pathogens (Flegel 1999; Flegel, 2006 a & b). The infected juveniles of shrimps displayed stunted growth, anorexia, reduced preening activity, increase surface and gill fouling, and occasional opacity of tail musculature (Lightner & Redman, 1985; Safeena *et al*., 2012).

Histological evidence shows necrosis and atrophy of hepatopancreas with large prominent basophilic, dense hypertrophied Fuelgen-positive nuclei in hepatopancreatic, predominantly in the rapidly dividing E-cells of tubule epithelial cells or hepatopancreatocytes (Lightner *et al*., 1983; Lightner, 1996).

Due to the need to differentiate strains of hepandensoviruses from different geographical areas and from different species, nucleic acid based diagnostics as more rapid and non-

destructive molecular tools have been developed to effectively diagnose hepandensoviruses infection in shrimp larvae, broodstock and potential carriers within shrimp aquaculture systems (Safeena *et al*., 2012). Conventional polymerase chain reaction (PCR), real-time PCR, loop-mediated isothermal amplification (LAMP), PCR-enzyme linked immunosorbent assay (PCR-ELISA), probe techniques, *in situ* hybridyzation, dot blot hybridization, and monoclonal antibody (MAb) based techniques are parts of the molecular tools which have been used to study hepandensoviruses (reviewed by Safeena *et al*., 2012).

2.2.4 Pathogenesis of the diseases

The hepandensoviruses enter the cells by pinocytocis, initially attaching to the microvilli before entering the host cell (Mari *et al*.,1995). Following this step, the matured virions in the nucleoplasm accumulate in the main inclusion bodies of their host cells (Pantoja & Lightner, 2001). During the last stage, the accumulation causes disruption of the nuclear membrane and the release of virions into the cytoplasm (Kawase *et al*., 1990). The whole intranuclear inclusion can be liberated into the hepatopancreatic tubule lumen, it is believed that the virus spreads to new host cells (Pantoja & Lightner, 2001).

2.3 STRAINS of HEPANDENSOVIRUSES

At present, 10 strains of hepandensoviruses have been described (Safeena *et al*., 2012). Of the known strains, 7 strains have been grouped as Decapod hepandensovirus1. The new term of species will be used to reflect the original name of hepatopancreatic parvovirus and the host, decapoda (Cotmore *et al*., 2013).

Not all the genomes of Decapod hepandensovirus1 strains have been fully sequenced and characterized. At present, only four complete genomes are available, including the genome of PmoHDV1 (Sukhumsirichart *et al*., 2006); PmeHDV (La Fauce *et al*., 2007c), PmoHDV4 (Safeena *et al*., 2010), and FchHDV (Jeeva *et al*., 2012), whilst the genomes of PchHDV PmoHDV2, and PmoHDV3 are only partially sequenced (Tang *et al*., 2008).

2.3.1 Thai P. monodon hepandensovirus

The first report of the hepandensovirus in the black tiger shrimp (*P. monodon*) was described in the past two decades (Flegel *et al*., 1992; Flegel *et al*., 1993). During the outbreaks, high level of the virus infection in early juvenile stages of shrimps was documented (Flegel *et al*., 1995; Lightner, 1996). Thai P. monodon hepandensovirus (PmoHDV1) or previously termed as P. monodon densovirus (PmDNV) (Sukhumsirichart *et al*., 2006) was also observed in the

PL of *P. monodon* in outdoor nurseries and from reared shrimps in ponds (Flegel *et al.,* 1992; Lightner, 1996; Flegel, 1999a). Sukhumsirichart *et al*. (1999) suggested that unknown carriers may present in these two rearing systems of shrimps.

After constructing a phylogenetic tree based on conserved amino acid sequences (119 aa). Sukhumsirichart *et al*. (2006) suggested that PmoHDV1 was closely related to other member of genera *Brevidensovirus* (IHHNV) and mosquito densoviruses (AaeDNV and AalDNV), but differs in the overall genomic organization and genome size.

The whole genome of PmoHDV1 is 6.321 kb long with overlapping of between NS1 and NS2, with a larger VP gene, and bigger genome size than those of other brevidensoviruses. Sukhumsirichart *et al*. (2006) confirmed that the ORF1 (NS2), ORF2 (NS1), and ORF3 (VP) on the complementary (plus) strand may code for 428, 579, and 818 amino acids (aa) (50, 68, and 92 kDa, respectively). The aa sequence similarities were 94.2, 97.1, and 87.3% with those FchHDV, respectively and have 89.0, 92.4, and 74.8% identity (Jeeva *et al*., 2012).

The 5’ and 3’ ends of the viral genome contained hairpin-like structure with lengths of approximately 222 and 215 bp, respectively but no inverted terminal repeat (ITR) was detected (Sukhumsirichart *et al*., 2006). The ORF2 of PmoHDV1 contained conserved replication initiator motif, NTP-binding and helicase domain similar to NS1 of other parvoviruses. Therefore, they are most likely encoded for by the major NS1. The ORF1 of PmoHDV1 encoded putative NS2 with unknown function whilst the ORF3 of the genome encoded a capsid protein (VP) of 92 kDa. Sukhumsirichart *et al*. (2006) suggested that it may be later cleaved after an arginine residue to produce a 57-kDa structural protein.

2.3.2 Tanzanian P. monodon hepandensovirus

In contrast with the reports of other strains of Decapod hepandensovirus1 that are widely described, the reports of Tanzanian of *P. monodon hepandensovirus* or PmoHDV2 which previously known as Tanzania HPV are very limited.

Nevertheless, Tang *et al*. (2008) has collected the Tanzanian HPV-infected PL of *P. monodon* from a farm near Dar es Salaam, to analyse and compare the nucleotide sequence with those of deposited HPV sequences from FchHDV, PmoHDV1, and PmeHDV. Tang *et al*. (2008) determined that the Tanzanian HPV isolate was closest to the Madagascan isolate with the pairwise distance between these two isolates approximately

12%. The phylogenetic tree by Tang *et al*. (2008) based on the comparison of NS1 sequences, and interestingly, Korean, Madagascan, Tanzanian isolate were grouped as one group of genotypes (bootstrap value of 75%). This grouping was consistent with that of Loh *et al*. (1997) and Jeeva *et al*. (2012).

The partial genome sequence of PmoHDV2 is available in GenBank (EU588991). The ORF1 and ORF2 of PmoHDV2 (Tanzanian isolate) was closest to PmoHDV3 (Madagascan isolate) with only 1% and 2% distance, respectively (Tang *et al*., 2008). The ORF3 of PmoHDV2 (Tanzanian isolate) was 18% different to with PmoHDV3 (Madagascan isolate) and FchHDV (Korean isolate). The PmoHDV2 NS2 was 344aa (incomplete), NS1 was 578aa, and VP was 821aa (Jeeva *et al*., 2012). More information is required to reveal the characterisation of the whole genome of this African strain of hepandensovirus.

2.3.3 Madagascan P. monodon hepandensovirus

The occurrence of hepatopancreatic parvovirus (HPV) in *P. monodon* was confirmed in Madagascar since the early 1990s by histology, without a negative effect on the shrimp growth observed (Tang *et al*., 2008). Nearly 100% of both wild and farmed populations of *P. monodon* in Madagascar are infected with the virus.

The transmission of HPV in hatcheries in Madagascar usually occurs during spawning as the shrimp eggs could be contaminated by faeces of the shrimp broodstock (Tang *et al*., 2008). When the virus infection was monitored by PCR in a farm in Madagascar in 2006, about 6 out of 13 *Penaeus monodon* were identified to be weakly positive. A year later, strong positive HPV was detected in all 11 samples from another shrimp farm (Tang *et al*., 2008). The infection of shrimp HPV in Madagascar was a serious concern as African stocks of *P. monodon* are often widely used as broodstock because the African penaeid was claimed to be free from the major infectious viruses such as WSSV, IHHNV, Taura syndrome virus (TSV), yellow head virus (YHV), and infectious myonecrosis virus (IMNV) (Tang *et al*., 2008).

The genome of Madagascan HPV (5.6 kb) from *P. monodon* or renamed as Madagascan P. monodon hepandensovirus or PmoHDV3 (Cotmore *et al*., 2013) was partially sequenced by Tang *et al*. (2008). The PmoHDV3 included a full length of ORF2 (578aa) and ORF3 (820 aa), and a partial ORF1 (349aa). The aa sequence similarities were 97.1, 89.8.1, and 95.1% with those FchHDV, respectively and they had 95.2, 88.5, and 83.3% identity (Jeeva *et al*., 2012). The ORF1 and ORF2 of PmoHDV3 had different reading frames. The 3’ end of ORF1

overlaps (16nt) with the ORF2. The ORF2 and ORF3 were in the same reading frame with a 424nt gap in between (Tang *et al*., 2008).

2.3.4 Indian P. monodon hepandensovirus

Hepandensovirus-related mortality in *P. monodon* PL in hatcheries in India was reported in the presence of multiple viral infection caused by monodon baculovirus (MBV) and white spot syndrome virus (WSSV) (Manivannan *et al*., 2002; Umesha *et al*., 2006). This fact often makes the specific diagnosis of hepandensovirus to be difficult (Safeena *et al*., 2010).

The complete genome sequence of Indian P. monodon hepandensovirus (PmoHDV4) was confirmed by Safeena *et al*. (2010). The BLAST results of 441 bp showed 99% similarity (only three nucleotide variation) with other hepandensovirus sequences submitted from India (AY497193 and AY497194). In addition, another part of this VP gene sequence showed 94% nucleotide similarity with partial sequence of hepandensovirus capsid gene from another Indian HPV isolate (DQ415282). This clearly indicates that there are sequence variations within the Indian strains of hepandensoviruses.

Safeena *et al*. (2010) confirmed that NS2 of PmoHDV4 is 1281 bp long with six nucleotides absent when compared to PmoHDV1 (1287 bp) at position between 186 bp and 191 bp. This ORF1 of PmoHDV4 encodes a protein of 426 amino acids, corresponding to a molecular weight of 50.04 kDa. Even though the NS1 of PmoHDV4 showed 94% amino acid similarity with that of PmoHDV1, it has 32aa substitutions in the protein and 2aa deletions (35th and 218th positions of the protein) (Safeena *et al*., 2010). There were 91aa substitutions in the VP sequence of PmoHDV4 when compared to its close relatives (Safeena *et al*., 2010). In addition, Safeena *et al*., (2010) identified an additional proline, in the VP protein sequence of PmoHDV4 compared to all other strains.

A conserved region of glycine-rich sequence was also identified in the downstream of alanine (amino acid 274) of the ORF2 PmoHDV4 (Safeena *et al*., 2010), similarly with that of Sukhumsirichart *et al*. (2006). It has been thought that amino acid changes in the capsid proteins of some parvoviruses have been linked to changes in pathogenicity (Tijssen *et al*., 1995; Fox *et al*., 1999). Thus, further studies are needed to investigate whether this extra aa in PmoHDV4 has any significance to the pathogenesis of the virus as reported for other parvoviruses (Truyen *et al*., 1995, Hemauer *et al*., 1996).

2.3.5 Chinese P. chinensis hepandensovirus

A hepatopancreatic parvovirus-related disease in China was first described from the PL of *P. chinensis* which was also known as *P. orientalis*, originating from Qingdao, China (Lightner & Redman, 1985). As it was described in Cotmore *et al*. (2013), this virus will be renamed as the Chinese P. chinensis hepandensovirus (PchHDV).

A non-destructive method was developed by Pantoja & Lightner (2000) for detection of PchHDV by applying a polymerase chain reaction (PCR) assay to faecal samples from the live virus-infected *P. chinensis* from the Yellow Sea, China. A pair of PCR primers, 1120F/1120R, which amplify a 592-base pair (bp) region from the virus genome, were designed from FchHDV (Korean isolate). However, it was unknown if the primers can accurately recognize PchHDV (Pantoja & Lightner, 2000).

Even though a nucleotide sequence of F. chinensis HPV from China (6.085kb) is available in GenBank (GU371276), it lacks the 3'- and 5'-terminal sequences compared to the other complete genome sequences of HPV (Kawase *et al*., 1990). However, in the proposed taxonomy by Cotmore *et al*. (2013), this HPV strain has not been listed as the member of Decapod hepandensovirus*s*1. The partial genome sequence of PchHDV (5.74 kb) is available in GenBank (AY008257), with NS2 is 348aa (incomplete), NS1 of 578 aa and VP of 821aa.

These sequence sizes are similar with those of Chinese F. chinensis HPV, except for the NS2, in that the incomplete NS2 of FchDNV from China is longer (427aa) (Jeeva *et al*., 2012). A very low nucleic acid variation (1%) between PchHDV (AY008257) and FchHDV (JN082231) indicates that the genetic diversity of hepandensovirus strains was only slightly affected by geographical distribution of the host. It could be due to the genetic adaptation of the virus to the different environmental factors in different geographical areas (Safeena *et al*., 2012). Since the whole genome of PchHDV has not been characterised yet, the information regarding the characteristic of the ORFs need to be confirmed.

2.3.6 Australian P. merguiensis hepandensovirus

In Australia, *P. merguiensis* together with other penaeids such as *P. esculentus, P. japonicus*, and *P. monodon* have been reported to be infected by hepandensovirus or previously known as shrimp HPV (Paynter *et al*., 1985; Lightner, 1996; Spann *et al*., 1997) but there was limited knowledge on whether the strain of the infectious agents are similar to each other or to those already described overseas (La Fauce *et al*., 2007c). La Fauce *et al*. (2007c)

proposed to name the shrimp HPV as P. merguiensis densovirus (PmergDNV) for the isolates from P. merguiensis based on the convention of the International Committee for the Taxonomy of Viruses (ICTV) guidelines. The virus will be colloquially described as the Australian strain *P. merguiensis* hepandensovirus (PmeHDV) (Cotmore *et al*., 2013). Recent studies by Walsh *et al*. (2017) have shown HDV strains in *P. esculentus* from Western Australia have a mix strains from around the Indian Ocean and the Western Pacific and also include east coast PmeHDV.

La Fauce *et al*. (2007c) successfully sequenced the whole genome of PmeHDV (approximately 6.3 kb). The study also documented three putative ORFs, in which the two NS proteins are overlapping, similarly to the organisation structure of the PmoHDV genomes where the two NS proteins are also overlapping but differs from the other prawn parvovirus (IHHNV) and the mosquito densoviruses (AaeDNV and AalDNV) (Afanasiev *et al*., 1991; Boublik *et al*., 1994; Shike *et al*., 2000; Sukhumsirichart *et al*., 2006). The consensus sequence which was reported by La Fauce *et al*. (2007c) contains the complete sequence of putative NS1, NS2 and VP1. La Fauce *et al*. (2007c) successfully sequenced the hairpin like structure on the 5′ end of the genome. However, the sequence the hairpin structure on the 3′ end remained elusive.

Apart from characterising the molecular properties, La Fauce *et al*. (2007b) developed a TaqMan based real-time PCR assay that was specific for the detection of PmeHDV as it did not detect related crustacean and canine parvoviruses from Australia. The very low homology of the target sequence with published sequences from PmoHDV1 and FchHDV and other prawn viruses such as WSSV, also suggested that the TaqMan based real-time PCR assay would be specific for PmeHDV (La Fauce *et al*., 2007b). The TaqMan RT- PCR assay was claimed by La Fauce *et al*. (2007b) as potential diagnostic tool for the detection and quantitation of low-grade infection of PmeHDV as it detected hepatopancreatic parvovirus in 22/22 wild-caught *P. merguiensis* clinical samples and 473/545 (87%) farmed *P. merguiensis*.

2.3.7 South Korean P. chinensis hepandensovirus

F. chinensis or previously named as *Penaeus chinensis* (common names, fleshy prawn, Chinese white shrimp or oriental shrimp) is the native shrimp of Korea (Jeeva *et al*., 2012). This species was a prime farmed species in farms from the west coast of the peninsula in the southern region of Korea (FAO, 2011) to the east coast of northern China, and is

characterised by long distance migration for over-wintering (Kim, 1973; Deng *et al*., 1983 *in* Jang *et al*., 2008).

The first record of hepandensovirus in Korea came from samples of *F. chinensis* in 1991 (Park, 1992; Jang & Jun, 2005). The hepandensovirus is believed as one of the most prevalent viral diseases that often cause disastrous losses to shrimp farming in the Yellow Sea (Pantoja *et al*., 2005). As the result, due to the damage, the Pacific white shrimp (*Litopenaeus vannamei*) has replaced *F. chinensis* as the cultured species in South Korea since 2003 (Jang *et al*., 2006). Jeeva *et al*. (2012) termed the hepandensovirus in *F. chinensis* as the Korean isolate of HPV (FcDNV).

According to the proposal by Cotmore *et al*. (2013), this virus' name will be changed to F. chinensis hepandensovirus or FchHDV. Jeeva *et al*. (2012) successfully characterized the genome of the Korean strain hepandensovirus. The entire genome of the FchHDV is 6,336 bp, with 36% A, 15% C, 25% G, and 24% T. This viral genome (JN082231) is considered the largest genome amongst the Decapod hepandensovirus1. The study by Jeeva *et al*. (2012) also confirmed similarity between the genomic organization of FchHDV and those for PmeHDV and PmoHDV1. Three putative ORFs (ORF1, ORF2, and ORF3) with a 15-nucleotide overlap between ORF1 and ORF2, which used different reading frames (namely, 1+ and 3+), were identified in FchHDV.

The ORF1 encodes NS2 of 425aa with a molecular weight of 50kDa. Whereas the ORF2 encodes NS1, containing 578aa with a molecular weight of 68kDa (Jeeva *et al*., 2012). Like other hepandensovirus NS1, FchHDV-NS1 contains a conserved motif showing homology to the replication initiator motifs involved in the initiation and termination of the rolling circle replication mechanism of parvovirus between aa 86 and 155 (Jeeva *et al*, 2012). Consistent with Shike *et al*. (2000), another conserved region located between amino acids 389 and 489 shares sequence homology to the NTP-binding and helicase domains of other parvoviruses was also identified in the NS1 of FchHDV (Jeeva *et al*., 2012).

The ORF3 encodes the viral protein (VP) of 820aa. A glycine-rich region involved in the externalization of the N termini of capsid protein subunits (Simpson *et al*., 1998) was identified in this VP ORF between aa 276 and 286, and a GGSG motif that is known to be essential for capsid assembly was present at position 281–284 (Jeeva *et al*., 2012). In addition, Jeeva *et al*. (2012) determined that the three ORFs encoded contain potential

promoters of their own with TATA boxes, transcription start signals (Inr-box) and downstream promoter element (DPE), similarly with those of Thai P. monodon hepandensovirus (PmoHDV1) (Sukhumsirichart *et al*., 2006).

A phylogenetic analysis by Jeeva *et al*. (2012) classified some of Decapod hepandensovirus1 into 3 genotypes based on the amino acid comparison of the NS1. Type I covers FchHDV, PmoHDV2, and PmoHDV3; Type II covers: PmoHDV1 and PmoHDV4; Type III covers PmeHDV and New Caledonian hepandensovirus isolate.

2.4 COMPARATIVE ANALYSES OF ENCODED PROTEINS AMONG Decapod hepandensovirus1

Sukhumsiricart *et al*. (2002) described that both the 5' and 3' terminal sequences of the genome contained palindromic sequences capable of forming hairpin-like structures by base pairing which is necessary for replication and encapsidation of viral genome. Even though the nucleotide sequence at 5' end could be folded into a hairpin-like structure it could not generate a typical Y-, T-, or J-form similar to other parvoviruses (Sukhumsirichart *et al*., 2002). Sukhumsiricart *et al.* (2002) also determined that three ORFs of Decapod hepandensovirus1 strains have their own potential promoters with TATA boxes, transcription start signals (Inrbox), and downstream promoter elements. In addition, the study also confirmed that there were three functional promoters namely p2, p22 and p48 upstream from the ORF1, ORF2 and ORF3 of the virus. The putative promoter (p22) found upstream to the ORF2 controls the transcription of the ORF2 and the third set of putative transcription regulatory sequences (p48) controls transcription of the right ORF3.

Of all the three encoded proteins of the autonomous parvoviruses, NS1 is the major replicative protein (Cotmore & Tattersall, 1987) which is highly conserved across the available genomes of hepandensoviruses. The NS1 sizes of PmoHDV1, PmoHDV4, PmeHDV and FchHDV is 1,740, 1,734, 1,737, and 1,737bp, respectively (Safeena *et al*., 2012).

The replication initiator motifs of parvoviruses are involved in initiation and termination of rolling circle replication (Shike *et al*., 2000). Highly conserved motifs of replication initiator and NTP-binding and helicase domains are identified in the two regions among hepandensovirus NS1. The presence of asymmetrical generation of flip and flop at the 5' and 3' ends of hepandensovirus genome leads to an assumption that this viral DNA may

replicate by using the modified rolling hairpin model as in case of autonomous parvovirus MVM (Astell *et al*., 1985).

NS2 of PmoHDV1, PmoHDV4, PmeHDV and FchHDV is 1.287, 1.281, 1.026 and 1.278 kb, respectively with unclear function. Previous studies by Naeger *et al*. (1990 and 1993), and Cotmore *et al*. (1997) reported that NS2 from a parvovirus, minute virus of mice (MVM) works in the assembly of the viral capsid and in the generation of viral ssDNA. Other reports by Naeger *et al*. (1990 and 1993) and Cater & Pintel (1992) confirmed that NS2 also works in the production of other viral replicative forms. In addition, NS2 appears to enhance NS1 associated parvovirus-induced cell killing in some cells.

VP1 of hepandensoviruses is encoded for ORF3. The length of VP1 of PmoHDV1, PmoHDV4, PmeHDV and FchHDV is 2,457, 2,460, 2,454, and 2,563 bp respectively (reviewed by Safeena *et al*., 2012). The VP1 also possess a highly conserved glycine-rich sequence with no appearance of phospholipase A2 (PLA2) signature sequence (Safeena *et al*., 2012).

2.5 STRUCTURES of TERMINAL SEQUENCES of Decapod hepandensovirus1

A common characteristic of parvoviruses is the presence of the hairpin-like loop structure of the termini, which plays an important role as a primer in replication by the rolling hairpin mechanism, which produces a head-to-head or tail-to-tail structure as an intermediate (Muzyczka *et al*., 2001). Jeeva *et al*. (2012) used a RACE-PCR to analyse the 5'- and 3'-terminal sequences of FchHDV, PmoHDV1, PmoHDV4, and PmeHDV for which complete genome sequences are available in GenBank. The noncoding region of the 5'-end length is 204bp, upstream of the AUG start codon for the NS2 gene. A hairpin structure of 124bp, with a Y-form, except for the first guanine residue at the 5'-end, is similar to the typical form of other parvoviruses during the prediction of secondary structure was also identified (Ranz *et al*., 1989; Dumas *et al*., 1992).

The multiple alignments by Jeeva *et al*. (2012) confirmed that the sequences of the 5' ends within the four hepandensovirus strains were highly similar. After about 45 nucleotides, a 12-bp gap is identified in FchHDV and PmeHDV, followed by a variable region and another region of high similarity. Considering the high sequence homology among the 5'-ends of the different hepandensovirus strains, the 5'-end guanine residue, which is not involved in the

formation of the hairpin structure, would appear to be genuine and further studies are required to analyse the dissimilarities (Jeeva *et al*., 2012).

The 3'-end of the genomic DNA is approximately 238 bp, starting after the UAA stop codon of the VP gene, at the position of 6,081-6,336bp. The 3'-noncoding regions of FchHDV is 36, 48, and 56 nucleotides longer than the corresponding regions of PmoHDV1, PmoHDV4 and PmeHDV, respectively (Jeeva *et al*., 2012). The secondary structure of the 3'-noncoding regions showed a potential hairpin-like structure of 238bp. In contrast to the 5'end, the 3'-end of the hepandensovirus strains did not show high sequence similarity at the 3'-terminus, although a homologous region was detected about 50bp upstream of the 3-end. In addition, no sequence homology was observed between the 5'- and 3'-ends of the FchHDV genome sequence (Jeeva *et al*., 2012).

The selected hepandensoviruses have differing secondary structures at their 5'- and 3'-ends, and some of them, such as the 5'-end of PmoHDV4 show no hairpin-like structure at the termini. This could be due to incomplete the sequencing on the structure differences require further studies to confirm the detailed mechanism of replication in hepandensoviruses with differing terminal structures and the functions of these terminal sequences (Jeeva *et al*., 2012).

2.6 NUCLEAR LOCATION SIGNALS (NLSs) of Decapod hepandensovirus1

The fact that parvoviruses have an obligate intranuclear site for their replication and assembly and that the viruses often need cells in rapidly dividing phase (S-phase) suggest there should be strong nuclear location signals (NLS) (Owens, 2013) linked to the parvovirus proteins to allow them entry through the NPC to be functional. NLSs are most often stretches of sequences of the basic amino acids lysine (K) and arginine (R) and can be preceded by helix-breaking neutral amino acids, proline (P), glutamine (Q) or glycine (G) and less commonly by the negatively charged aspartic acid (D) and glutamic acid (E) (Cokol *et al*., 2000).

NLSs can be classic monopartite (e.g. SV-40T-antigen) when they are hexapeptides (6aa) with at least 4aa basic and neither acidic nor bulky amino acids (Boulikas, 1994; Owens, 2013) and preceded by a helix-breaking residue (P, Q or G) (e.g. PKRKKVR) (Cokol *et al*., 2000; Owens, 2013). NLS can also be bipartite when 2 basic aa separated by at least 9aa from a cluster of at least 3 basics aa ((e.g. DNA helicase Q1) or non-classical (e.g. proline–

tyrosine (P–Y)). In addition, NLS can be a Chelsky sequence when 4aa, three of which are basic, starting dibasic e.g. K-K/R-x-K/R (Chelsky *et al*., 1989; Owens, 2013).

Transportation into the nucleus and viral synthesis are absolutely required during the virus replication. Specific proteins called importins (Imp) types α/β or β alone mediate the transportation of the viral proteins. Upon the binding of NLSs to the Imp, viral proteins are transported in or out of nuclei (Jans *et al*., 2000).

A bioinformatical analysis by Owens (2013) demonstrated that all NLSs of Decapod hepandensoviruses1 strains are at the carboxyl ends of the ORFs. That study also has identified various characteristics of the shrimp virus NLSs.

2.6.1 NLSs in Decapod hepandensovirus1 non-structural protein2 (NS2)

The Thai strain of P. monodon hepandensovirus (PmoHDV1 (DQ00873)) and Indian strain of P. monodon hepandensovirus (PmoHDV4 (FJ410797)) have a potential classic human a1, DNA helicase Q1 (KK-15aa-KKRK) NLS starting at aa 267 (266–268). DNA helicase Q1s are heavily involved in unwinding DNA duplexes in a 3’ to 5’ direction and may have DNA repair capabilities. This enzyme may be involved with separation of DNA templates during the synthesis of rolling replication (Owens, 2013).

Most other hepandensovirus strains such as Madagascan P. monodon hepandensovirus (PmoHDV3 (EU588991)), Chinese P. chinensis hepandensovirus (PchHDV (AY008257)), South Korean F. chinensis hepandensovirus (FchHDV (JN082231)) and Australian P. merguiensis hepandensovirus (PmeHDV (DQ458781)) have only 14 amino acids between the bipartite repeats of the lysine (KK-14aa-KKRK) missing an A (alanine) at position aa 274. In addition, the PmeHDV potentially has slightly different basic aa sequences (KR-14aa-KKTK). Of interest in PmoHDV1 to PmoHDV4 (PASKKRK) and PchHDV (PANKKRK), is that the second part of the bipartite signal could almost operate as a monopartite signal defined as 6 amino acids, four of which are basic, proceeded by a helix-breaking proline (PxxKKRK). The PmeHDV (PPSKKTK) almost fits with one less basic aa (the T), but a double proline. There are a few unimportant changes between the strains in the spacing stretch of 15aa.

2.6.2 NLSs in Decapod hepandensovirus1 non-structural protein1 (NS1)

NS1 only slightly overlaps with the 3′ end of NS2. It has a potential classic Chelsky sequence (K-K/R-x-K/R) (Chelsky *et al*., 1989) preceded by a disruptive negatively charged aspartic acid (D), starting at aa 558/9 KKFK or RKFK.

Twenty-four aa upstream in all PmoHDVs is a 533/4RK signal or in PchHDV and PmeHDV, a KK signal, making this a possible bipartite NLS compensating for the negative D. This pattern is similar to the Dorsal (RRPS-22 aa-RRKRQK) (Jans *et al*., 2000), but in the PmeHDV sequence the serine (S) is absent in the upstream fragment of the bipartite NLS and is experimentally unconfirmed as a functioning NLS at the moment (Owens, 2013). Another potential bipartite NLS starting at aa 172 KK-20aa-KRDK also was identified. However, the negative D in the middle of the Chelsky signal is possibly disruptive as there is no known NLS that has D within the Chelsky signal as opposed to be a terminator of the signal.

2.6.3 NLSs in Decapod hepandensovirus1 viral protein1 (VP1)

In all PmoHDVs and PmeHDV, the VP1 has a very strong NLS (PKKKKKYK) starting at aa 808 or 809 which is strikingly similar to SV40 T-antigen (PKKKRKV). In PchDV and FchHDV, the leading proline (P) has been replaced with a glutamine (Q), otherwise the sequences are similar. The very strong NLS in all hepandensovirus VP1s would certify nuclear transport (Owens, 2013). Therefore, the VP1s tend to allow the strong purple or blue colouration of the inclusion body in the infected cells when stained with Mayer's haematoxylin and eosin staining. Also, based on electron micrographs, virions always exist in the inclusion bodies of hepandensovirus nuclei (Owens, 2013).

2.7 IVERMECTIN AS a POTENTIAL ANTI-NLS DRUG

2.7.1 General application of ivermectin

Ivermectin (22, 23-dihydroavermectin B_{1a} + 22, 23-dihydroavermectin B_{1b}), is a broad-spectrum anti-parasitic drug that belongs to the avermectin family, which are macrolytic lactones derived from the bacterium *Streptomyces avermitilis* (Woodruff & Burg, 1986; Davies & Rodger, 2000). The drug is commercially available with brand names of Heartgard, Sklice, Stromecol, Ivomec, Mectizan, Ivexterm, to name but few. During the screening program at a drug company called Merck, the drug is encoded with MK-933 (Pampiglione *et al*., 1985). Traditionally, ivermectin has been used against parasitic worms. More evidence

supports that this wonder drug was used to kill parasitic arthropods and insects (reviewed by Geary, 2005). The drug kills by interfering with the nervous system and muscle function of the parasites (Campbell *et al*., 1983).

For medical purposes, ivermectin has been approved to treat patients with onchocerciasis (Babalola, 2011) and also to treat worm infestation-related diseases, such as strongyloidiasis, ascariasis, trichiasis, filariasis, anaerobiosis, and some skin diseases, including scabies (Victoria & Trujillo, 2001; Geary, 2005). For veterinary medical use, ivermectin is often administered as the mixture with other medications to treat a broad parasite spectrum in cattle, horses, sheep, swine, cats and dogs. The drug is also often injected or sprayed as an acaricide in reptiles. Even though the use of ivermectin in aquaculture is not licensed, the drug is effectively used as oral treatment against sea lice (*Lepeoptheirus salmonis* and *Caligus elongates* in Atlantic salmon (*Salmo salar* L.) farms due to the low solubility of the compound (Davies & Roger, 2000; Mladineo *et al*., 2006; Heberer *et al*., 2009).

While ivermectin works well as therapeutics in some cases, care must be taken as several concerns on bioavailability of the drug. Ivermectin is excreted mainly without changing form from the gut and the concentration are maintained in the body fluids for prolonged periods (Barragry, 1987). The long degradation time in sediment (> 28 days) of ivermectin may significantly cause harmful impacts to species in pelagic environment. In addition, field studies have demonstrated the dung of animals treated with ivermectin supports a significantly reduced diversity of invertebrates, and the dung persists longer (Iglesias *et al*., 2006). Ivermectin has been shown to be toxic to some benthic infaunal species in single species tests, but there is no evidence that treatment of fish with ivermectin has affected multispecies benthic communities in the field (reviewed by Davies & Rodger, 2000).

The sensitivity of crustaceans to ivermectin has been documented. Calcott & Fatig (1984) reported that avermectin causes potential loss of action in the neuron, loss of functional motoric and eventual paralysis in the brine shrimp (*Artemia salina*). Wislocki *et al*. (1989) determined that mysid shrimp (*Mysidopsis bahia*), was sensitive at 96 h LC_{50} 0.022 µg/l, whilst the no-observed effect concentration (NOEC) was 4ng/l, but the 96 h LC_{50} for pink shrimp *Penaeus duorarum* was 1.6 µg/l. The study by Davies et al. (1997) demonstrated that the mysid, *Neomysis integer* showed a 96h LC_{50} of 70 (44–96, 95% CI) ng/l. Burridge & Haya (1993) determined that sand shrimps (*Crangon septemspinosa*) could tolerate ivermectin in water at the maximum concentration of 21.5 µg/l.

The drug becomes toxin when ivermectin supplemented feed was ingested by the shrimps (96h LC_{50} was 8.5 µg/g food). This indicates that ivermectin kills the sand shrimps via digestive tracts but not via the gills. The no observable effect concentration (NOEC) for shrimp was 2.6 µg/g food (Haya *et al*., 2001). This indicates that shrimps may tolerate at this recommended concentration (Burridge & Haya, 1993; Allen *et al*., 2007). Nevertheless, much remains to be learned about the lethal risks of ivermectin for shrimps.

While negative effects of ivermectin have been reported as above, the effects of viruses on shrimp production are severe enough that the potential positive effects of use ivermectin as an NLS blocking agent should be considered and a judgement made regarding its use.

2.7.2 Ivermectin as a non-specific inhibitor of viral replication

The commercially available inhibiters such as leptomycin B (LMB), ratjadone, peptide-based inhibiters and several small-molecule inhibiters (Flint *et al*., 2005; Kau *et al*., 2003; Kau & Silver, 2003; Meissner *et al*., 2004 Mutka *et al*., 2009) exclusively work to inhibit nuclear export.

Due to critical role played by viral protein nuclear import, the interaction between NLS and Imp is a very attractive target for the development of small-molecule inhibitor (Wagstaff *et al*., 2011). Thus, the new and specific nuclear import inhibiter is urgently required. Wagstaff *et al*. (2011) identified ivermectin as a potential general Imp α/β-mediated nuclear import inhibiter through an amplified luminescent proximity homogeneous assay (AlphaScreen®) technology. However, ivermectin did not appear to interfere with Imp β1-mediated nuclear import. Importantly, the subsequent research by Wagstaff *et al*. (2012) identified ivermectin as a potent inhibiter of heterodimer Imp α/β, with no effect on a range of other nuclear import pathways, including that mediated independent Imp β1. Perhaps, ivermectin binds to the NLS-binding pocket of Imp α, preventing the recognition NLS-containing cargo proteins (Wagstaff *et al*., 2012).

Equally important, Wagstaff *et al*. (2012) provided evidence that ivermectin is a non-specific inhibitor of viral replication (HIV-1 and dengue virus or DENV), both of which strongly rely on Imp α/β for nuclear import, with respect to the HIV-1 integrase and NS5 polymerase proteins, respectively. Therefore, the opportunity to evaluate ivermectin in future research of protein nuclear import, as well as the development of ivermectin as antiviral agents is widely open.

2.7.3. *In vivo* effectiveness of ivermectin against a crustacean parvovirus

Owing to the studies by Wagstaff *et al*. (2011 & 2012) coupled with the bioinformatics analysis of NLSs in crustacean hepandensoviruses by Owens (2013), Nguyen *et al*. (2014) recently examined ivermectin *in vivo* against viruses for the first time and showed that ivermectin did dramatically block some parvoviruses, possibly by interactions with cellular importins (Imp)s. Histological examination by Nguyen *et al*. (2014) demonstrated a protective effect of ivermectin. As doses increased up to 7 µg/kg (injected intramuscularly), so did survival. Ivermectin at 7µg/kg dramatically (~68%) decreased the number of hypertrophied nuclei in pre-infected crayfish expressing parvo-like virus in the gills.

Even though the information on how antiviral chemicals act on the putative parvovirus is not reported elsewhere, Nguyen *et al*. (2014) suggested that the mode of action of ivermectin in the putative gill parvovirus in crayfish would be similar with that proposed by Wagstaff *et al*. (2012). Ivermectin probably blocks the NLS-binding pocket of Impα limiting nuclear importation of viral cargo proteins NS1 and NS2 of the crayfish putative parvovirus (Nguyen *et al*., 2014). This suggestion is supported by the evidence that the DNA helicase Q1 NLS (Owens, 2013) of crustacean hepandensoviruses interacts with Imp α (Seki *et al*., 1997).

The study by Nguyen *et al*. (2014) suggested therapeutic benefit in delivering ivermectin to crayfish in general and in some cases of infection caused by viruses which perhaps use Imp α/β-mediated nuclear import. Hence, it is important to consider a potential therapeutic role for ivermectin in viral reduction in broodstock in crustacean aquaculture.

2.8 CONCLUSIONS

Decapod hepandensoviruses1 was previously known as the shrimp hepatopancreatic parvoviruses or densoviruses. The three ORFs of hepandensoviruses from 5’ to 3’ end are ORF1, which carries NS2; ORF2 (NS1 gene); and ORF3 (VP gene).
The comparison of encoded proteins and structure of terminal sequences among Decapod hepandensovirus1 demonstrated that this virus pose replication properties which are similar with those of other parvoviruses.
Previous studies showed that in some parvovirus capsid proteins, the discrepancy in the number of aa has been associated with the different level of pathogenicity of the

parvoviruses. Hence, it is worthy to consider that the variety of replication characteristics in Decapod hepandensovirus1 may also associate with the pathogenesis.

The characterization of putative NLSs of crustacean hepandensovirus by Owens (2013) coupled with the *in vivo* study of injecting ivermectin intramuscularly to crayfish (*C. quadricarinatus*) by Nguyen *et al*. (2014) lead to a consideration of a therapeutic role for ivermectins in viral reduction in crustacean aquaculture.

CHAPTER 3
General Materials and Methods

The general materials and methods described in this chapter contain techniques that have been used in more than one thesis chapter. Materials and methods applied only once are mentioned in the appropriate section.

3.1 CELL CULTURE PREPARATION AND MAINTENANCE

Cell lines from mosquito larvae (C6/36), African monkey's kidney (Vero) and mouse macrophage (RAW-Blue™ ISG) that were frozen stored in a liquid nitrogen dewar at JCU Townsville, Australia were retrieved and used in this research. For further cultivation, thawing was carried out as quickly as possible. Cells were taken from the liquid nitrogen tanks, quickly defrosted in sterile warm water (37 ºC) and mixed with fresh medium to eliminate the effect of frozen medium. Gentle centrifugation (1800 rpm for 5 mins) was applied to pellet the cells. The pellets were suspended in completed appropriate culture medium and developed in flasks until used. C6/36 was cultured in Roswell Park Memorial Institute-1640 (RPMI) and Minimum Essential Medium Eagle's (MEM). Vero and RawBlue™ ISG were grown in Dulbecco Modified Essential Medium (DMEM).

All medium used were supplemented with 10% foetal bovine serum (FBS), penicillin (200 U/ml), streptomycin (200 µg/ml), kanamycin (80 µg/ml), polymyxin B (30 U/ml) and 1x Amphotericin B. Normocin (100 µg/ml) and L-glutamine (2mM) were added into RAW-Blue™ ISG. C6/36 was incubated at 28 ºC. Whereas Vero and RAW-Blue™ ISG were incubated at and 37 ºC with media changed when required.

The cell lines were passaged when monolayers performed. For passaging, old medium in the TC flasks were aseptically decanted and the cells were washed with 1x PBS twice. C6/36 and Vero were detached by diluting the cells with 1ml prewarmed trypsin solution. Whereas RAW-Blue™ ISG was detached using cell scrapers. After 75 – 80 % cells were detached, the cells then were suspended in fresh medium and were equally distributed in new culture flasks.

For cells restocking, the suspended cells were centrifuged at 1000 rpm for 5 mins and then were diluted in 90 % FBS supplemented with 10 % DMSO as freeze medium. The cells were put into 1 ml vials and kept in a Mr Frosty™ freezing container (Thermo Fisher Scientific,

Australia) and the cells were slowly frozen at a -80 °C freezer for 24 hrs, before being transferred to a cryogenic container liquid nitrogen dewar for very slow cooling the stocks.

3.2 DAILY OBSERVATION

Daily observation of the cultured cells under Olympus CK2 and CKX41 microscopes were made to confirm the cells growth and to ensure that cells were free from contaminants. Digital images of cells were processed using QCapturePro 6.0. Media changing was conducted once or twice a week as required.

The cells were sub-cultured when monolayers formed. For passaging, the old medium was aseptically decanted, and the cells were washed with 1x phosphate buffer saline (PBS) before being trypsinised with 0.25% trypsin/EDTA solution (0.25 % trypsin and 0.2 % EDTA in PBS). The cells then were re-suspended in supplemented fresh medium and were equally distributed in two new culture flasks. The passaging ratio for all cell lines in this study was 1:3.

3.3 CELL VIABILITY COUNTS

Equal amount of cells suspension and 0.14 % Trypan Blue in PBS were mixed, loaded into the counting chamber of a haemocytometer and examined using a light microscope Olympus CX31 (Olympus, Japan). The live (white) and dead with disrupted cellular membranes (stained blue) cells were counted. The number were computed into live cells/ml and dead cells/ml. The percentage of cell viability (%) was calculated by dividing the number viable cells by the total number of cells and the result was multiplied by 100.

3.4 VIRUS SUSCEPTIBILITY

The culture media was decanted when cell lines reach confluence approximately 70 – 80 % monolayer. After media removal, cells were divided into 2 groups (infected and non-infected). The infected group received 1mL virus inoculum of interest and the non-infected group received 1 ml of 1x PBS. The cells were incubated for 1hr before fresh media were added. Following this, the flasks were incubated for several days at the desired temperatures.

3.5 MAYER’S HAEMATOXYLIN & EOSIN (H & E)

The collected cell samples were fixed in a fixative solution and stained with H & E to observe cellular morphological changes (Drury and Wallington *in* Hayakijikosol & Owens, 2013).

3.6 DETERMINATION OF DNA CONCENTRATION

The DNA concentration was determined using a spectrophotometer (Eppendorf. Australia). Absorbance reading were recorded at wavelengths of 260nm (A_{260}) and 280 nm (A_{280}) with 1cm path length. The concentration of nucleic acid in the sample was calculated from A_{260}, based on the assumption that 1 absorbance unit is equivalent to 50 µg/mL dsDNA. The A_{260}:A_{280} ratio provided an estimate of the purity of the nuclei acid, with values of 1.8-2.0 representing pure preparations (Sambrook *et al*., 1989).

3.8 PCR PRODUCT ANALYSES

To analyze PCR products, 10 µl of PCR reaction mixture was used for electrophoresis on 2.0 % agarose gels containing 0.05 ml/ml GelRed (Biotium, USA). A UV trans-luminating box (SynGene USA) linked to Gensnap software was used to view and photograph gel images.

CHAPTER 4

In vitro Propagation of Penaeus merguiensis hepandensovirus in C6/36 Cell Line

4.1. INTRODUCTION

Viruses within the family *Parvoviridae* can infect vertebrates and invertebrates with the viruses of the subfamily *Densovirinae* infecting invertebrates especially arthropods (Cotmore *et al*., 2013).

Parvoviral diseases have been reported as a major threat to penaeid culture due to their ability to cause slow growth and mass mortality of infected prawns (Flegel *et al*., 1992, 1999a &b, 2006a, 2012; Safeena *et al*., 2012). Penaeus merguiensis hepandensovirus (PmeHDV) (GenBank accession No. DQ458781) is a shrimp hepatopancreatic parvovirus (HPV), an Australian strain of the species Decapod hepandensovirus1, in the genus Hepandensovirus, subfamily *Densovirinae* (Bergoin & Tijssen, 2010; Cotmore *et al*., 2013). Based on the analysis of the complete genome sequence among different strains of hepandensoviruses, the sequence variation between the Indian strain of Decapod hepandensovirus1 a.k.a. P. monodon hepandensovirus (PmoHDV4) and PmeHDV is 22.1%, which is unexpectedly high, showing that these Hepandensovirus strains are markedly genetically different (Safeena *et al*., 2012).

Control of the parvoviral diseases is crucial. However, the knowledge of the pathogens and their host defence responses is relatively poor. The major problem of understanding the replication dynamics of shrimp viruses is hampered by the lack of any continuous cell line.

Mosquito cell lines (C6/36) have been examined for *in vitro* propagation of shrimp viruses. Sudhakaran *et al*. (2007) reported that Macrobrachium rosenbergii nodavirus (MrNV) and extra small virus (XSV) were successfully propagated in C6/36 by serial passaging of the culture supernatant. Sriton *et al*. (2009) found weak positive results for PmoHDV in C6/36 cells by PCR three days post-viral inoculation and strong positive results on day 4 and 5 but the virus could not be further passaged. Sriton *et al*. (2009) demonstrated that the C6/36 mosquito cells exposed to other shrimp viruses, including white spot syndrome virus (WSSV) and yellow head virus (YHV) followed by serial passaging could lead to insect cell cultures in which 100% of cells persistently expressed viral antigens. Sriton *et al*. (2009) determined that the supernatant of the infected cell cultures was free from virions, suggesting that the

genomic material remained in the cultures and it transferred from mother to daughter cells during the cell's cultivation. Also, they hypothesized that the viral genomic material must have replicated to support antigen production as the infected cells remained 100% antigen positive for more than 100 passages.

Gangnonngiw *et al*. (2010) demonstrated that C6/36 persistently expressing YHV antigens could serve as YHV inoculum to infect shrimp. The authors determined that the fifth passage of the infected cell cultures caused mortality to exposed shrimp, while the 15th passage of the infected cells did not, even though immunohistochemically, the challenged shrimp were positively infected by YHV antigens. Arunrut *et al*. (2011) reported the successful production of C6/36 that persistently expressed Taura syndrome virus (TSV) antigens as previously described for WSSV and YHV. The authors also claimed the successful infection of *P. vannamei* with homogenates of TSV-infected cells. They obtained positive reverse transcriptase PCR (RT-PCR) results for TSV in the immune-positive mosquito cell cultures and the successful infection of *P. vannamei* with homogenates of TSV-immuno-positive mosquito cell cultures from passage 15. None of these studies proved that the virus actually replicated to patency or that the shrimp in bioassays that died were not dying from the original inoculum placed in the cell cultures. Indeed, the results of Sriton *et al*. (2009) and Gangnonngiw *et al*. (2010) suggest this is exactly the scenario being observed. Partial translation could result in positive immunoassays.

Hayakijkosol & Owens (2013) observed mortality in virus-exposed mosquito cell lines but using quantitative PCR, found no patent replication of Australian isolate of MrNV, suggesting that C6/36 are non-permissive for this virus. Madan *et al*. (2013) reported that C6/36 cultures were susceptible to PmoHDV4 and the infected cultures showed cytopathic effect (CPE) in the form of vacuole formation and cell shrinkage. Madan *et al*. (2013) confirmed the infection by PmoHDV4 in C6/36 cultures based on cytology, PCR, reverse transcriptase PCR (RT-PCR) and Western blotting. The CPE in the virus-exposed cell cultures was continuously detected for five cell passages. The infected cell cultures were PCR positive for PmoHDV4, producing a 441 bp amplicon.

The RT-PCR detected that the RNA specific to capsid gene of HPV was in infected C6/36 cells at different days post-infection. Further investigation to quantify the PmoHDV4-load was conducted using quantitative PCR (qPCR), ELISA and immunocytochemistry. qPCR and ELISA indicated an increase in viral nucleic acid and viral protein in the infected C6/36 cells

within 12 days of exposure (Madan *et al*., 2013). In addition, immunofluorescence was observed in the PmoHDV4-exposed cells. Madan *et al*. (2013) determined that immersion of postlarval *P. monodon* into water containing cell culture fluids from after the twentieth passage of PmoHDV4 in C6/36 cell line caused very slow, total mortality of postlarvae (PL) by 7 weeks post-exposure.

This present study aims to investigate the susceptibility of the mosquito cell line (C6/36) to P. merguiensis hepandensovirus (PmeHDV) which has 22% nucleotide difference to PmoHDV4 and also examine the replication of the virus in the mosquito cells. Differential staining was undertaken to understand cellular events occurring during PmeHDV infection. Confirmation of the viral exposure was conducted by PCR. A quantitative, specific PCR assay (TaqMan qPCR) for PmeHDV (Owens *et al*., 2015) was used to detect any viral replication of the virus and calculate the viral copies in the infected cell cultures.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of viral inoculum

PmeHDV inoculum were prepared from hepatopancreata of frozen, infected (inclusion body positive) *P. merguiensis* stored at the College of Public Health, Medical and Veterinary Science laboratory, James Cook University (JCU) Townsville, Australia. The hepatopancreata were thawed and homogenized in 25 % Tris-HCl, NaCl (TN) buffer (0.4 M NaCl, 0.02 M Tris-HCl, pH7.4). The homogenate was centrifuged twice for 10 mins at *720 g and 4500 g*. The final supernatant was subsequently filtered through 0.45 mm and 0.22 mm membranes. The presence of PmeHDV was confirmed by PCR using a diagnostic primer set (Section 4.2.7) (Owens *et al*., 2015). The filtrate was stored at 20°C until used.

4.2.2 Cell line and maintenance

The C6/36 mosquito cells, derived from frozen stocks at JCU Townsville, Australia were used to propagate the virus. The cell lines were grown in Roswell Park Memorial Institute-1640 (RPMI) and Minimum Essential Medium Eagle (MEM) media supplemented with 10% foetal bovine serum (FBS), penicillin (200 U/ ml), streptomycin (200 mg/m), kanamycin (80 mg/ml), polymyxin B (30 U/ml) and 1x Amphotericin B (Sigma Aldrich, Australia). The cells were propagated at 28°C with media changed when required.

The cells were sub-cultured when monolayers formed. For passaging, the old medium was aseptically decanted, and the cells were washed with 1x phosphate buffer saline (PBS). The cells were detached by incubating them with 1 mL pre-warmed 0.25 % trypsin/EDTA solution for 3–5 min or until the 75% of the cells detached. The cells then were re-suspended in complete fresh medium and were equally distributed in two new culture flasks. Live cell images were taken using an inverted microscope (Olympus, Japan) equipped with a digital camera and Q Capture P software (Olympus, Japan).

4.2.3 Viral susceptibility

T25 mL flasks (Sarstedt, Germany) were used to culture C6/36 cells (approximately 10^5 cells/ml) at 28°C to give a confluence of 70–80 %. After removing the medium, 1 ml of viral inoculum per flask was inoculated on the surface of the C6/36 cells in 4 flasks, and allowed to adsorb for 1 hr (Hirt, 1967). The 4 negative control flasks received 1 ml 1x PBS for each flask. After incubating for 1 h at 28°C, 9 ml of fresh media were added into all flasks. The trial was conducted in triplicate. Passaging and sample collection from each flask was conducted when cells were in log phase (5 days after passage). In this present study, C6/36 cells that were exposed to viral inoculum are considered as the infected cell culture groups while the uninfected C6/36 cells were considered as the negative control cell cultures.

At the time of passaging, 50% of each (uninfected and infected) cell culture was collected and subjected to various analyses for PmeHDV infection and replication. After sampling, the remaining collected cells from the control, non-infected cell cultures were added into each infected cell culture for on growing of the virus. This ensured that the necessary dividing cells were available for replication of the parvovirus. Both infected and uninfected control cells were re-grown at 28°C until the next passage. Four replicate samples of infected cells from each culture flask, serially passaged four times were used for the PCR analyses at each passage (Section 4.2.7 & 4.2.8). Negative control PCRs (including qPCR) were from four non-infected cell cultures, passaged 4 times.

4.2.4 Cytology

For each stain, a twenty-four well plate was used to culture C6/36. The cells were seeded (approximately 10^5 cells/ml) onto cover slips in 16 wells of a 24 well plate for maximum 24 hrs to give a confluence of 70–80 %. Cells in the first 8 wells were exposed to the prepared

inoculum PmeHDV (100 µl/well) while the uninfected groups received medium (100 µl/well). After the viral inoculation, the plates were incubated at 28 °C for 1hr and following this, fresh medium was added (900 µl/well) into both infected and uninfected cell cultures.

The presence of cytopathic effect (CPE) was examined daily under an inverted microscope (Olympus DP21, Japan). Staining of both uninfected (negative control cells) and infected cells was conducted on days 2, 4, 7, and 14 days post infection (dpi) with four replicate samples. Cells were washed and fixed with different fixatives according to each staining protocol.

4.2.4.1 Mayer's Haematoxylin & Eosin (H & E)

Cells were fixed in Bouin's solution and stained with H & E (Appendix 1) to observe cellular morphological changes (Drury and Wallington, 1980 in Hayakijkosol and Owens, 2013).

4.2.4.2 Giemsa stain

Cells were fixed with 100% methanol before staining with Giemsa stain overnight (Appendix 1). After overnight staining, the cells were rinsed with distilled water containing 0.5 % aqueous glacial acetic acid (Kiernan, 2010). Observations were conducted using a microscope (Olympus, Japan) equipped with a digital camera and Q Capture P software (Olympus, Japan).

4.2.4.3 Acridine orange

Acridine orange (AO) was used to observe nucleic acid proliferation in cells. Cells were fixed at 7 days with 100 ml of 95 % ethanol for 30 min and rehydrated with 80%, 70% and 50% ethanol, respectively, for 10 s each and then rinsed with water. One percent acetic acid (100 mL) was added for 6 s and then removed. One hundred ml of 0.1% AO solution was used to stain the cells for 3 mins before they were washed with McIlvain citric acid and M/10 calcium chloride (Culling *et al*., 1985). The C6/36 cells were examined under an epifluorescent microscopes (Olympus, Japan and Zeiss, Australia), Zeiss Imager Z1, (Zeiss, Australia) using 475/530 nm and 560/650 nm excitation/emission filters. Photographs of cells were taken using a digital camera equipped with AxioVision version 4.8.2 SP3 software.

4.2.5 Counting of disrupted cell membranes (presumptive dead cells)

Equal amounts of cells suspension and 0.14% trypan blue in 1x PBS (Appendix 1) were mixed, 20 µl of the mixture was loaded into the counting chamber of a haemocytometer (Neubauer, UK) and examined using a light microscope, Olympus CX31, (Olympus, Japan). The live (translucent) and presumptive dead (stained blue) cells were counted in the eight corners of the haemocytometer. The percentage of cell viability (%) was calculated by dividing the number viable cells/mL by the total number of cells/ml and the result was multiplied by 100.

4.2.6 Water Soluble Tetrazolium-1 (WST-1) assay for cell proliferation

The stable tetrazolium salt WST-1 (Sigma Aldrich, Australia) is cleaved to a soluble formazan by a complex cellular mechanism that occurs primarily at the cell surface. This bio-reduction is largely dependent on the glycolytic production of NAD(P)H in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture.

Twelve replicates of infected and control C6/36 cells were prepared in a 96 well plate. Fifty microliters of complete cell medium and 50 µl of viral inoculum were placed in each appropriate well producing 12 replicates. Fifty microliters of cell suspension were added into all wells. An extra 50 µl medium was added into the non-infected, control wells. The insect cells were grown at 28 °C and analyzed on 2, 4, 7, and 14 dpi. On each examination day, ten microliters of WST-1 reagent (Roche, Germany) were added into the 24 wells and the plate was re-incubated at 28°C for 4 h prior to the absorbance reading. Plates were shaken for 1 min and the absorbance of the cells was measured at 450 nm on a Varioskan flash reader version 2.4.3 (Thermoscientific, Australia).

4.2.7 Confirmation of PmeHDV exposure by PCR

At each passage, PCR was conducted to confirm the presence of PmeHDV in the C6/36 cells using diagnostic primer sets (Table 4.1). DNA was extracted from both infected and control cells with a Genomic Isolate-II kit (Bioline, Australia), following the manufacturer's instructions. Cells and supernatant were collected from the flasks and spun down at 583g for 5 min. The pellets were resuspended in 1x PBS before being extracted. The concentration of

DNA in each sample was estimated using a spectrophotometer (Eppendorf, Australia) by measuring the absorbance towards UV light at 260 nm (A_{260}).

Table 4.1. Primers and probe used for PCR to confirm the infection and replication of PmeHDV in C6/36.

Assays	Primer/ probe	Nucleotides (5'-3")	Annealing temperature (°C)
PCR & TaqMan qPCR	HPV140F	CTA CTC CAA TGG AAA CTT CTG AGC	63.5
PCR	HPV140R	GTG GCG TTG GAA GGC ACT TC	62.5
TaqMan qPCR	HPV4507R	GGC ACT TCC TGT ATT TTT CCC G	62.1
TaqMan qPCR	Probe	FAM-TAC CGC CGC ACC GCA GCA GC-TAMRA	66.7

Fluorogenic probe FAM (6-carboxyfluoresin); quenching dye TAMRA (6 carboxytetramethylrhodamine (La Fauce *et al*., 2007b).

Each PCR reaction using MyFi master mix (Bioline, Australia) consists of 5 µl of reaction buffer, MyFi Taq polymerase (2 m/µl), 1 µl of each primer HPV140F and HPV140R, 2.5 µl of DNA template and nuclease-free water to a final volume of 25 µl. Amplification was performed in a thermal cycler (Applied Biosystems, USA). PCR profile consisted of an initial denaturation at 95 °C for 1 min, then 35 cycles of 95 °C denaturation for 15 s, 54 °C annealing for 20 s and extension at 72 °C for 5 mins. Samples were polymerized for 7 mins at 72 °C following the last cycle and held at 10 °C. The amplified products were run on a high-speed gel electrophoresis system (Biokeyston Co. USA) and visualized on to 2 % agarose gel containing 0.05 ml/ml GelRed (Biotium USA). Ten microliters of PCR reaction mixture of each sample were used for the electrophoresis. A UV trans illuminating box (SynGene USA) linked to Gensnap software was used to view and photograph gel images.

4.2.8 Quantification of PmeHDV copy number by TaqMan real-time PCR

TaqMan qPCR based on the capsid gene was conducted to determine the copy number and C_T (cycle threshold) value of PmeHDV in the infected C6/36 cell cultures. Cells at each passage, every 5 days up to 20 days, were sampled for viral copy numbers by qPCR. Prior to the qPCR assay, a standard curve was generated using serial dilutions from $1x10^9$ to $1x10^0$ copies of a positive control plasmid to determine the sensitivity of the TaqMan assay according to the protocol described by La Fauce *et al*. (2007b). A forward primer (HPV140F)

and a TaqMan probe used were those of La Fauce *et al*. (2007b) whereas a newly designed reverse primer (HPV4507R) (Owens *et al*., 2015) was used in this assay (Table 4.1).

Each TaqMan qPCR reaction for generating standard curve using a prepared pDNA (labelled 6C) of positive control PmeHDV at JCU Australia (the standard curve can be seen in Appendix 4) and quantitation of viral copy number was carried out in a 20 ml volume containing 10 ml of 2 buffer (Applied Biosystems, Australia), 5 pmol of probe, 20 pmol of each primer 140F and HPV4507R (Macrogen, Korea), 1 ml of DNA template, and nuclease-free water to a final volume of 20 mL. The Taqman cycle pattern consisted of initial incubation at 95°C for 3 min, followed by a two – step cycle pattern consisting of 40 cycles at 95 °C for 10 s, and 60 °C for 45 s. The data acquisition and analysis were performed using a Corbett Rotor-Gene Q (Qiagen, Australia).

4.2.9. Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 19.

4.3 RESULTS

4.3.1. Characteristics of the infected cells

Examination of live cell images (Figure 4.1) showed only minor differences in characteristics between control and viral infected cells during the four passages, with infected cells appearing slightly more clumped than the cells of control groups.

4.3.2. Cytology

Vacuole formation was observed in both infected and control C6/36 cells stained with H & E and Giemsa. Even though enlarged nuclei (anisonucleosis) were observed in the infected cells stained with Giemsa, no viral inclusion bodies were detected in the infected cells (Figs. 2 and 3).

Acridine orange (AO) staining of both infected and control groups demonstrated nucleic acids (Figure 4.4). No red/orange fluorescence was detected using 460/650 nm filters in either cell groups or they stained predominantly green under examination with 475/530 nm

filters. The green fluorescence of infected cells was more intense and a brighter green colour in the cytoplasm than in the uninfected control cells.

4.3.3 Counting of cells with disrupted membranes (presumptive dead cells)

The number of cells stained by trypan blue indicating disrupted cell membranes (presumptively dead cells) of both virus infected and control cell groups fluctuated over the serial passages (Figure 4.5). The highest percentage of dead cells (7.6 %) was detected in the infected groups at the 2nd passage while the control groups had the lowest percentage of dead cells at the 2nd passage ($F = 9.554$, d.f. (1, 22), $p < 0.05$) (see Appendix 3).

4.3.4 Cell proliferation

Different light absorbances between virus infected and control cells were recorded by the WST-1 assay (Figure 4.6). Light absorbance of infected cells was significantly lower ($F = 6.879$, d.f. (1, 94), $p < 0.05$) than that of control groups for 2 weeks (see Appendix 3), suggesting less metabolically active cells i.e. cell death.

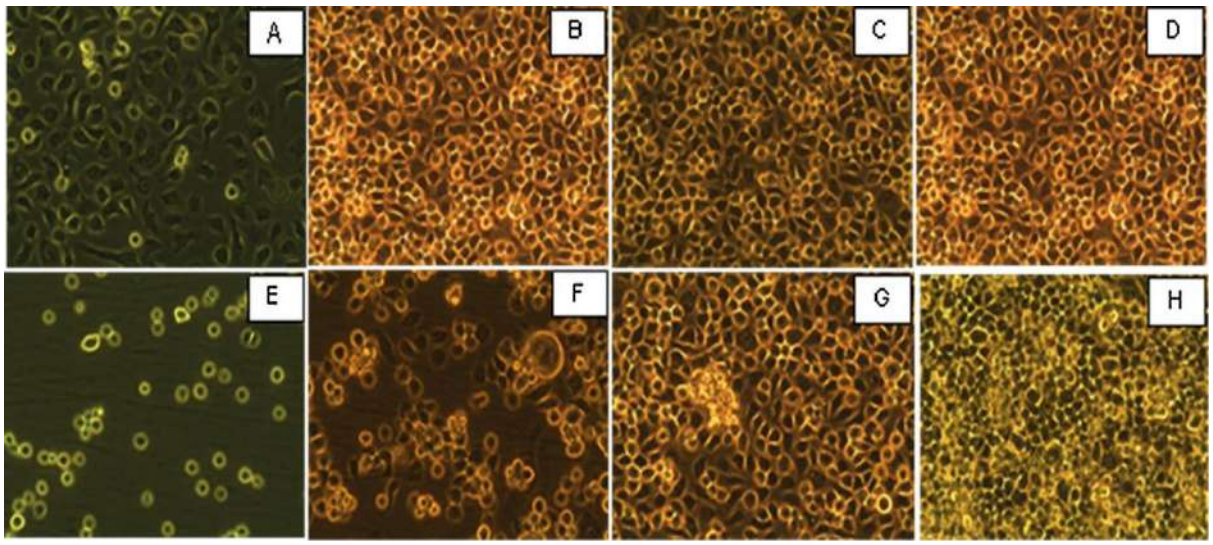

Figure 4.1. Uninfected (A-D) and PmeHDV- infected C6/36 (E-H) from the first to fourth passages. Bars 20μm.

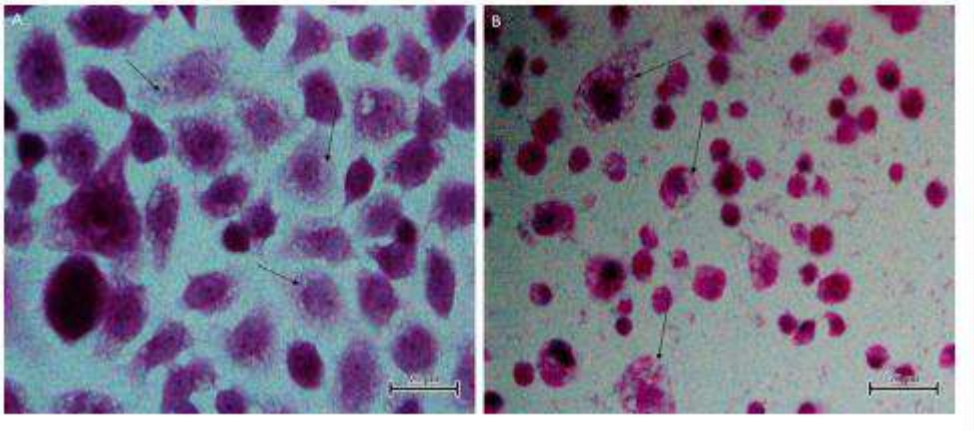

Figure 4.2. H & E staining of uninfected (A) and PmeHDV-infected C6/36 (B) 1-week post infection (p.i.) (both cell group showed multiple vacuoles). Bars 20µm.

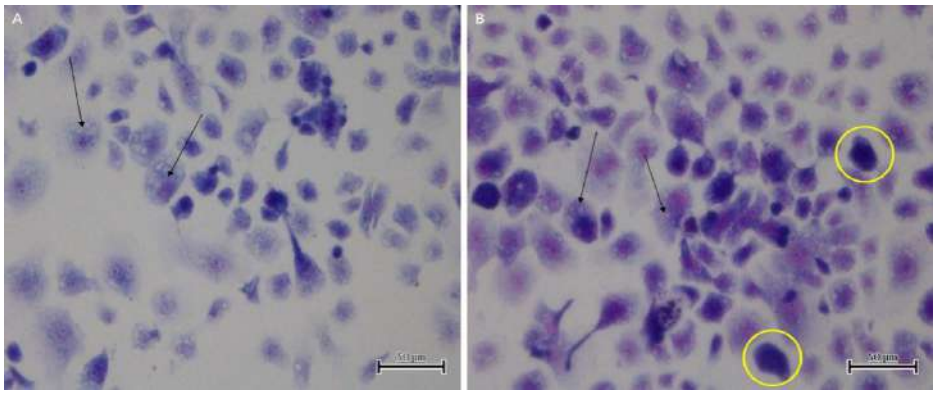

Figure 4.3. Giemsa stain of uninfected (A) and PmeHDV-infected C6/36 1 wpi. Vacuolation (arrows) and possible anisonucleosis (circles). Bars 50 µm.

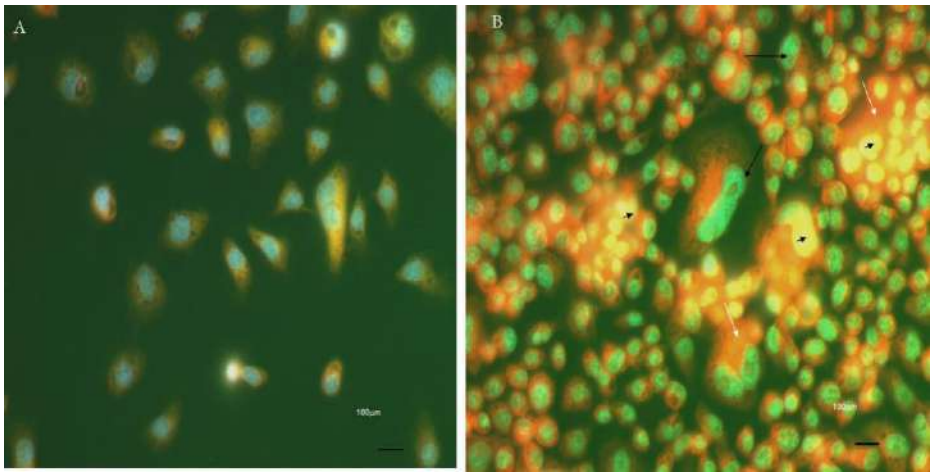

Figure 4.4. AO staining of uninfected C6/36 (A) and PmeHDV-infected C6/36 (B) at 1 wpi. Red/orange fluorescence indicates the presence of single stranded nucleic acid (cytoplasmic mRNA; white arrows); yellow fluorescence shows dsNA (probable hybrid ssDNA + mRNA

transcription; short black arrows); green fluorescence shows nuclear dsDNA (long black arrows). Bars 100 µm.

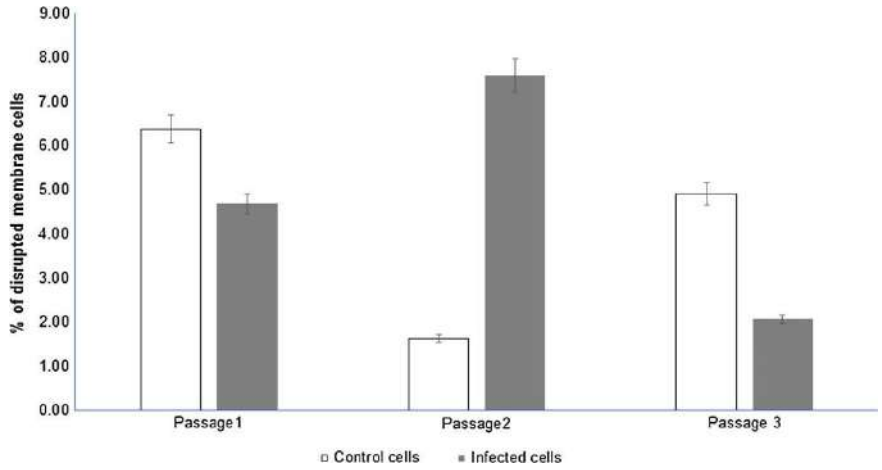

Figure 4.5. Average number (±STDEV) of disrupted membranes (presumptive dead cells) between uninfected and PmeHDV-infected C6/36

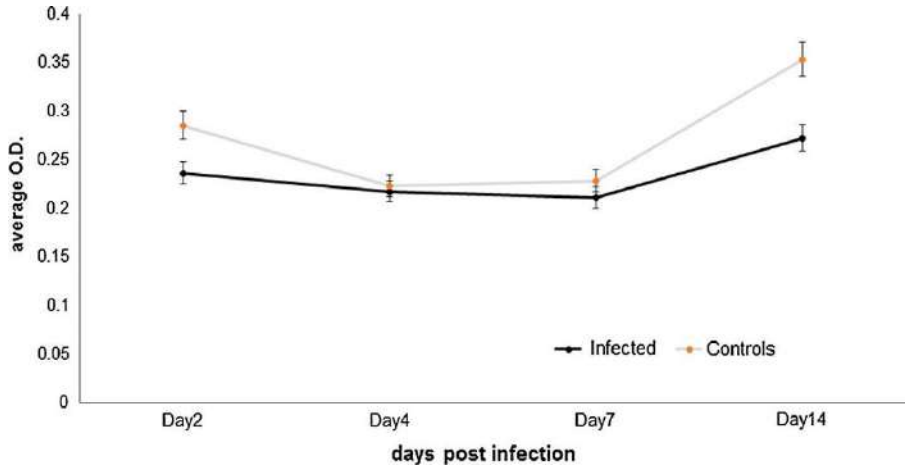

Figure 4.6. WST-1 assay. Light absorbance of uninfected and PmeHDV-infected C6/36 observed over 2 wpi.

4.3.5 Molecular studies

4.3.5.1 PCR

Using a specific PCR primer set for PmeHDV (Table 4.1), the infected C6/36 samples showed weak positive 140 bp amplicons (not shown).

4.3.5.2 TaqMan qPCR

The TaqMan qPCR results in this study demonstrated a dropping copy number in each subsequent passage of the infected C6/36 cells. The average viral copy numbers declined from 3.8 x 10^5 to 5.69 x 10^2 copies/ml and the mean of cycle times increased from 19.26 to 27.63 during four serial passages (Table 4.2), suggesting that the PmeHDV could not use the C6/36 for complete replication. With a cut off C_T value of 38 cycles, uninfected control cells had no measurable C_T values i.e. any virus present was below detection limits.

Table 4.2. The calculated concentration (number of copies) and threshold (C_T) values of PmeHDV in four flasks with replicate assays of C6/36 cell lines during serial passage

Samples	Passage 1		Passage 2		Passage 3		Passage 4	
	(x 10^5) Copies/µl	C_T	(x 10^4) Copies/µl	C_T	(x 10^3) Copies/µl	C_T	(x 10^2) Copies/µl	C_T
1	4.70	18.92	4.55	21.92	4.64	24.86	8.62	27.02
2	4.94	18.86	4.53	21.93	5.68	24.60	7.13	27.27
3	4.88	18.87	4.32	21.99	5.01	24.76	9.10	26.95
4	3.46	19.31	9.61	20.96	8.21	24.12	4.50	27.86
5	3.79	19.20	9.35	21.00	7.65	24.21	4.49	27.86
6	3.52	19.29	8.19	21.17	8.36	24.10	3.32	28.25
7	3.52	19.82	5.22	21.74	5.87	24.55	6.61	27.36
8	2.42	19.77	5.53	21.67	6.81	24.36	6.05	27.35
9	2.26	19.86	5.00	21.80	5.37	24.67	6.02	27.48
10	4.44	19.18	8.13	20.18	5.89	24.55	3.49	28.19
11	4.55	19.04	1.55	20.34	7.59	24.22	3.84	28.06
12	4.35	19.02	8.79	21.07	5.66	24.60	4.53	27.85
Mean	**3.80**	**19.26**	**7.39**	**21.40**	**6.39**	**24.47**	**5.69**	**27.63**

4.4 DISCUSSION

4.4.1 Negative results in this current study

The lack of an established crustacean cell line is one reason why researchers have looked extensively for alternative cell lines to understand the interaction between host cells and shrimp viruses. As it was mentioned earlier, some research groups have published on the successful replication of different shrimp viruses in mosquito cell cultures (C6/36) which encourages future research on understanding the mechanism of the viral infection.

This present study demonstrated that the Australian strain of Decapod hepandensovirus1 (PmeHDV) cannot replicate to patency in C6/36 cell cultures. The cell cultures were affected by PmeHDV as demonstrated by the H & E and Giemsa stainings (Figures 4.1–4.4). Examination of live C6/36 cells showed differences between control and PmeHDV-

infected cell cultures (Figure 4.1), in that cells were clumped upon viral inoculation but other changes in both infected and control cell cultures were similar. PCR results indicated that the cell cultures were exposed to virus. However, TaqMan qPCR clearly showed a dropping number of viral copies and increasing C_T values with serial passages indicating no replication to patency.

CPE such as vacuole formation was found in both infected and uninfected cell cultures as observed by H & E and Giemsa staining (Figures 4.2 & 4.3), suggesting the microscopical examinations were not a sensitive enough technique to determine the infection of PmeHDV. The presence of vacuoles in both infected and control cells was also described by Sudhakaran *et al*. (2007) and Hayakijkosol & Owens (2013).

Acridine orange (AO) was used to observe nucleic acid changes. AO is a metachromatic dye which differentially stains double-stranded (ds) and single-stranded (ss) nucleic acids. Under a fluorescent microscope, AO emits orange or red fluorescence when it interacts with single stranded nucleic acid upon excitation at wavelength 480–490 nm. An increasing yellow/green colour in infected C6/36 cells (Figures 4.4) might suggest an increasing double stranded (ds) nucleic acid production, either dsDNA or a mRNA/DNA hybrid that occurs in the densovirus rolling cycle propagation. Notably the red/ orange colours of ssDNA in the nucleus of cells from the patent densovirus were not observed. There was no indication that the inoculation of PmeHDV into C6/36 enhanced the viral ssDNA production as none was visualized by AO staining.

The positive results via PCR following gel electrophoresis is an indication that the cells were exposed to PmeHDV. However, quantification of viral copy numbers using TaqMan qPCR is required to confirm the viral replication. TaqMan qPCR results (Table 4.2) demonstrated dropping viral copy number from 3.85×10^5 to 3.32×10^2 copy/ml and the mean of cycle times increased from 18.86 to 27.36 during the four serial passages, suggesting PmeHDV was not able to use C6/36 cell cultures to multiply their viral DNA even though fresh, rapidly dividing cells (S-phase) were added into the infected flasks at each subsequent passage. Uninfected cell cultures were negative proving the PCR primers did not react non-specifically with the C6/36 cells.

One interpretation of the present data is that the initial stages of viral decapsidation, and transcription of early genes might be occurring as evidenced in these present assays, but the late genes (VP) failed to produce the capsid and therefore, viable viruses. Similarly, observations of partial CPE and failure for complete virogenesis were seen with MrNV in the

piscine SSN1 cells (Hernandez-Herrera *et al*., 2007) and with C6/36 cells (Hayakijkosol & Owens, 2013). Also of note are similar results in Sriton *et al*. (2009) and Gangnonngiw *et al*. (2010) with regard to WSSV and YHV, and YHV alone, respectively. This phenomenon of partial replication of parvoviruses is not new. E.g. in cells non-permissive for human parvovirus B19, there is a large build-up of NS1 and NS2 transcripts but low quantities of the capsid transcript (Liu *et al.*, 1992). In cells permissive for B19, these transcripts are in equal amounts.

4.4.2 Differences in hepandensoviruses that caused contrary results between Madan *et al*. (2013) and the current study

Differences in this present study compared to that of the successful propagation of PmoHDV4 in C6/36 cell culture could be due to the 22.1 % genetic variation between the PmoHDV4 and PmeHDV (Safeena *et al*., 2012). Of interest, the qPCR product in the capsid area of this current report completely incorporates the polyglycine motif. Only PmeHDV from Australia and New Caledonia, have a serine-free, polyglycine signal in the capsid protein (Owens, 2013). All other sequenced isolates have a serine in this polyglycine motif.

Polyglycine motifs form capsid pores for nucleic acid entry on assembly and exit on decapsidation. The pore can also be used for cell ingress in some viruses. Does the lack of a serine affect the permissiveness of the C6/36 cell lines and could this be responsible for the different results between Madan *et al*. (2013) and the current report? This seems unlikely, as the qPCR indicating the falling copy number is based on the late gene for the capsid and it is not being replicated, so the block to replication appears to be occurring before this step in virogenesis not at the assembly stage or cell infection stage. This needs to be confirmed experimentally.

The ORF-1 (NS2) protein of PmoHDV4 is larger than that of PmeHDV recorded at 1281 and 1026 bp, respectively (Safeena *et al*., 2012). The NS2 protein of parvoviruses is important in viral assembly, in the production of viral ssDNA (Cotmore *et al*.,1997; Naeger *et al*., 1990; 1993) and it is involved in the production of other viral replicative forms (Cater & Pintel, 1992; Naeger *et al*., 1990, 1993). Equally important, it is suggested that NS2 protein enhances NS1-associated parvovirus-induced cell killing in some cell lines (Brandenburger *et al*., 1990; Caillet-Fauquet *et al*., 1990; Legrand *et al*., 1993). At present, we can find no data on why the size difference of the NS2s could cause differences in replication of these two strains.

4.5 CONCLUSIONS

This study examined the susceptibility of the mosquito cell lines (C6/36) to P. merguiensis hepandensovirus (PmeHDV) using cytological and molecular analyses (PCR). Even though cytological and PCR analyses showed that PmeHDV could cause changes in C6/36 in vitro, the TaqMan qPCR showed that the number of viral copies declined over four serial passages. Further research on trying different cell lines or adapting the PmeHDV to different clones of C6/36 may be necessary to successfully replicate PmeHDV. We suggest there should be more rigorous criteria applied to cell lines before the cell lines are classified as susceptible to viruses. qPCR for late genes for enumerating viral copies with a subsequent specificity check on amplicons should be the minimal acceptable evidence.

CHAPTER 5

Transfection of Vero Cell Lines with Putative NLSs of Penaeus merguiensis **Hepandensoviruses**

5.1 INTRODUCTION

Cellular processes such as cell differentiation and development require protein transport between the nucleus and cytoplasm. The transportation of proteins is also critical to some viral disease and oncogenesis (Hogarth *et al*., 2006; Moseley *et al*., 2007; Pryor *et al*., 2007). In order to gain entry into the cell nucleus via the nuclear pore complexes (NPCs), proteins with molecular mass greater than 45kDa generally need a nuclear location signal (NLS).

Viruses within the subfamily *Densovirinae* are intranuclear and require cells in their S-phase when DNA is synthesized for all or most of their replication and assembly. Transportation into the nucleus is absolutely required for viral replication. Specific chaperone proteins called importins (Imp) types α/β or β alone mediate the transportation of the viral proteins into the nuclei. Upon the binding of NLSs to the Imp, viral proteins are transported in or out of the nuclei (Jans *et al*., 2000).

Some stretches of basic aa such as lysine (K) and arginine (R) are commonly the main components of an NLS and sometimes an NLS sequence is headed by helix-breaking neutral amino acids, proline (P), glutamine (Q) or glycine (G) and less commonly by the negatively charged aspartic acid (D) and glutamic acid (E) (Cokol *et al*., 2000). There are three main group of NLSs. When a NLS contains 6 aa (hexapeptides) with at least 4 of them are basic aa without acidic or bulky aa, it can be termed classic monopartite NLS (e.g. SV-40T-antigen) (Boulikas, 1994; Owens, 2013). The classic monopartite NLS can be preceded by aa P, Q or G as a helix-breaking residue (e.g. PKRKKVR) (Cokol *et al*, 2000; Owens, 2013). Whereas when 2 basics aa separated by at least 9 aa from a cluster of at least 3 basics aa (e.g. DNA helicase Q1) or non-classical (e.g. proline–tyrosine (P–Y)), an NLS can be bipartite. The third group is a Chelsky NLS, which is when three of 4 aa are basic and the dibasic K-K/R-x-K/R are found at the beginning of the NLS (Chelsky *et al*., 1989; Owens, 2013).

PmeHDV (DQ458781) had been demonstrated has putative NLSs in all of the three ORFs (Table 1) and suggested that the NLSs affected the nature of inclusion bodies of PmeHDV to be basophilic and the site of encapsidation to be nucleus (Owens, 2013).

Table 5.1. Putative NLSs in PmeHDV used in transfection of Vero cell line.

Coding domains, ORF	Putative NLS sequences	Functional homology
NS2, ORF1	181 **KR**DQQKT (-) EKKDDE**PPKKKTK**	DNA Helicase Q1
NS1, ORF2	534 **KK**NPELTQFVLASMQYVHSNYMDKPDRKFK	Dorsal
VP1, ORF3	809 **PKKKKK**YR	SV40T_antigen

Bold letters indicate conserved basic amino acids (aa); blue underlined are the helix-breaking prolines; and those in red could be disruptive (Owens, 2013).

PmeHDV NS2 has a potential classic human a1, DNA helicase Q1 (KK-15aa-KKRK) NLS, starting at amino acids (aa) 181. This putative NLS in PmeHDV contains only 14 amino acids between the bipartite repeats of the lysine (KK-14aa-KKTK) without an A (alanine) at position aa 274 (Table 1). DNA helicase Q1s are heavily involved in unwinding DNA duplexes in a 3' to 5' direction and may have DNA repair capabilities. This enzyme may be involved with separation of DNA templates during the synthesis of rolling replication (Owens, 2013).

NS1 has a potential classic Chelsky sequence preceded by a disruptive negatively charged aspartic acid (D), starting at aa 559 RKFK. A possible pattern of the Dorsal (RRPS-22-aa-RRKRQK) (Jans *et al*., 2000) is observed in NS1 with a bipartite NLS compensating for the negative D might exist as 20 aa upstream there is a KK signal (Table 5.1). The NLS was suggested functional particularly with an upstream bipartite partner. However, an experimental investigation is needed to prove the functioning of the bipartite NLS as serine (S) of dorsal is absent in the upstream segment of the bipartite NLS (Owens, 2013).
In addition, the capsid protein or VP1, ORF3 shows a very strong NLS (PKKKKKYK) starting at aa position 809. This aa sequence is noticeably very similar to SV40-T-antigen (PKKRKV) (Owens, 2013). The very powerful NLS would ensure nuclear transport (Owens, 2013).

Various techniques have been developed for decades for efficient introduction of DNA into cultured eukaryotic cells and have allowed expression of various genes cloned in an expression vector for the studies of gene regulation. Although many experiments can be performed with cells transiently expressing foreign genes, it is often necessary to isolate cell lines that permanently express foreign genes. Especially when large quantities of a product are required for the experiments, the success of the experiments depends on the isolation of a cell line highly expressing the foreign gene.

This present study aims to determine experimentally if any of the three putative NLSs of PmeHDV (Owens, 2013) are functioning by transfecting NLS-inserted-plasmid DNAs into

mammalian cell culture using a transfection reagent. Originally, it was planned to use C6/36 mosquito cell lines which should have supported the growth of PmeHDV but it was proved that C6/36 was unsuitable for PmeHDV (Syahidah *et al.*, 2017). Each plasmid has been synthetically constructed and inserted with each sequence of the putative NLSs and a fluorescent protein marker. The presence of the NLS in the cell nucleus and cytoplasm was screened under a fluorescent microscope.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of Synthetic Plasmid DNA (pDNA) from Glass Microfiber Filters

Each protein NLSs sequence was inserted into a plasmid by DNA2.0, USA which has now changed their name to ATUM (www.atum.bio). In this present study, IP-Free© Mammalian transient expression vectors (pD603 and pD673) were designed to express the NLSs protein sequences. Each plasmid contains a fluorescence protein (FP) such as Dasher Green, Cayenne, Yukon, and Match Yellow with different Excitation (Ex) and Emission (Em) wavelengths (nm), to express the location signals (Table 5.2). The neomycin resistance gene (neo-r) was also inserted into each plasmid. A neo-r gene is included as a selectable marker for successful insertion of the plasmid and confers resistance to neomycin and kanamycin in prokaryotes and eukaryotes. The map of pD603-Dasher-VP_NLS can be seen in Figure 5.1. and the maps of rest pDNAs are provided in Appendix 6.

Table 5.2. NLS sequences inserted in the plasmid for transfection of Vero cell line.

Plasmids	**Amino acids (aa) NLS sequences**	**Ex**	**Em**
pD603-Dasher-VP_NLS	PKKKKKYR	505	525
pD603-Yukon-NS2_NLS	KRDQQKTEKKDDEPPKKKTK	550	563
pD673-Cayenne-NS1_NLS	KKNPELTQFVLVLASMQYVHSNYDKPDRKFI	554	590
pD603-MatchYFP (negative control plasmid)	None	520	542
pD673_Cayenne-PfDNV**_NLS (positive control plasmid)	RRRRRR	554	590

Underlined aa are the NLS sequences.
** Periplanata fuliginosa densovirus (Zhou *et al.*, 2009).

The synthetized DNA plasmids that were stored on glass microfiber grade GF/C filter papers, the presence of the NLS-fluorescent hybrids protein and the selection marker, neomycin, were assessed following the manufacture's guidance. Briefly, the filter papers were placed on sterile and clean surfaces separately. One hundred µl of 10 mM Tris-HCl pH 7.5 were added directly into the center of each filter. The filters than were incubated at room temperature for 2 mins. After being incubated, the GF/C filters were placed in 0.6mL tubes that were punctured using a needle-tipped syringe. Each 0.6 ml tube was placed in a 1.5 ml tube and centrifuged at 11,000 *g* to transfer the DNA liquid, approximately contain 2 µg plasmid DNA or 20 ng/µl from the 0.6ml tube into the 1.5 ml tube.

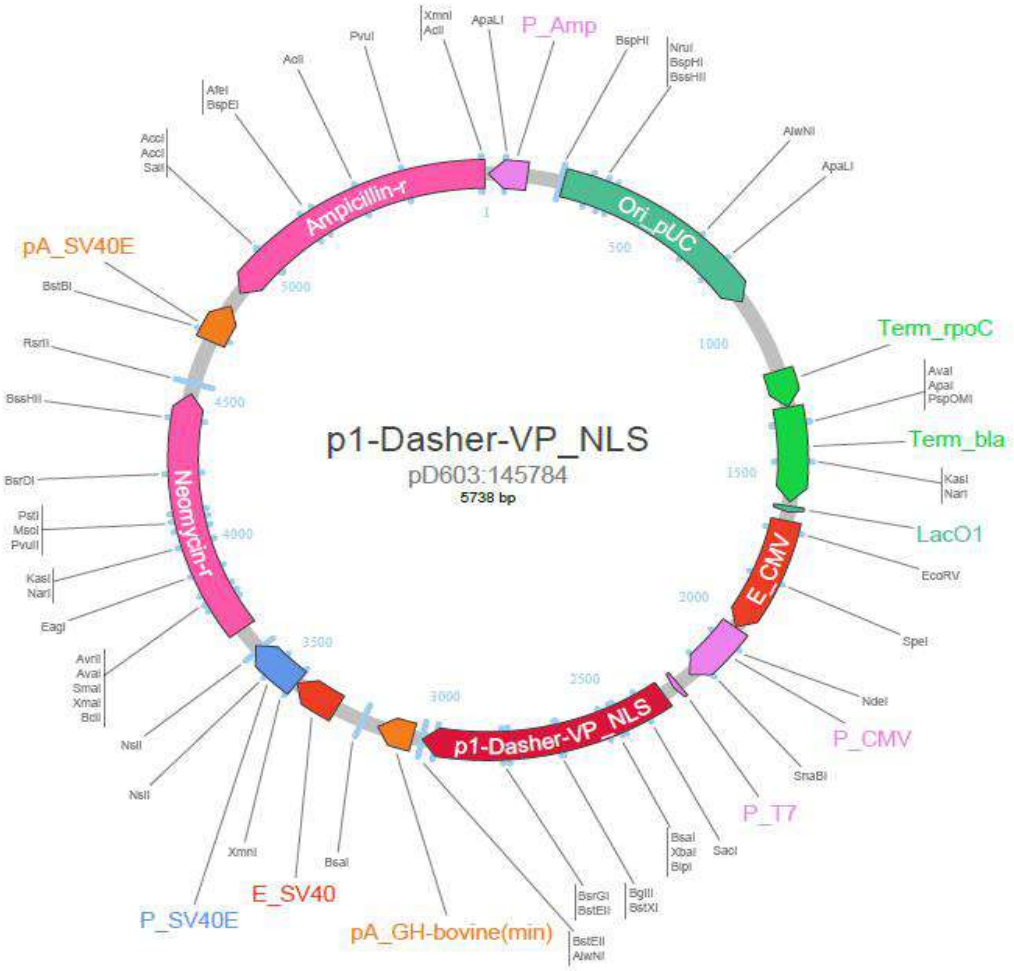

Figure 5.1. The map of pD603-Dasher-VP_NLS (the feature of the map and the sequence is provided in Appendix 5).

5.2.2 Cloning of pDNAs

To perform pDNA cloning, α-select commercially competent *E. coli* cells (Bioline, Australia) were used to transform the synthetized pDNA following the manufacturer's instructions. Each plasmid (5 L) was added into 50µl competent cells in each chilled tube. The mixtures were incubated on ice for 30 mins and heat shocked at 42 C for 30 secs and returned to ice for 2 mins. Bacterial colonies were grown on medium containing ampicillin at 100 µg/ml. pDNAs were extracted using ISOLATE II Plasmid minikit (Bioline, Australia) following the manufacturer's protocol. The concentrations of pDNAs were measured using a spectrophotometer (IMPLEN, Germany) at the absorbance length of 260 nm. The isolated DNA was cloned for usage and further analyses.

5.2.3 Preparation of Mammalian Cell Lines

After the failure of C6/36 to support the growth of PmeHDV (Syahidah *et al*., 2017), we decided to use Vero (African green monkey kidney) cell lines to test the NLSs (Table 5.2) as Vero cells are robust when manipulated and interferon deficient so they are less likely to reject a plasmid when transfected. Vero cells were obtained from the frozen stocks at JCU Australia. The cells were cultured in Minimum Essential Modified (MEM) medium supplemented with 10 % FBS, 2 mM glutamine, 1 % non-essential amino acids and antibiotics (penicillin (200 U/ml), streptomycin (200 mg/ml), kanamycin (80 mg/ml), polymyxin B (30 U/ml) and 1x amphotericin B (Sigma Aldrich, Australia)) in 37 °C incubator. At 24 hrs prior to transfection, cells were seeded in 24 well plates. Viafect™ (Promega, Australia; lipofection reagent) was used per manufacturer's instructions to transfect five different pDNAs into Vero cells.

A selectable marker gene confers a type of reporter gene to determine the success of transfection to introduce the desired gene into a cell. Selectable markers are often antibiotic resistance genes. Ampicillin-r and neomycin-r were fused in the plasmids as selectable markers. Ampicillin-r is considered useful for selectable markers for *E. coli*. Selection of transfected Vero cells was conducted using an analogy of neomycin sulphate, G418 selection antibiotic (Thermo Fisher, Australia) at concentration of 0.5 mg/µl which was added into the cell medium.

5.2.4 Transfection Both a Non-NLS Inserted pDNA and NLS-Inserted pDNAs Into Vero cells

One day before transfection, Vero cells were plated in 16 wells of a 24 well plates in complete growth medium at 37 C to reach 70-80 % confluence. On the day of transfection, the freeze dried constructed plasmid was diluted per 50 l of OPTI-I reduced serum MEM (Life Technologies, Australia) and mixed well. The transfection was performed using ViaFect™ (Promega, Australia) at 2 µl per well.

5.2.5 Cell visualization post transfection

Four days after transfection, growth medium was removed and cells in each well were incubated in 1x PBS for 10 secs and subsequently fixed with acetone for 10 secs. Coverslips with cells were removed from the wells and dried for 10 mins at room temperature. Following this, coverslips with cells stained with DAPI (4',6-diamidino-2-phenylindole), a fluorescent stain that binds strongly to A-T rich regions in DNA, were mounted and kept away from light. The presence of nuclear location signals was observed under a fluorescent microscope (Olympus, Japan) using different combination of filter sets of DAPI-blue, FITC-green and TEXAS-red with different excitation (Ex) and emission (Em) wave lengths, namely 358/461, 488/520, and 591/618 nm, respectively.

5.3. RESULTS

Transfection of Vero cell cultures with plasmids (Table 5.2) was assessed by growing on neomycin containing media. Only cells with the neomycin-resistance plasmids should have survived. The NLS was assessed by the cell's ability to fluoresce at the appropriate wavelength (nm). Three fluorochrome filters (FITC-Green, DAPI and Texas Red) were selected to identify the fluorescence within the infected Vero cells 4 days post transfection (dpt).

All transfected cells in this present study demonstrated poor to no noticeable translocation of signals in the nuclei even though there was a build-up of fluorescence ringing the outside of the nuclear membrane and in the cytosol. The pictures were similar, so examples are shown (Figure 5.2 & 5.3) of the exposure to the plasmid pD603-Dasher-VP_NLSs. Other constructed pDNA can be seen in Appendix 6.

The visualization of transfected cells allowed us to identify the location of the NLSs. Ideally, the cell nucleus or cytosol will glow indicating the co-location of the fluorescent signals. There are differences between transfected Vero cells with pD603-Dasher-VP_NLSs and

non-transfected Vero cells (Figure 5.2). In transfected Vero, screening using a single filter failed to show the green fluorescence in nucleus (N) but green granola (foci) was identified in cytosol (c). The merged images between using the two filter sets also showed no fluorescence within the nuclei of the cells as all nuclei were blue, stained with DAPI. Whereas non-transfected Vero cells showed low dasher green intensity. The addition of the red filter and combination all three filters seems light up the fluorescent spots within nuclei in some transfected Vero cells (Figure 5.3).

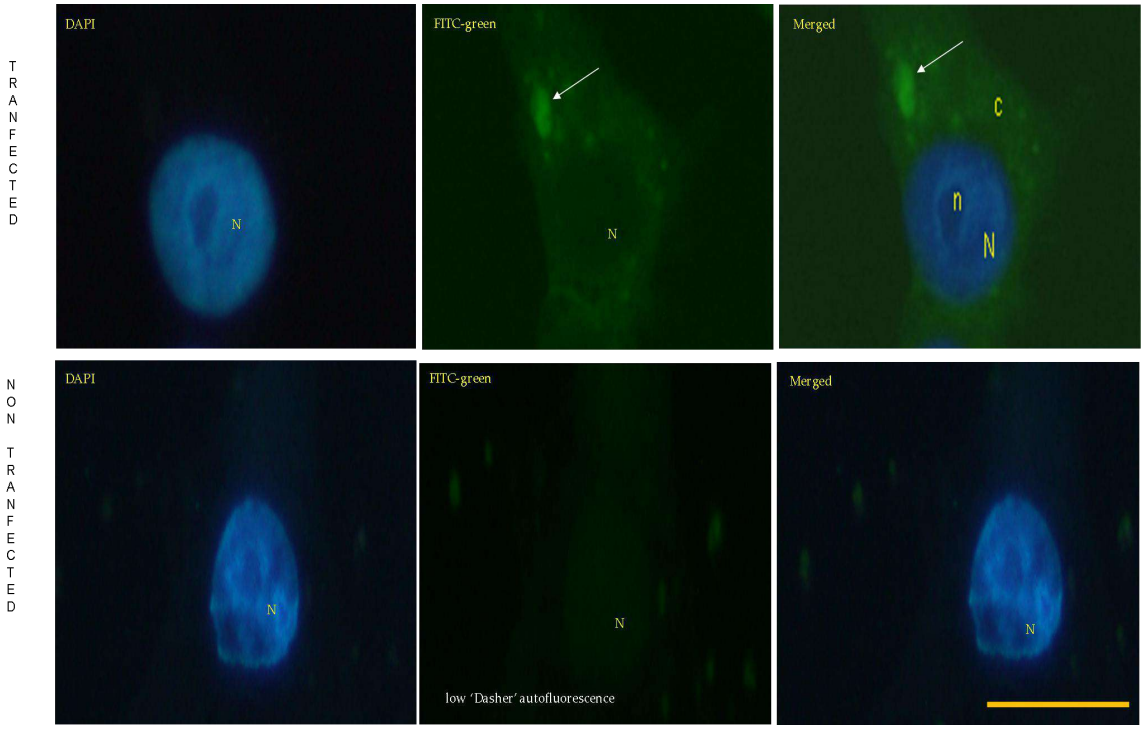

Figure 5.2. Comparison between transfected Vero cells with pD603-Dasher-VP_NLS and non-transfected Vero, using combination of different filter sets (Ex358/Em461 (blue); Ex488/Em520 (green)); N: nucleus; n: nucleolus; c: cytosol; Arrows: Green foci. Bar: 20μm.

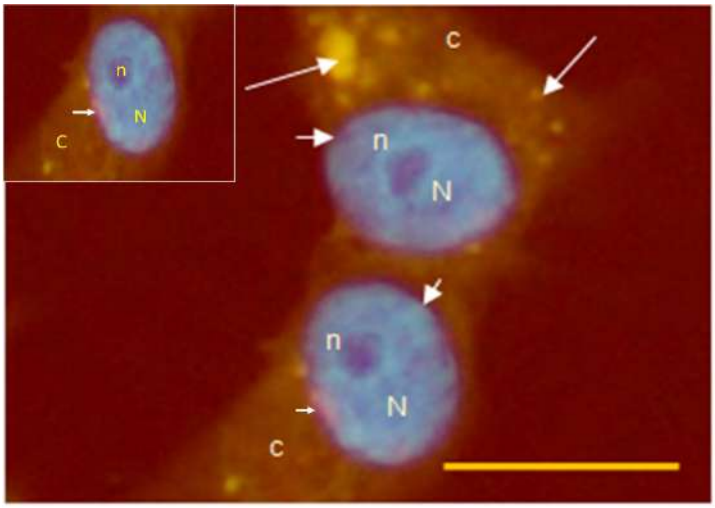

Figure 5.3. Transfected Vero cells with pD603-Dasher-VP_NLS of PmeHDV, 4 dpt using combination of three filters (Ex358/Em461 (blue), Ex 488/Em 520 (green), Ex 591/Em618 (red)). c: cytosol; Long arrows: green foci; Short arrows: red-purple fluorescence. Bar 20 µm.

5.4 DISCUSSION

The research on the mechanism of the interplay between hepandensovirus and the host's cells remains at the bioinformatics analysis stage (Owens, 2013). A major impediment to the study of densoviruses including hepandensoviruses is the lack of suitable cell lines that support the replication of the virus to high titre, despite many attempts to *in vitro* propagating some viruses in cell cultures (Sudhakaran *et al.*, 2007; Sriton *et al.*, 2009; Gangnonngiw *et al.*, 2007; Arunrut *et al.*, 2011). It was reported that C6/36 cell cultures were successfully infected with MrNV but there was no evidence to show complete viral propagation (Hayakijkosol & Owens, 2013). Success for hepandensovirus was reported only for P. monodon hepandensovirus (PmoHDV4) in C6/36 (Madan *et al.*, 2013). However, this was not repeatable for PmeHDV (Syahidah *et al.*, 2017). To date, no experimental investigation on the role of nuclear location signals (NLSs) of PmoHDV4 during viral infection has occurred.

The NLS assists in the navigation of viruses to the nucleus and leads to further nuclear translocation. The bioinformatical analysis that highlighted many possible NLSs of prawn parvoviruses that have not been recognized before and needed to be tested in experimental systems (Owens, 2013). Subsequent analyses would also shed light on functionality. In this present study, the synthetic plasmids (Table 5.2) were transfected into Vero cell cultures hoping to identify their expression and functionality.

Cell transfection and fluorescent analyses have been widely used in previous studies to understand the mechanisms of NLSs during viral infection. As a good example, NS1 of Periplenata fuliginosa densovirus (PfDNV) was successfully localized to both the cytosol and nucleus of cockroach (*P. fuliginosa*) haemocytes (Zhou *et al*., 2009) (Figure 5.4). The NS1 protein was also ectopically (relative to the nucleus) expressed in non-*P. fuliginosa* insect cells such as Schneider line 2 (S2) of *Drosophila melanogaster* and *Aedes albopictus* (C6/36) cell lines, but the NS1 remained outside of the nucleus staying in the cytosol of the non-*P. fuliginosa* insect cells. We had hoped to see similar results and indeed the fluorescence is outside the nucleus in the Vero cell lines thus the present results are identical to the results for the insect cells lines (S2 and C6/36) but not successful as in the host cockroach haemocytes (see in Figure 5.4).

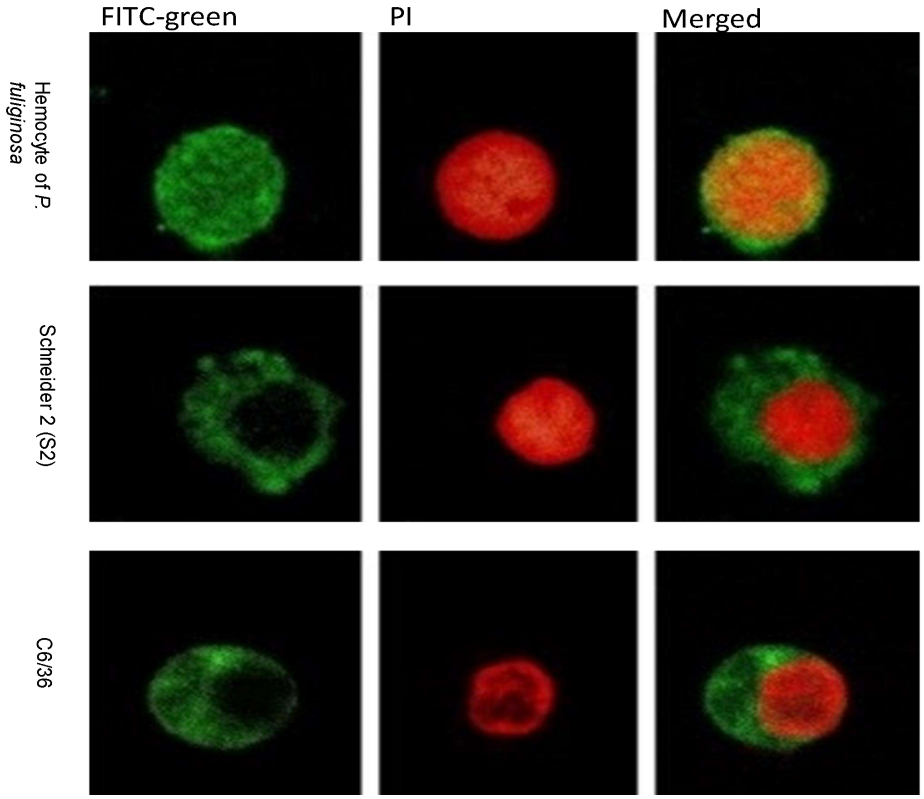

Figure 5.4. Expression of NS1 in PfDNV-infected cell lines (*P. fuliginosa* nymph haemocytes, S2, and C6/36). Cells were counterstained with propidium iodide (PI, red fluorescence) to visualize nucleus morphology, and antibody conjugated fluorescein isothiocyanate (green fluorescence) (Zhou *et al*., 2009). Notes: the fluorescent signals are only in the nucleus of the host hemocytes and not in the insect cell lines (S2 & C6/36).

Parvovirus DNA replication takes place in the cell nucleus and is strictly dependent on the activities of the major viral non-structural protein, NS1. NS1 protein is a multifunctional nuclear phosphoprotein which is absolutely required for parvovirus replication both *in vivo* and *in vitro* (Yang *et al*., 2006). Parvoviral NS1 carries out several obligatory roles in virus replication that require NS1 translocation into the nucleus (Raab *et al*., 2001; Young *et al.*, 2002). Since the nuclear pores in nuclear envelope have a functional radius of 4.5 nm (Paine *et al.*, 1975), it is thought that proteins with a molecular mass of less than 67 kDa can passively diffuse through these nuclear pores (Paine *et al*., 1975). NS1 at 61 kDa could theoretically, passively diffuse into the nucleus and therefore in transfection trials, the presence of NS1 should be clearly detected in nucleus.

An insight into the journey of an NLS of a crustacean virus was recently reported. The location of Macrobrachium rosenbergii nodavirus capsid protein (*Mr*NVc) was successfully detected in insect cell cultures, *Spodoptera frugiperda* (Sf9) (Hanapi *et al*., 2017). The fluorescent assay and in a sub-cellular fraction, the *Mr*NVc was shown to enter Sf9 cells and located in the nucleus. Small granules appeared in Sf9 cell cultures incubated with fluorescein-labelled capsid proteins of MrNV virus-like-particles (F-*Mr*NVc VLPs) (Hanapi *et al*., 2017) (Figure 5.5), similar to green granola patterns (foci) identified in the cytosol of transfected Vero cell cultures in this present study (Figure 5.2).

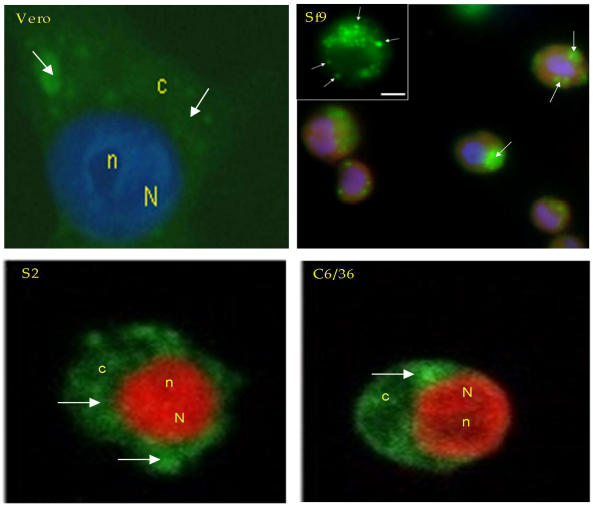

Figure 5.5. Comparison between transfected Vero cells with pD603-Dasher-VP_NLS of PmeHDV in this present study, 4 dpt using combination of three filters (Ex358/Em461 (blue), Ex 488/Em 520 (green), Ex 591/Em618 (red)) and the previous studies of transfected Sf9 (Hanapi *et al*., 2017), S2 and C6/36 (Zhou *et al*., 2009); N: nucleus; n: nucleolus; c: cytosol; arrows: green foci.

Despite the high virulence and infectivity of for their natural hosts, densoviruses are not known to infect mammals. Previous experiments conducted to infect or transfect vertebrate or vertebrate cells line with densoviruses were unsuccessful (El-Far *et al.*, 2004). Similarly, there is no report on the successful propagation and transfection of Decapod hepandensovirus 1 in/into mammalian cells to date. Therefore, an artificial plasmid construct was attempted in this present study. Transfecting NLSs in this present study failed to show clearly the cellular changes upon the transfection of pDNAs in Vero cell lines. The result indicates that techniques in this present study may not be sensitive enough to be confident that intranuclear fluorescence is real and therefore cannot conclusively test the NLS of PmeHDV.

A recent concept of moonlighting function of importin beta-1 (Imp -1) in human cells during the cell cycle highlighted the Imp -1 acting as a nuclear transport receptor during the interphase, when it accumulated at the nuclear envelope (Figure 5.6B) (Verrico *et al*., 2016). In some of Vero cells in this present study, light fluoresce signals were identified at the nuclear membranes in the merged pictures (see in Figure 5.3 & 5.6).

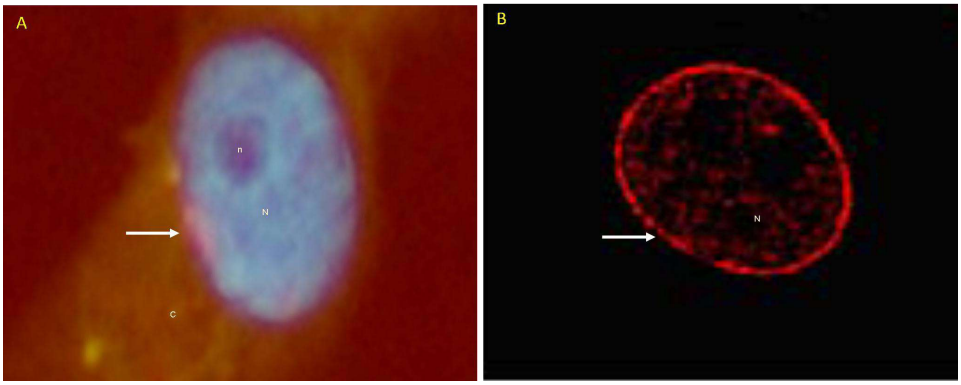

Figure 5.6. Comparison between transfected Vero cells with pD603-Dasher-VP_NLS of PmeHDV, 4 dpt using combination of three filters (Ex358/Em461 (blue), Ex 488/Em 520 (green), Ex 591/Em618 (red)) in this present study (A) and the previous study of human cells (B) (showed Imp -1 (red) accumulates at the nuclear membrane indicated by arrows, encircling the nucleus (blue). -tubulin stained green during interphase in the cytoskeleton (Verrico *et al*., 2016).

It is possible that the Vero cell lines might be inappropriate for these NLS but there does seem to be cytoplasmic signals at least, a build-up of fluorescence at the nuclear membrane like the experimental protein is conjugated to importin beta-1 (Imp -1) (Verrico *et al*., 2016). The presence of fluorescent spots within the cytoplasm as well as at the nuclear membranes

of the transfected Vero (Figure 5.4 & 5.6, respectively) suggested that some signals, perhaps entered the nuclei. Incubating transfected Vero cells longer (>4 days) before being observed might have improved the results.

5.5 CONCLUSIONS

This present study found that the nuclear location signals (NLSs) potentially did enter the nuclei, but this present fluorescent study was not sensitive enough to be confident in detecting differences in NLS-transfected-cells under different filters. Strong fluorescence appears to be in the cytoplasm of transfected Vero were similar with that of previous study using S2 & C6/36 (Zhou *et al*., 2009) and that of using Sf9 (Hanapi *et al*. 2017). Light red purple at the nuclear membrane of transfected Vero nuclei **might be** importin/NLSs complex similarly with that was detected in human cancer cells (Verrico *et al*., 2016). However, we are not convinced enough to state that the fluorescences were related to the NLSs of PmeHDV. Therefore, Vero cell lines are not sensitive enough to identify the location of NLSs of PmeHDV. The study of virus-host interaction using proxy cell cultures as models remains a major challenge.

CHAPTER 6

Experimental Infection of Raw Cell Line With *Chequa iflavirus* and *Athtab bunyavirus*

6.1 INTRODUCTION

Viruses within the genus *Iflavirus* belong to family *Iflaviridae* (ICTV, 2009), under the order Picornavirales (Le Gall *et al*., 2008). Iflaviruses are originally discovered in economic important insects such as honeybees and silkworms. Previously, iflaviruses have been isolated from Lepidoptera, Hymenoptera, Hemiptera, and Arachnida. Iflaviruses are non-enveloped viruses, appear as icosahedral particles, approximately 30 nm and possess a single-stranded RNA genome of positive polarity. In general, iflavirus replication and accumulation occurs in the cytoplasm, as has been concluded from the incorporation of RNA precursors and often found densely packed in cytoplasmic areas in the infected cells. More and more iflavirus sequences are being described, but molecular, virological, and biological studies so far are still restricted to insect iflaviruses. Studies of host range have hardly been performed for iflaviruses, which makes it difficult to state that the organisms from which the virus was isolated is the original host in which it evolved (van Oers, 2010).

Viruses within the genus *Bunyavirus* belong to the family *Bunyaviridae* appear as spherical particles, approximately 90-100 nm and enveloped with glycoprotein surface projections. The virions contain three unique segments of negative-sense, single-stranded RNA in the form of circular ribonucleoprotein complexes (nucleocapsids) and a transcriptase enzyme. Viruses have the ability to interact genetically with certain other closely related viruses by genome segment reassortment. Like iflavirus, bunyavirus replication occurs in the cytoplasm of the infected cell and mature by budding into smooth-surface vesicles in or near the Golgi region (Bishop *et al*., 1980). The majority of the Bunyaviridae are transmitted by biting arthropods; bunyaviruses by mosquitoes and gnats, nairoviruses and uukuviruses by ticks, and phleboviruses by sandflies (phlebotomines) and gnats (Bishop & Shope, 1979) and bunyaviruses has been associated with some diseases infecting humans (Shope (1985) *in* Elliott, 1990). Numerous bunyaviruses isolated from crustaceans are present across order Bunyavirales without formal taxonomical description (Bojko, *et al*., 2019).

Recently, isolated crustacean viruses, including *Chequa iflavirus* and *Athtab bunyavirus*, were discovered in redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Queensland, Australia (Sakuna *et al*., 2017a & 2018). These discoveries provide important

insights into the potential diversity, taxonomy and evolution within the family *Iflaviridae* and within the family *Bunyaviridae*. These viruses might be responsible for the stress-related mortalities, which caused mortality up to 20–40% after about three weeks subsequent to a stress event. Virus-free animals are needed to confirm River's postulates on viral cause and effect. Also, an understanding of how viruses replicate and cause asymptomatic or symptomatic conditions in a species-specific model is required to prevent viral disease outbreaks (Nomaguchi & Adachi, 2010). Relationships between viruses in insects (Robles-Sikisaka *et al*., 2001), particularly crickets (Mari *et al*., 2002, La Fauce and Owens, 2008) and crustaceans have been reported. However, due to a lack of *Chequa iflavirus* and *Athtab bunyavirus*-free crayfish (preliminary testing shows > 85% infection rate) (Sakuna *et al*., 2017c) and with no cell cultures, an alternative cell culture to propagate the viruses is needed.

It is possible that a lack of correct cell surface receptors on non-host cell lines could be a barrier for viruses to enter the cell. Macrophages are phagocytic, which may bypass the requirement for the correct cell surface receptor. As such, an alternative cell culture from mammalian cells could be considered such as mouse macrophage Raw-Blue™ ISG. This cells line is a very common mammalian cell line used to study mechanism involved during virus infection and replication in host cells and for the identification of Interferon type I, with the goals of finding out precise drug interference (Sooreshjani *et al*., 2018; Rees and Lowly, 2018) and proven to demonstrate high efficiency of infection in some previous studies.

In this study, RAW-Blue™ ISG cells (from murine RAW 264.7 macrophage cell line) was used to propagate *Chequa iflavirus* and *Athtab bunyavirus*. In previous chapters (see Chapter 4 and 5), an insect cell line (C6/36) and a mammalian cell line (Vero) were examined to study crustacean viruses. Both studies demonstrated negative results. In this chapter, we will describe an experimental infection of *Chequa iflavirus* and *Athtab bunyavirus* to mouse macrophage reporter cells or RAW-Blue cells.

6.2 MATERIALS AND METHODS

6.2.1 Preparation of viral inoculum

Viral inoculum was collected by withdrawing haemolymph from the arthrodial membrane under chephalothorax of some known *Chequa iflavirus* and *Athtab bunyavirus* infected redclaw crayfish (*C. quadricarinatus*). The area was swabbed or flushed with 70 % ethanol to

sterilize the surface and a 1 ml gauge sterile hypodermic needle and syringe was used to extract around 1 ml of haemolymph. The collected haemolymph samples were stored at -20 °C until used. An anticoagulant (citrate/EDTA anticoagulant, consisting of 0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA; pH 5.4) was drawn (1:10) into the 1 ml syringe prior to taking haemolymph, so that the haemolymph could be drawn directly into the anticoagulant.

6.2.2 Cells preparation and maintenance

Mouse macrophage interferon (IFN) reporter cells or Raw-Blue™ ISG (InvivoGen, USA) were recovered from the stock and sub-cultured following the supplier's guidelines. The cells were used to propagate *Chequa iflavirus* and *Athtab bunyabvirus*. The cell cultures were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Australia) supplemented with 10 % foetal bovine serum (FBS), penicillin (200 U/ml), streptomycin (200 µg/ml), kanamycin (80 µg/ml), polymyxin B (30 U/ml) and 1x amphotericin B (Sigma-Aldrich, Australia), Normocin (100 g/ml) and L-glutamine (2 mM). The cells were propagated in 37 C with 5 % $C0_2$ with media changed when required.

The cells were sub-cultured when monolayers formed. For passaging, the old medium was aseptically decanted, and the cells were washed with 1x PBS twice. The cells were detached by scraping them using cell scrapers (Life Sciences, Australia) for 2 mins until 75 % of the cells detached. Fresh DMEM without supplement was added into the flask and the cells were pipetted up and down to break any clumps. The cells then were homogenized in complete fresh medium and were equally distributed in two new culture flasks. Live cells images were taken using an inverted microscope (Olympus, Japan) equipped with a digital camera and Q Capture P Software (Olympus, Japan).

6.2.3. Virus susceptibility

Six T75 flasks (Sarstedt, Germany) were used to culture RAW-Blue cells (approximately 10^5 cells/mL) to give confluence approximately 70% monolayer. After the media removal, cells were scraped and resuspended with 5 mL fresh media and 1 ml of viral inoculum were added into 3 flasks (labelled as infected) and the other 3 flasks received 1 ml 1x PBS (labelled as non-infected). Cells were incubated at 30 °C. Passaging and samples collection from each flask were conducted at 2, 4, and 7 days post infection (dpi).

6.2.4. Collection of cell samples

Raw-Blue™ ISG cells were harvested, centrifuged and re-suspended with 1x PBS (1ml), to a concentration approximately 5x10 ^{5} cells/ml. Approximately, 800 µl of cell suspension from each flask was prepared for molecular assay by PCR and the remaining cells suspension (200µl) was added on to each slide chamber. The chambers were centrifuged at 500 rpm for 5 min using Cytospin4 Cytocentrifuge (Thermo, Australia).

6.2.5 Mayer's haematoxylin & eosin (H & E)

Cytospinned cells were air-dried for 1min, fixed in methanol and stained with routine H & E to observe cellular morphological changes (Drury & Wallington, 1980 *in* Hayakijikosol & Owens, 2013).

6.2.7 Confirmation of *Chequa iflavirus* and *Athtab bunyavirus* exposure by RT-PCR

RT-PCR was conducted to examine the presence of *Chequa iflavirus* and *Athtab bunyavirus* using primer sets designed by Sakuna *et al*. (2017b) (Table 1). RNA was extracted from both infected and non-infected RAW-Blue cells with reverse transcriptase PCR (RT-PCR) one step SensiFast SYBR no Rox kit (Bioline, Australia) according to manufacturer's instructions. Cells and supernatant were collected from the flasks and spun down at 583*g* for 5min. The concentration of DNA in each sample was estimated using a spectrophotometer (Eppendorf, Australia) by measuring the absorbance of UV light at 260 nm (A_{260}).

Table 6.1. Primers used in PCR to confirm the infection of *Chequa iflavirus* and *Athtab bunyavirus* in RAW-Blue™ISG cells.

Virus tested	**Primers (nucleotides) (5'- 3')**	**Expected amplicon length (bp)**	**Annealing temperature (C)**	**References**
Chequa iflavirus	865F CTCCTTCTGGGTGCGTTTA 967R ATACTCTGGCGCATGCTCTC	104	59	Sakuna *et al.* (2017a & b)
Athtab bunyavirus	2889F GATCCGCAGAATACGAGGG 3095R ACAACTGTCTGGCTATGGC	207	58	Sakuna *et al*., (2018)

Each RT-PCR reaction using MyFi master mix (Bioline, Australia) consisted of 5µl of reaction buffer (5x), MyFi Taq polymerase (2 µl/ml), 1 µl of each primer F & R (Table 6.1), 2.5 µl of

DNA template and nuclease-free water to a final volume of 25 µl. Viral inoculate (8.41 x 10^8 copies/µl) from infected crayfish was used as a positive control, non-infected RAW-Blue cell cultures (from 2, 4 and 7 dpi) were used as a negative control, and no reverse transcriptase (NRT) samples were used as the negative controls for RT-PCR. A 50 bp marker was used for visualization of gel electrophoresis. The RT-PCR profile consists of 45 C (20 mins) reverse transcription; 95 C (1 min) polymerase activation; and 40 cycles of 95 C for 10s, 60 C for 10s; 72 C for 30s.

6.2.7. Quantitative assay of *Chequa iflavirus* by RT-qPCR

Prior to the RT-qPCR assay, a standard curve was generated using a serial dilutions from $1x10^9$ to $1x10^0$ copies of positive control plasmid to determine the sensitivity of the RT-qPCR assay for *Chequa iflavirus* as developed by Sakuna *et al*., 2017b. A set of forward and reverse primers used was that of Sakuna *et al*., 2017b. The standard curve can be seen in Appendix 7. Each RT-qPCR reaction for generating standard curve using SensiFAST SYBR No Rox one step kit (Bioline Australia) consist of 10 µl SensiFAST SYBR No Rox one step kit (2x), 0.8 µl of each primer for *Chequa iflavirus* (Table 6.1), 0.2 µl of reverse transcriptase, 0.4 µl RiboSafe RNAse inhibitor, 5 µl of RNA template and DEPC water to a final volume of 20 µl.

A quantitative assay for measuring *Chequa iflavirus* load was conducted by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Amplification was performed in a thermocycler with 3 step cycling (RotorGeneQ, Qiagen, Australia). The RT-qPCR profile consist of an initial holding at 95 °C, 3 mins, 3 steps cycling of the first hold at 95 °C, 5s, the second hold at 59 °C, 10s and final hold (acquiring) at 72 °C,10s (repeated 40 times), and a melt step (ramp from 50 °C to 99 °C, held for 90 secs in the first step). The amplified products were also run on a high-speed gel electrophoresis system (BioProduct, Biotec Australia) and visualized on to 2% agarose gel containing 0.05 µl/ml GelRed (Biotium USA) linked to Gensnap software.

6.3 RESULTS

6.3.1 Cytology

Examination of cell images following H&E staining, showed vacuole formation in both uninfected and infected RAW-Blue cells (see Figure 6.1. below).

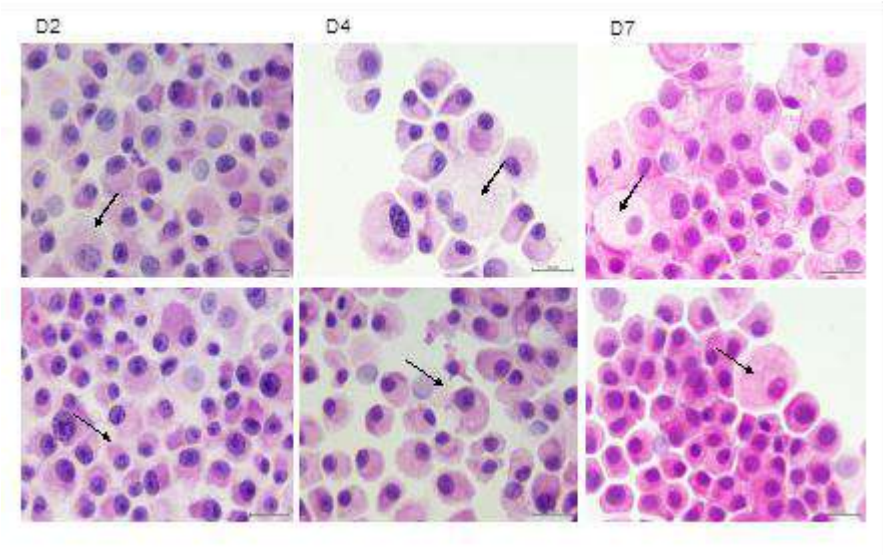

Figure 6.1. H & E staining of infected (top row) and (*Chequa iflavirus* & *Athtab bunyavirus*)-uninfected RAW-Blue™ ISG (bottom row) of cell culture (40x), visualised at day 2, 4 and 7 dpi. Vacuolations were identified in both cell groups (see the arrows).

6.3.2 Molecular Studies (RT-PCR and RT-qPCR)

Molecular assays by RT-PCR for both *Chequa iflavirus* and *Athtab bunyavirus* showed that the primers used work to detect the presence of the viruses in RAW cells (Figure 6.2). The quantitative assay by RT-qPCR for *Chequa iflavirus* indicated that the replication of the virus occurred within 2-4 dpi (Table 6.2).

Table 6.2. The average threshold (C_T) values and viral copy numbers for *Chequa iflavirus* (copies/µl) in RAW-Blue™ ISG cells.

Samples	2 dpi		4 dpi		7 dpi	
	X 10^9 Copies/µl	C_T	X 10^9 Copies/µl	C_T	X 10^9 Copies/µl	C_T
1	1.04	4.96	3.01	3.68	2.26	4.06
2	1.16	4.83	3.01	3.68	1.141	4.04
3	1.17	4.81	3.02	3.67	1.95	4.59
Mean	1.12	4.87	3.01	3.68	1.95	4.23

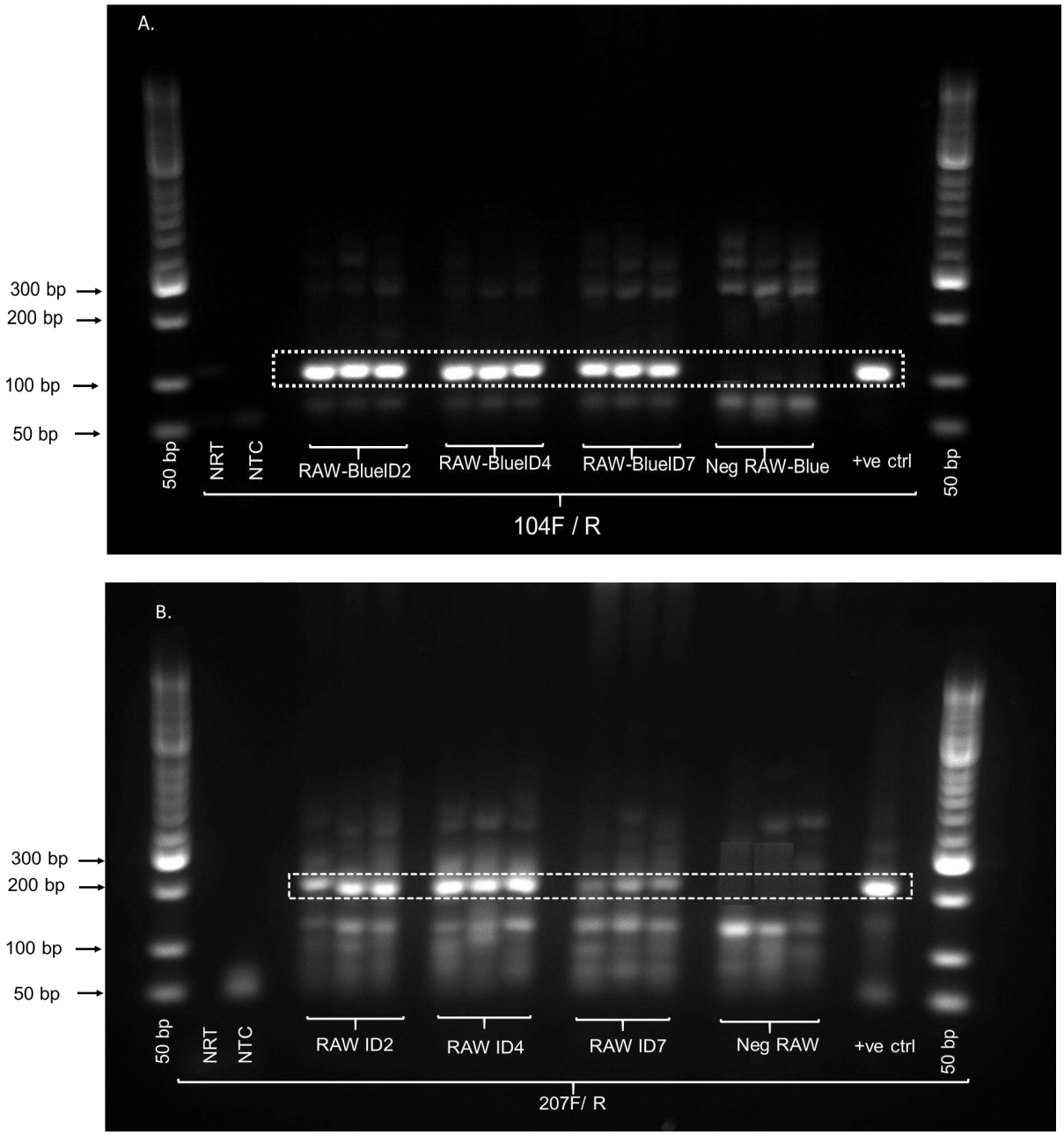

Figure 6.2. RT-PCR of *Chequa iflavirus* in RAW-Blue™ ISG using 104F/R (A) and *Athtab bunyavirus* in RAW-Blue™ ISG using 207F/R (B). The expected band size (bp) for each RT-PCR is shown in the white dashed boxes. ID: infected day.

6.4 DISCUSSION

In this study, the susceptibility of RAW-Blue™ ISG cell culture to *Chequa iflavirus* and *Athtab bunyavirus* from redclaw crayfish (*C. quadricarinatus*) was examined. Initially, H&E staining was conducted to attempt to identify the presence of viruses' infection, following by RT-PCR and RT-qPCR assays.

Cytological assay using H & E staining failed to confirm the presence of cytopathic effect (CPE) in the infected RAW-Blue cell line (Figure 6.1). RT-PCR using primer set for both viruses (Table 6.1) suggested that both *Chequa iflavirus* and *Athtab bunyavirus* can infect RAW-Blue™ ISG cells (Figure 6.2) and can maintain their survival in the cells until 7dpi.

Chequa iflavirus and *Athtab bunyavirus* positive RAW-Blue cell lines produced specific amplification in the RT-PCR (Figure 6.2). Whereas, negative control RAW-Blue cells and NRT control were negative for both RT-PCR and RT-qPCR (Figure 6.2). Possibly the stronger amplicons at 2 and 4 dpi on the RT-PCR (Figure 6.2 B), showed amplification of *Athtab bunyavirus* due to virus immediate early (IE) gene expression. The *Athtab bunyavirus* PCR was for RNA-dependent RNA polymerase (an immediate-early gene (IEG), detecting mRNA as well as viral genome, but mRNA of RNA polymerase expression shuts down >D4 when other genes are then transcribed. Immediate early genes are a class of genes activated rapidly and transiently and constitute an important mechanism for specific early genomic responses to signalling cascades (Poirier *et al*., 2008).They represent a standing response mechanism that is activated at the transcription level in the first round of response to stimuli, before any new proteins are synthesized. Therefore, it is important to design new PCRs to look at a later genes & see if PCR amplicons of these increase.

The assay by RT-qPCR in this present study only carried out for *Chequa iflavirus* as the quantitative assays (RT-qPCR) was only available for *Chequa iflavirus* (Sakuna *et al*., 2017b) at the time this study was undertaken. The chequavirus RT-qPCR gave a low limit of detection and high diagnostic sensitivity and it has the melt curve as a confirmation step. Gel visualisation of *Chequa iflavirus* post RT-PCR was unclear to state the status of virus replication between 4 and 7 dpi (Figure 6.2 A). RNA-dependent RNA polymerase (RdRp) domain (nt 8383 – 9873) of *Chequa iflavirus* was chosen as the target gene (Sakuna *et al*., 2018).

RT-qPCR (Table 6.2) to calculate viral copy number of *Chequa iflavirus* confirmed a significant replication (approximately 300%) of *Chequa iflavirus* from 2 dpi to 4 dpi, accounting from 1.12 $x10^9$ to 3.08 $x10^9$ copies per µl and declined in number (by30%) at 7 dpi to 1.95 x 10^9 copies/µl (Table 6.2). The declining copy number **might** suggest that between 4-7 dpi, a pattern recognition of the virus to stimulate the interferon gene in RAW-Blue cell to destroy and leave no viable virus or enough traces of RNA to be detected by RT-PCR.

The result of quantitative assay for *Chequa iflavirus* in RAW-Blue™ISG cell culture by RT-qPCR of in this study has similar pattern with that of quantitative assay for Penaeus merguiensis hepandensovirus (PmeHDV) in C6/36 cell culture by TaqMan qPCR, in that both examined viruses showed initial replication in cell culture in less than 7 dpi (see in Table 4.2 in chapter 4 and Table 6.2 in this chapter).

6.5 CONCLUSIONS

The cellular changes visualized via the H & E staining were not dramatic enough to confidently detect viruses in mouse RAW-Blue™ISG cell cultures. Molecular analyses by RT-PCR suggested that RAW-Blue cell line was potentially susceptible to *Chequa iflavirus* and *Athtab bunyavirus*. The research to use RAW-Blue cell line to propagate crustacean viruses and/or the nuclear location signals (NLSs) needs further investigation.

CHAPTER 7
General Discussion

According to FAO statistics in 2017, the global production of crustaceans for 2018, including cultured prawns, was expected to be around 8.63 million tonnes (Shinn et al., 2018). Following the establishment of crustacean industry and aquaculture, known viral pathogens, newly identified, and emerging viruses will pose future threats to crustacean aquaculture.

Crustaceans are particularly vulnerable to viral diseases, as their immune system lacks the antigen/antibody system of vertebrates, and consequently, classic vaccination against lethal viruses is not possible. To understand and ultimately control the viral diseases in crustacea, the tools to investigate these pathogens must be developed. Given the tremendous advances achieved in human and veterinary virology, such great feats in crustacean virology greatly depends on the development of cell cultures that permit *in vitro* cultivation of intracellular pathogenic agents (Claydon & Owens, 2008).

Research studies on viral pathogens of crustaceans have attracted interests for decades with a growing number of published reports since the last 10 years. Different viruses, particularly of freshwater crayfish, including Cherax quadricarinatus hepatopancreatic reovirus (Hayakijikosol & Owens; 2011), Pacifastacus leniusculus bacilliform virus (PlBV) (Longshaw *et al.*, 2012), white spot syndrome virus (WSSV or WSBV) (Zuo *et al*., 2015), infectious pancreatic necrosis virus (IPNV) (Yu *et al*., 2014), infectious hypodermal and haemotopoietic necrosis virus (IHHNV) (Chen *et al*., 2017), *Chequa iflavirus* (Sakuna *et al*., 2017a), *Athtab bunyavirus* (Sakuna *et al*., 2018), to name but few.

The cell cultures used in these studies were originally derived from mosquito larvae (C6/36), green monkey kidney (Vero), and mouse macrophage interferon reporter cells (RAW-Blue™ ISG) and they were used to study the interaction between crustacean viruses and their host cells employing different methods.

The logic of the use of the C6/36 cell line to attempt the culture of HDV was as follows:

The mosquito (Aedes albopictus) C6/36 cell line has previously been examined for *in vitro* propagation of crustacean viruses, including Macrobrachium rosenbergi nodavirus (MrNV) and extra small virus (XSV) (Sudhakaran *et al*., 2007; Hayakijikosol & Owens, 2013), Penaeus monodon hepandensovirus (PmoHDV) (Sriton *et al*., 2009; Madan *et al*., 2013), white spot syndrome virus (WSSV) and yellow head virus (YHV) (Sriton, 2009; Gangnonngiw,

2010) and taura syndrome virus (TSV) (Arunrut *et al*., 2011). Of these, only Madan *et al*. (2013) purported to confirm the success of *in vitro* propagation of a crustacean virus (PmoHDV) in C6/36. This apparent success needed to be replicable for the *in vitro* propagation of other hepandonsoviruses (HDV) strains within subfamily Densovirinae.

The logic for the use of plasmids for transfection of inserted putative NLSs of PmeHDV-pDNA into African green monkey kidney (Vero) cell culture was as follows;

The Densovirinae are intranuclear viruses that require cells in S-phase for all or most of their replication and assembly. In order to gain entry into the cell nucleus via the nuclear pore complexes (NPCs), the viruses need to bind their nuclear location signals (NLSs) to importins (Imp) to transport in and out of the nuclei (Jans, *et al*., 2000). The NLSs aid navigation of viruses to the nucleus and leads to further translocation. The research on the interaction between hepandensoviruses and the host cells remains at the bioinformatics analysis level (Owens, 2013) except for a single therapeutic study using ivermectin to block densovirus NLS by Nguyen *et al*. (2014).

The bioinformatical analyses of NLSs of crustacean viruses, to date was described only from hepandensoviruses and their functioning should be experimentally confirmed (Owens, 2013). The only crustacean virus NLSs identified to date, was in insect cells of Spodoptera frugiperda (Sf9) and the protein was Macrobrachium rosenbergii nodavirus capsid protein or MrNVc (Hanapi *et al*., 2017).

The logic for the use of mouse macrophage cells (RAW-Blue™ ISG) to attempt culture of *Chequa iflavirus* and *Athtab bunyavirus* was as follows. One of the main blocks to viruses infected cells is the lack of appropriate cell surface receptors to allow ingress of the virus. The phagocytic nature of macrophages allows the by-passing of the correct viral receptor, thus allowing cellular infection. Therefore, this was worth at least a try.

The different approaches trialled had variable success.

Although, the *in vitro* propagation of PmeHDV in C6/36 was not successful, new observations of hepandensovirus were made, in that, genetic variation and the length of the ORF-1 (NS2) protein within hepandensoviruses might have contributed to the severity of infection and to the viruses' replicative forms. This thesis also proposes a more rigorous requirement for claims of successful propagation of viruses in cell lines. More scrutiny should be applied to cell cultures before being reported as virus-susceptible cell cultures. The

minimal acceptable evidence should be qPCR for late genes for enumerating viral copies with a subsequent specificity check on amplicons.

A critical method to detect the presence of NLSs in cell cultures is transfection, by which the introduction of foreign DNA into cell culture is conducted using vector pDNA via a transfection reagent. The techniques used in this present study of transfecting pDNA into Vero cell culture may not be sensitive enough to be confident that intracellular fluorescence is real and therefore this study was not able to test the NLS of PmeHDV using vector pDNA. We suggest that the strong fluorescence that appears to be in the cytoplasm of the transfected Vero cells were similar to that seen in a previous study using S2 & C6/36 cells (Zhou *et al*., 2009) and to that seen when using Sf9 (Hanapi *et al*. (2017). Light red-purple at the nuclear membrane of transfected Vero nuclei might be importin/NLSs complexes similar to those detected in human cancer cells (Verrico *et al*., 2016). The investigation of interaction between viruses and their host's cells using proxy cell cultures remains a major challenge.

This experimental exposure of RAW-Blue™Interferon Stimulated Gene (ISG) cell lines to *Chequa iflavirus* and *Athtab bunyavirus* paved ways to further investigation of NLSs of crustacean viruses. In this present study, H & E staining failed to show the signs of vacuoles (CPE) in cells. Interestingly, RT-PCR results demonstrate that both *Chequa iflavirus* and *Athtab bunyavirus* can infect RAW-Blue™ ISG cells and can survive in the cells until 7 dpi. The stronger amplicons at 2 and 4 dpi showed amplification of *Athtab bunyavirus* due to the virus immediate early (IE) gene expression. The RNA-dependent RNA polymerase on which the RT-PCR was based turned on mRNA of the viral genome. However, the mRNA of RNA polymerase expression shuts down after 4 dpi, after which other genes would be transcribed if replication was continuing. Further assessment of RAW-Blue™ISG cell cultures for studying nuclear location signals (NLS) of *Chequa iflavirus* and *Athtab bunyavirus* would be valuable as this was a promising outcome.

These three approaches targeted different characteristics of the virus and cell line research, specifically; testing reported successful cell lines for crustacean viruses, utilising an understanding of replication requirements to test a single step of replication with plasmid expressed proteins and utilising a characteristic of a cell line to bypass an identified issue with viral infection in general. This diverse approach was valuable given the lack of existing crustacean cell lines indicates this is a tough nut to crack.

The first and most general lesson to come from this thesis, that should be more broadly applied to all future cell culture infection work, is that there are flaws in accepting reports of

successful use of cell culture for replication without late gene analysis. Given gene selection for PCR also limited the analysis of the testing of the RAW-Blue™ ISG cell cultures, all future work using qPCR to confirm replication should utilise late gene analysis. The use of pDNA vectors requires improvements in sensitivity to confirm possible NLS interaction with host cells. However, once this technology matures a bit more, this is an area that has good potential for studying the stages of replication. Overall, the most promising work utilised RAW-Blue™ ISG cell cultures and, with redesigned gene targets, more work should be done using a variety of crustacean viruses with this cell line. Ultimately understanding the underlying mechanisms involved during virus infection and replication could someday allow us to block virus infection in crustacean aquaculture, which makes continued work in this difficult area of research worthwhile.

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Appendices

Appendix 1

Staining Protocols

Haematoxylin and Eosin (H &E)

1.	Xylene	2 mins
2.	Xylene	2 mins
3.	Ethanol	2 mins
4.	Ethanol	1 min
5.	Ethanol	1 min
6.	Water Wash	1 min
7.	Mayer's haematoxylin	4 min
8.	Water Wash	30 secs
9.	Scott's Tap water substitute	30 secs
10.	Water Wash	1 min
11.	Eosin (Young's)	2 mins
12.	Differentiate Eosin by washing tap water until cells appear red purple approximately	1 min
13.	Ethanol	10 dips
14.	Ethanol	10 dips
15.	Ethanol	1 min
16.	Xylene	1 min
17.	Xylene	2 mins
18.	Xylene	1 min
19.	Mount with DPX	
20.	Cover slipped	

Giemsa

1.	Take section to water	30 secs
2.	Place in 10% Giemsa stain	overnight
3.	Rinse in tap water	1 min
4.	Decolourise with 2% Acetic acid, greatly diluted	
5.	Air dry, mount with DPX and cover slipped	

Trypan Blue 0.4 %

1.	Mix 0.4% Trypan Blue and cell suspension (1:1)	
2.	Allow mixture to incubate at room temperature	2 mins
3.	Count cells in a haemocytometer under a microscope (Dead cells: stained blue)	

Appendix 2

Reagents

TN Buffer

Tris-HCl (20mM)	1.2 g
NaCl (400nM)	11.69 g

Make up fresh with deionised water to 500mL,
adjust pH to 7.4, autoclave and store at room temperature.

TE Buffer

Tris-HCl (10mM)	0.606 g
EDTA (0.1mM)	0.019 g

Make up with deionised water to 500mL, adjust pH to 8.0,
sterilise and store at room temperature.

1x Phosphate Buffered Saline (1x PBS)

Dissolve the following in 800 ml distilled H_2O

Potassium chloride (KCl)	0.2 g
Sodium chloride (NaCl)	8 g
Sodium hydrogen phosphate (Na_2HPO_4)	1.44 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.24 g

Adjust pH to 7.4 with HCl

Adjust volume to 1 l with additional distilled H_2O

Sterilise by autoclaving

Anticoagulant solution AC-1

0.45 M Sodium chloride (NaCl)	26.39 g
0.1 M glucose	18.1 g

30 mM trisodium citrate	8.823 g
10 mM citric acid	5.462 g
10 mM Ethylenediaminetetraacetic acid (EDTA)	2.9224 g

pH 7.0 (adjust with HCl or NaOH)

Makes 1000 ml

Appendix 3

Statistical Results

1. Table shows the statistical analysis of counting of cells with disrupted membranes (presumptive dead cells) (Chapter 4.3.3)

Between-Subjects Factors

		Value Label	N
Treatment	1.00	PmeHDV infected C6/36	12
	2.00	Uninfected C6/36	12
Passage	1.00		8
	2.00		8
	3.00		8

Tests of Between- Subjects Effects

Dependant variable: no. of cells

Source	Type III Sum of Squares	df	Mean Square	F	Sig
Corrected model	98923.133[a]	5	19784.627	13.797	.000
Intercept	624295.527	1	642295.527	435.365	.000
Treatment	31190.460	1	31190.460	21.751	.000
Day	40332.726	2	20166.363	14.063	.000
Treatment * Day	27399.948	2	13699.974	9.554	.001
Error	25881.280	18	1433.960		
Total	749029.940	24			
Corrected Total	124734.413	23			

a. R squared = .793 (Adjusted R squared = .736)

2. Table shows the statistical analysis of cell proliferation assay (Chapter 4.3.4)

Between-Subjects Factors

		Value Label	N
Treatment	1.00	PmeHDV infected C6/36	48
	2.00	Uninfected C6/36	48
Day	2.00	D2	24
	4.00	D4	24
	7.00	D7	24
	14.00	D14	24

Tests of Between- Subjects Effects

Dependant variable: OD

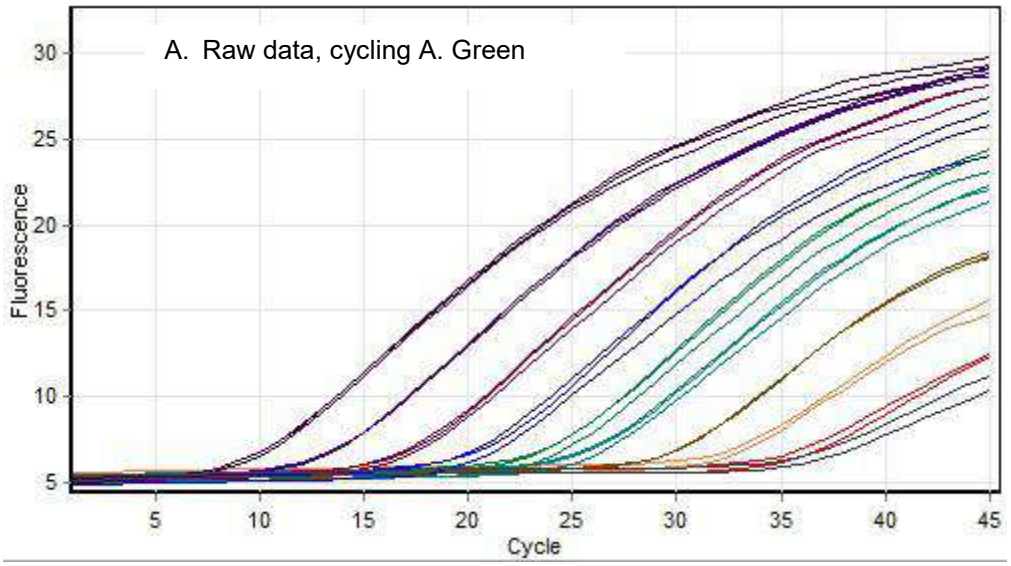
Source	Type III Sum of Squares	df	Mean Square	F	Sig
Corrected model	.196[a]	7	.028	27.935	.000
Intercept	6.175	1	6.175	6162.058	.000
Treatment	.035	1	.035	34.959	.000
Day	.140	3	.047	46.648	.000
Treatment * Day	.021	3	.007	6.879	.000
Error	.088	88	.001		
Total	6.459	96			
Corrected Total	.284	95			

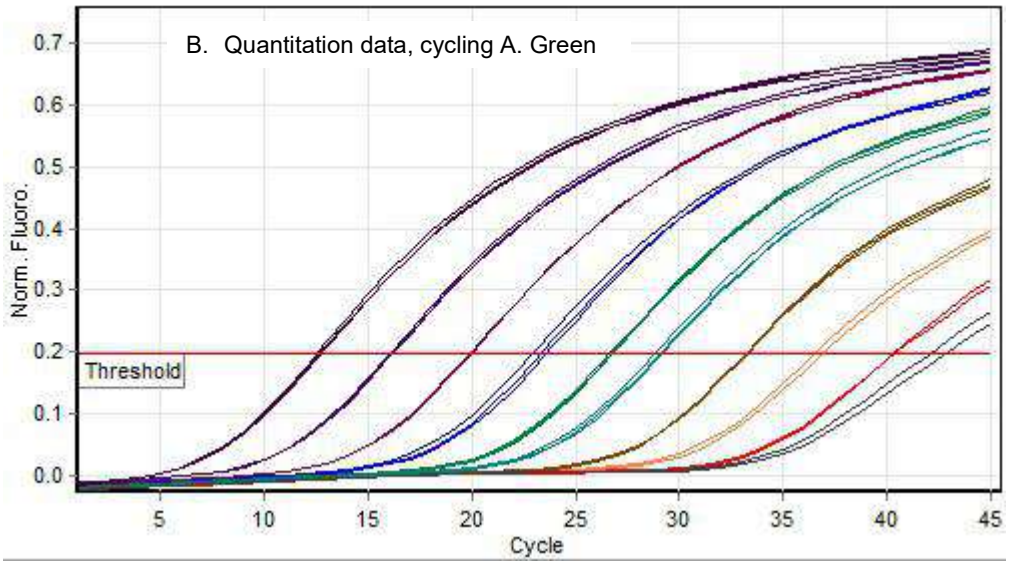
a. R squared = .690 (Adjusted R squared = .665)

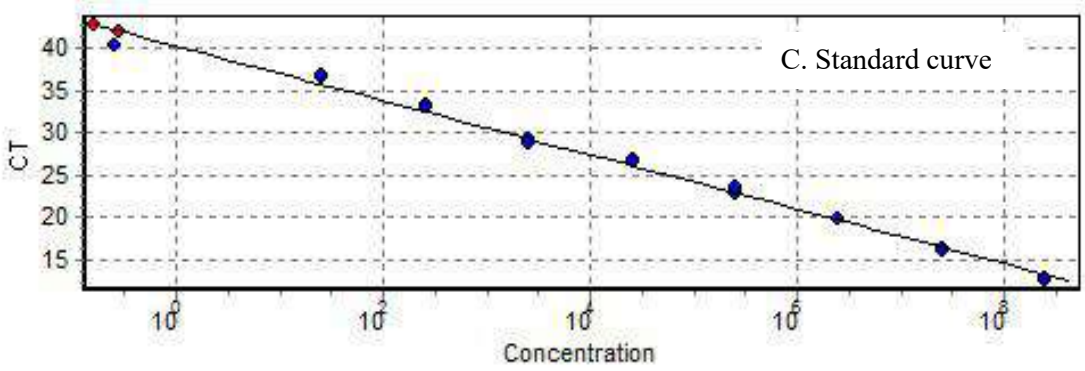
Appendix 4

qPCR Standard Curve for PmeHDV

Threshold	0.19754
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	conc= 10^(-0.312*CT + 12.552)
Standard Curve (2)	CT = -3.205*log(conc) + 40.231
Reaction efficiency (*)	(* = 10^(-1/m) - 1) 1.05113
M	-3.20519
B	40.23105
R Value	0.99618
R^2 Value	0.99237
Start normalising from cycle	4
Noise Slope Correction	No
No Template Control Threshold	% 0
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	




B. Quantitation data, cycling A. Green
Norm. Fluoro.
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Threshold
5
10
15
20
25
30
35
40
45
Cycle


C. Standard curve
CT
40
35
30
25
20
15
Concentration

Appendix 5

The feature, restriction and sequences of pD603-Dasher-VP_NLS (ATUM, USA)

1. The Feature of pD603-VP-NLS

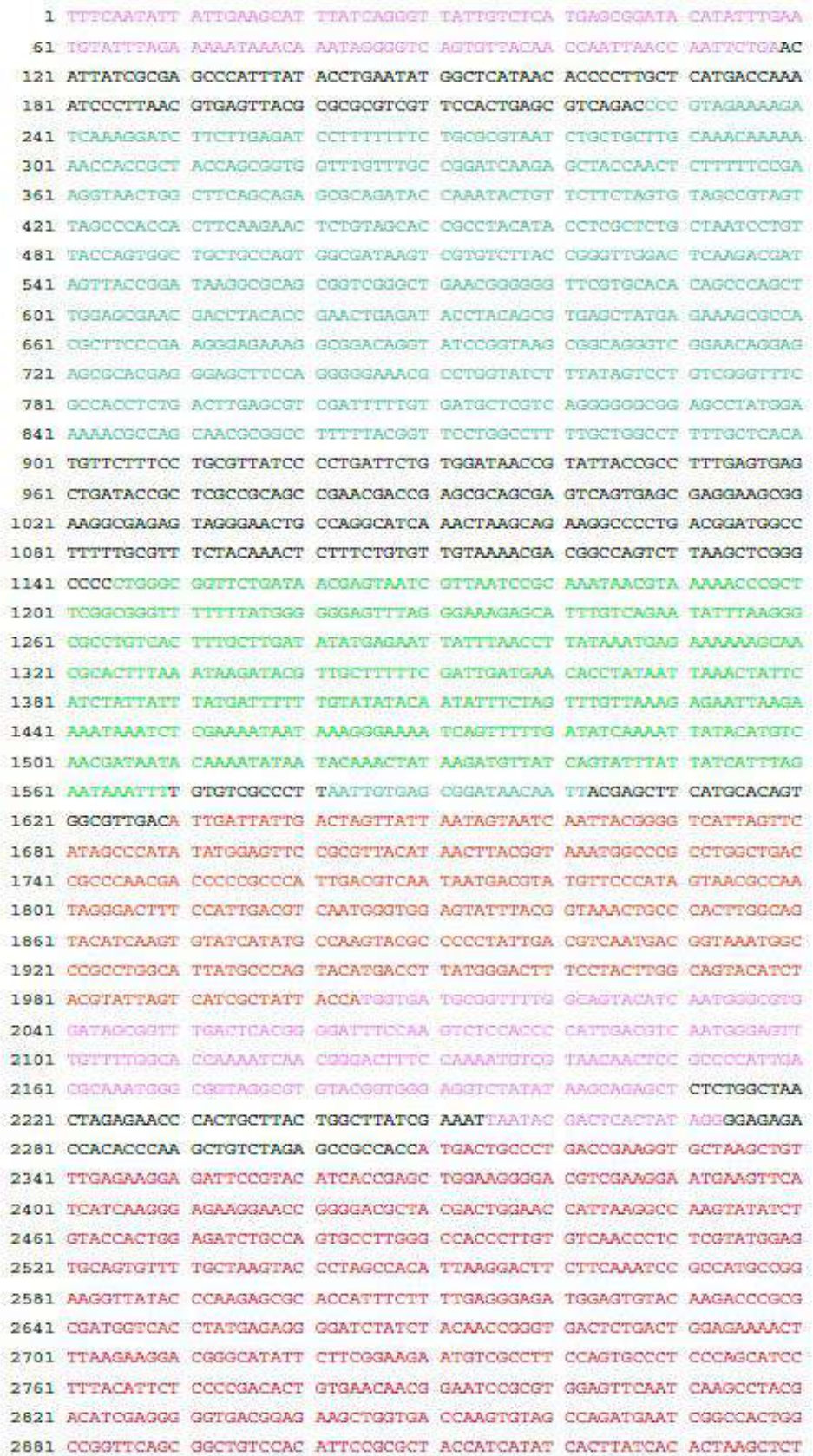
Name	Start	End	Direction
Insert: 145784	2310	3068	Forward
P_Amp	118	1	Reverse
Ori_pUC	228	900	Forward
Term_rpoC	1145	1264	Forward
Term_bla	1265	1569	Forward
LacO1	1582	1602	Forward
E_CMV	1630	2004	Forward
P_CMV	2005	2210	Forward
P_T7	2255	2273	Forward
pA_GH-bovine(min)	3097	3207	Forward
E_SV40	3343	3486	Forward
P_SV40E	3487	3659	Forward
Neomycin-r	3726	4517	Forward
pA_SV40E	4694	4822	Forward
Ampicillin-r	5728	4871	Reverse

2. The restriction map

Name	Sequence	5' Cut Positions
AccI	GTMKAC	4825, 4832
AclI	AACGTT	5167, 5540
AfeI	AGCGCT	4991
AlwNI	CAGNNNCTG	489, 2892
ApaI	GGGCCC	1142
ApaLI	GTGCAC	584, 5608
AseI	ATTAAT	1649, 2254, 3315, 5113
AvaI	CYCGRG	1135, 3865
AvrII	CCTAGG	3644
BclI	TGATCA	3695
BglI	GCCNNNNNGGC	1146, 1732, 1854, 1925, 5081
BglII	AGATCT	2471
BlpI	GCTNAGC	2332
BsaI	GGTCTC	2271(C), 3081
BspEI	TCCGGA	5027
BspHI	TCATGA	38, 170
BsrBI	CCGCTC	44(C), 969, 1590(C), 4467, 4521(C)
BsrDI	GCAATG	4087
BsrGI	TGTACA	2626
BssHII	GCGCGC	200, 4251
BstBI	TTCGAA	4536
BstEII	GGTNACC	2645, 2846

Name	Sequence	5' Cut Positions
BstXI	CCANNNNNNTGG	2485
BtsI	GCAGTG	2230(C), 2529, 4769(C), 5341, 5361(C)
EagI	CGGCCG	3760
EcoRV	GATATC	1482
HincII	GTYRAC	1500, 1626, 2503, 4833
KasI	GGCGCC	1259, 3853
MscI	TGGCCA	3936
NarI	GGCGCC	1260, 3854
NcoI	CCATGG	2002, 3551, 4286
NdeI	CATATG	1876
NruI	TCGCGA	127
NsiI	ATGCAT	3394, 3466
PciI	ACATGT	898, 1494, 2968
PspOMI	GGGCCC	1138
PstI	CTGCAG	3907
PvuI	CGATCG	5311
PvuII	CAGCTG	3960
RsrII	CGGWCCG	4370
SacI	GAGCTC	2210
SalI	GTCGAC	4831
SmaI	CCCGGG	3667
SnaBI	TACGTA	1982
SpeI	ACTAGT	1641
SphI	GCATGC	3392, 3484, 4259
SspI	AATATT	7, 1251, 1412
XbaI	TCTAGA	2295
XmaI	CCCGGG	3665
XmnI	GAANNNNTTC	3316, 5540
Acc65I	GGTACC	no cuts
AgeI	ACCGGT	no cuts
AscI	GGCGCGCC	no cuts
AseI	GCGATCGC	no cuts
BamHI	GGATCC	no cuts
BbsI	GAAGAC	no cuts
BsiWI	CGTACG	no cuts
BsmBI	CGTCTC	no cuts
ClaI	ATCGAT	no cuts
EcoRI	GAATTC	no cuts
FseI	GGCCGGCC	no cuts
HindIII	AAGCTT	no cuts
HpaI	GTTAAC	no cuts
KpnI	GGTACC	no cuts
MfeI	CAATTG	no cuts
MluI	ACGCGT	no cuts
NheI	GCTAGC	no cuts
NotI	GCGGCCGC	no cuts
PacI	TTAATTAA	no cuts
PmeI	GTTTAAAC	no cuts
PmlI	CACGTG	no cuts
PpuMI	RGGWCCY	no cuts
PshAI	GACNNNNGTC	no cuts
PspXI	VCTCGAGB	no cuts
SacII	CCGCGG	no cuts
SanDI	GGGWCCC	no cuts
SapI	GCTCTTC	no cuts
SbfI	CCTGCAGG	no cuts
SfiI	GGCCNNNNNGGCC	no cuts
SwaI	ATTTAAAT	no cuts
XhoI	CTCGAG	no cuts

3. The sequences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121 ATTATCGCGA GCCCATTTAT ACCTGAATAT GGCTCATAAC ACCCCTTGCT CATGACCAAA
181 ATCCCTTAAC GTGAGTTACG CGCGCGTCGT TCCACTGAGC GTCAGACCCC GTAGAAAAGA
241 TCAAAGGATC TTCTTGAGAT CCTTTTTTTC TGCGCGTAAT CTGCTGCTTG CAAACAAAAA
301 AACCACCGCT ACCAGCGGTG GTTTGTTTGC CGGATCAAGA GCTACCAACT CTTTTTCCGA
361 AGGTAACTGG CTTCAGCAGA GCGCAGATAC CAAATACTGT TCTTCTAGTG TAGCCGTAGT
421 TAGCCCACCA CTTCAAGAAC TCTGTAGCAC CGCCTACATA CCTCGCTCTG CTAATCCTGT
481 TACCAGTGGC TGCTGCCAGT GGCGATAAGT CGTGTCTTAC CGGGTTGGAC TCAAGACGAT
541 AGTTACCGGA TAAGGCGCAG CGGTCGGGCT GAACGGGGGG TTCGTGCACA CAGCCCAGCT
601 TGGAGCGAAC GACCTACACC GAACTGAGAT ACCTACAGCG TGAGCTATGA GAAAGCGCCA
661 CGCTTCCCGA AGGGAGAAAG GCGGACAGGT ATCCGGTAAG CGGCAGGGTC GGAACAGGAG
721 AGCGCACGAG GGAGCTTCCA GGGGGAAACG CCTGGTATCT TTATAGTCCT GTCGGGTTTC
781 GCCACCTCTG ACTTGAGCGT CGATTTTTGT GATGCTCGTC AGGGGGGCGG AGCCTATGGA
841 AAAACGCCAG CAACGCGGCC TTTTTACGGT TCCTGGCCTT TTGCTGGCCT TTTGCTCACA
901 TGTTCTTTCC TGCGTTATCC CCTGATTCTG TGGATAACCG TATTACCGCC TTTGAGTGAG
961 CTGATACCGC TCGCCGCAGC CGAACGACCG AGCGCAGCGA GTCAGTGAGC GAGGAAGCGG
1021 AAGGCGAGAG TAGGGAACTG CCAGGCATCA AACTAAGCAG AAGGCCCCTG ACGGATGGCC
1081 TTTTTGCGTT TCTACAAACT CTTTCTGTGT TGTAAAACGA CGGCCAGTCT TAAGCTCGGG
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1261 CGCCTGTCAC TTTGCTTGAT ATATGAGAAT TATTTAACCT TATAAATGAG AAAAAAGCAA
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1561 AATAAATTTT GTGTCGCCCT TAATTGTGAG CGGATAACAA TTACGAGCTT CATGCACAGT
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1861 TACATCAAGT GTATCATATG CCAAGTACGC CCCCTATTGA CGTCAATGAC GGTAAATGGC
1921 CCGCCTGGCA TTATGCCCAG TACATGACCT TATGGGACTT TCCTACTTGG CAGTACATCT
1981 ACGTATTAGT CATCGCTATT ACCATGGTGA TGCGGTTTTG GCAGTACATC AATGGGCGTG
2041 GATAGCGGTT TGACTCACGG GGATTTCCAA GTCTCCACCC CATTGACGTC AATGGGAGTT
2101 TGTTTTGGCA CCAAAATCAA CGGGACTTTC CAAAATGTCG TAACAACTCC GCCCCATTGA
2161 CGCAAATGGG CGGTAGGCGT GTACGGTGGG AGGTCTATAT AAGCAGAGCT CTCTGGCTAA
2221 CTAGAGAACC CACTGCTTAC TGGCTTATCG AAATTAATAC GACTCACTAT AGGGGAGAGA
2281 CCACACCCAA GCTGTCTAGA GCCGCCACCA TGACTGCCCT GACCGAAGGT GCTAAGCTGT
2341 TTGAGAAGGA GATTCCGTAC ATCACCGAGC TGGAAGGGGA CGTCGAAGGA ATGAAGTTCA
2401 TCATCAAGGG AGAAGGAACC GGGGACGCTA CGACTGGAAC CATTAAGGCC AAGTATATCT
2461 GTACCACTGG AGATCTGCCA GTGCCTTGGG CCACCCTTGT GTCAACCCTC TCGTATGGAG
2521 TGCAGTGTTT TGCTAAGTAC CCTAGCCACA TTAAGGACTT CTTCAAATCC GCCATGCCGG
2581 AAGGTTATAC CCAAGAGCGC ACCATTTCTT TTGAGGGAGA TGGAGTGTAC AAGACCCGCG
2641 CGATGGTCAC CTATGAGAGG GGATCTATCT ACAACCGGGT GACTCTGACT GGAGAAAACT
2701 TTAAGAAGGA CGGGCATATT CTTCGGAAGA ATGTCGCCTT CCAGTGCCCT CCCAGCATCC
2761 TTTACATTCT CCCCGACACT GTGAACAACG GAATCCGCGT GGAGTTCAAT CAAGCCTACG
2821 ACATCGAGGG GGTGACGGAG AAGCTGGTGA CCAAGTGTAG CCAGATGAAT CGGCCACTGG
2881 CCGGTTCAGC GGCTGTCCAC ATTCCGCGCT ACCATCATAT CACTTATCAC ACTAAGCTCT

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2941 CCAAAGACCG CGATGAGAGG AGAGATCACA TGTGCCTGGT GGAAGTGGTC AAGGCCGTCG
3001 ATCTCGATAC CTATCAGATG GGCGGCGGCG GCTCCGGCCC AAAGAAGAAG AAGAAGTACA
3061 GATAGTGAGG TTGAGGTCTC TAAAATCAGC CTCGACTGTG CCTTCTAGTT GCCAGCCATC
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3181 TTCCTAATAA AATGAGGAAA TTGCATCACA ACACTCAACC CTATCTCGGT CTATTCTTTT
3241 GATTTATAAG GGATTTTGCC GATTTCGGCC TATTGGTTAA AAAATGAGCT GATTTAACAA
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3361 GCTCCCCAGC AGGCAGAAGT ATGCAAAGCA TGCATCTCAA TTAGTCAGCA ACCAGGTGTG
3421 GAAAGTCCCC AGGCTCCCCA GCAGGCAGAA GTATGCAAAG CATGCATCTC AATTAGTCAG
3481 CAACCATAGT CCCGCCCCTA ACTCCGCCCA TCCCGCCCCT AACTCCGCCC AGTTCCGCCC
3541 ATTCTCCGCC CCATGGCTGA CTAATTTTTT TTATTTATGC AGAGGCCGAG GCCGCCTCTG
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3661 AGCTCCCGGG AGCTTGTATA TCCATTTTCG GATCTGATCA AGAGACAGGA TGAGGATCGT
3721 TTCGCATGAT TGAACAAGAT GGATTGCACG CAGGTTCTCC GGCCGCTTGG GTGGAGAGGC
3781 TATTCGGCTA TGACTGGGCA CAACAGACAA TCGGCTGCTC TGATGCCGCC GTGTTCCGGC
3841 TGTCAGCGCA GGGGCGCCCG GTTCTTTTTG TCAAGACCGA CCTGTCCGGT GCCCTGAATG
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3961 CTGTGCTCGA CGTTGTCACT GAAGCGGGAA GGGACTGGCT GCTATTGGGC GAAGTGCCGG
4021 GGCAGGATCT CCTGTCATCT CACCTTGCTC CTGCCGAGAA AGTATCCATC ATGGCTGATG
4081 CAATGCGGCG GCTGCATACG CTTGATCCGG CTACCTGCCC ATTCGACCAC CAAGCGAAAC
4141 ATCGCATCGA GCGAGCACGT ACTCGGATGG AAGCCGGTCT TGTCGATCAG GATGATCTGG
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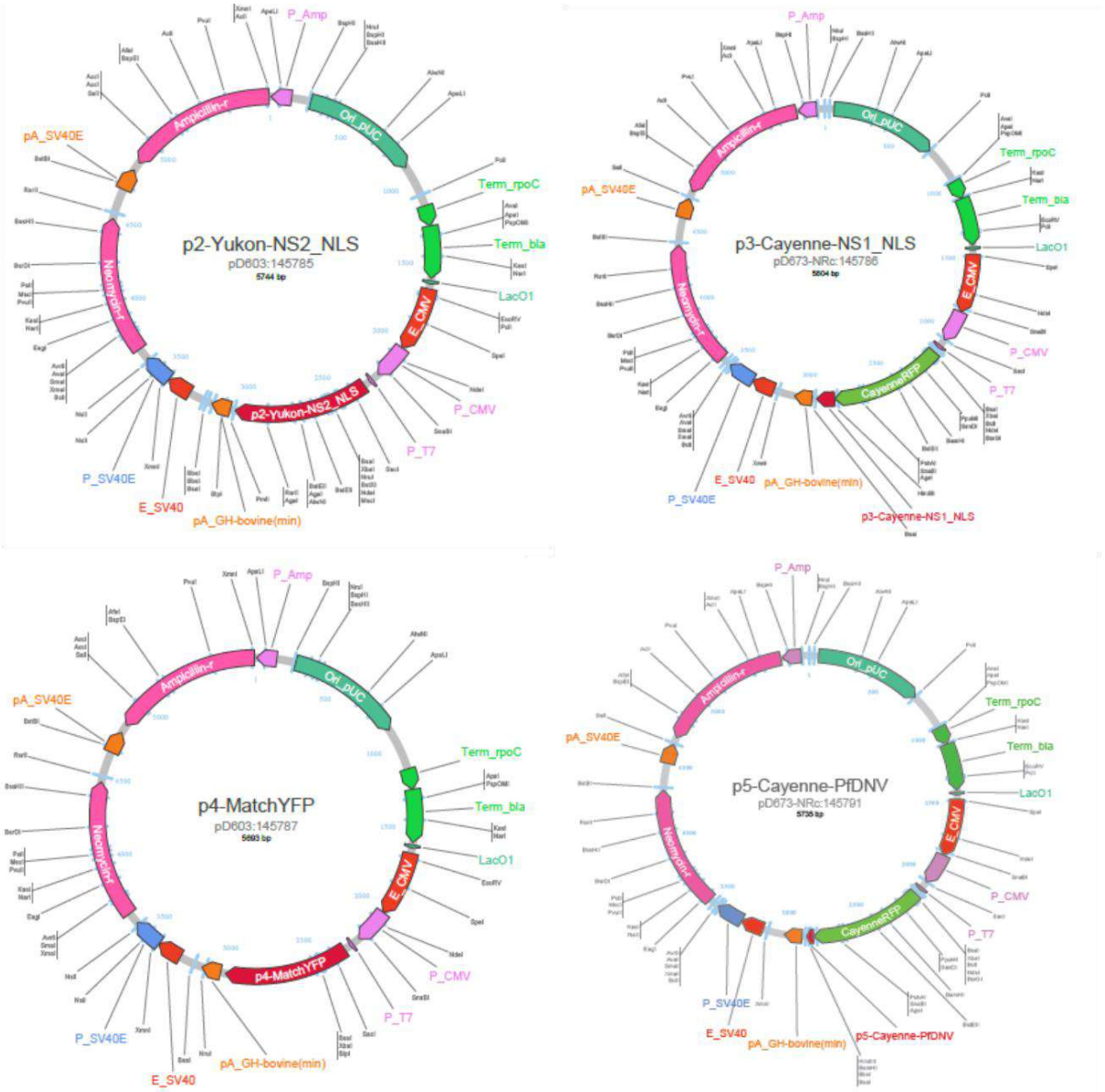
4261 CCGACGGCGA GGATCTCGTC GTGACCCATG GCGATGCCTG CTTGCCGAAT ATCATGGTGG
4321 AAAATGGCCG CTTTTCTGGA TTCATCGACT GTGGCCGGCT GGGTGTGGCG GACCGCTATC
4381 AGGACATAGC GTTGGCTACC CGTGATATTG CTGAGGAACT TGGCGGCGAA TGGGCTGACC
4441 GCTTCCTCGT GCTTTACGGT ATCGCCGCTC CCGATTCGCA GCGCATCGCC TTCTATCGCC
4501 TTCTTGACGA GTTCTTCTGA GCGGGACTCT GGGGTTCGAA ATGACCGACC AAGCGACGCC
4561 CAACCTGCCA TCACGAGATT TCGATTCCAC CGCCGCCTTC TATGAAAGGT TGGGCTTCGG
4621 AATCGTTTTC CGGGACGCCG GCTGGATGAT CCTCCAGCGC GGGGATCTCA TGCTGGAGTT
4681 CTTCGCCCAC CCCAACTTGT TTATTGCAGC TTATAATGGT TACAAATAAA GCAATAGCAT
4741 CACAAATTTC ACAAATAAAG CATTTTTTTC ACTGCATTCT AGTTGTGGTT TGTCCAAACT
4801 CATCAATGTA TCTTATCATG TCTGTATACC GTCGACCTCT AGCTAGAGCT TGGCGTAATC
4861 ATGGTCATTA CCAATGCTTA ATCAGTGAGG CACCTATCTC AGCGATCTGT CTATTTCGTT
4921 CATCCATAGT TGCCTGACTC CCCGTCGTGT AGATAACTAC GATACGGGAG GGCTTACCAT
4981 CTGGCCCCAG CGCTGCGATG ATACCGCGAG AACCACGCTC ACCGGCTCCG GATTTATCAG
5041 CAATAAACCA GCCAGCCGGA AGGGCCGAGC GCAGAAGTGG TCCTGCAACT TTATCCGCCT
5101 CCATCCAGTC TATTAATTGT TGCCGGGAAG CTAGAGTAAG TAGTTCGCCA GTTAATAGTT
5161 TGCGCAACGT TGTTGCCATC GCTACAGGCA TCGTGGTGTC ACGCTCGTCG TTTGGTATGG
5221 CTTCATTCAG CTCCGGTTCC CAACGATCAA GGCGAGTTAC ATGATCCCCC ATGTTGTGCA
5281 AAAAAGCGGT TAGCTCCTTC GGTCCTCCGA TCGTTGTCAG AAGTAAGTTG GCCGCAGTGT
5341 TATCACTCAT GGTTATGGCA GCACTGCATA ATTCTCTTAC TGTCATGCCA TCCGTAAGAT
5401 GCTTTTCTGT GACTGGTGAG TACTCAACCA AGTCATTCTG AGAATAGTGT ATGCGGCGAC
5461 CGAGTTGCTC TTGCCCGGCG TCAATACGGG ATAATACCGC GCCACATAGC AGAACTTTAA
5521 AAGTGCTCAT CATTGGAAAA CGTTCTTCGG GGCGAAAACT CTCAAGGATC TTACCGCTGT
5581 TGAGATCCAG TTCGATGTAA CCCACTCGTG CACCCAACTG ATCTTCAGCA TCTTTTACTT
5641 TCACCAGCGT TTCTGGGTGA GCAAAAACAG GAAGGCAAAA TGCCGCAAAA AAGGGAATAA
5701 GGGCGACACG GAAATGTTGA ATACTCATAT TCTTCCTT
```

Appendix 6

Other Four Constructed Plasmids in This Study (ATUM, USA)

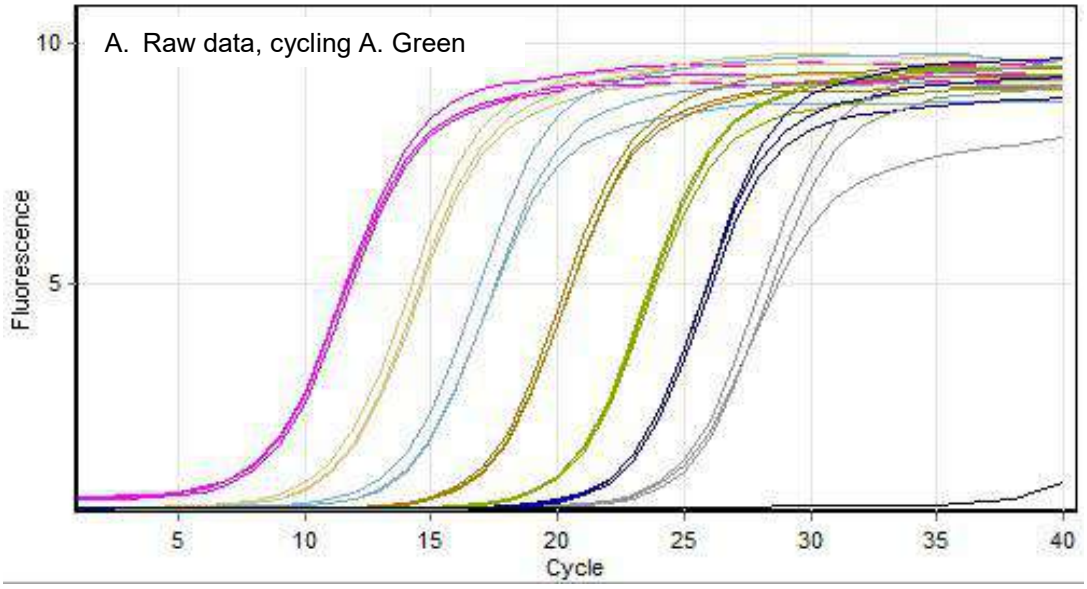


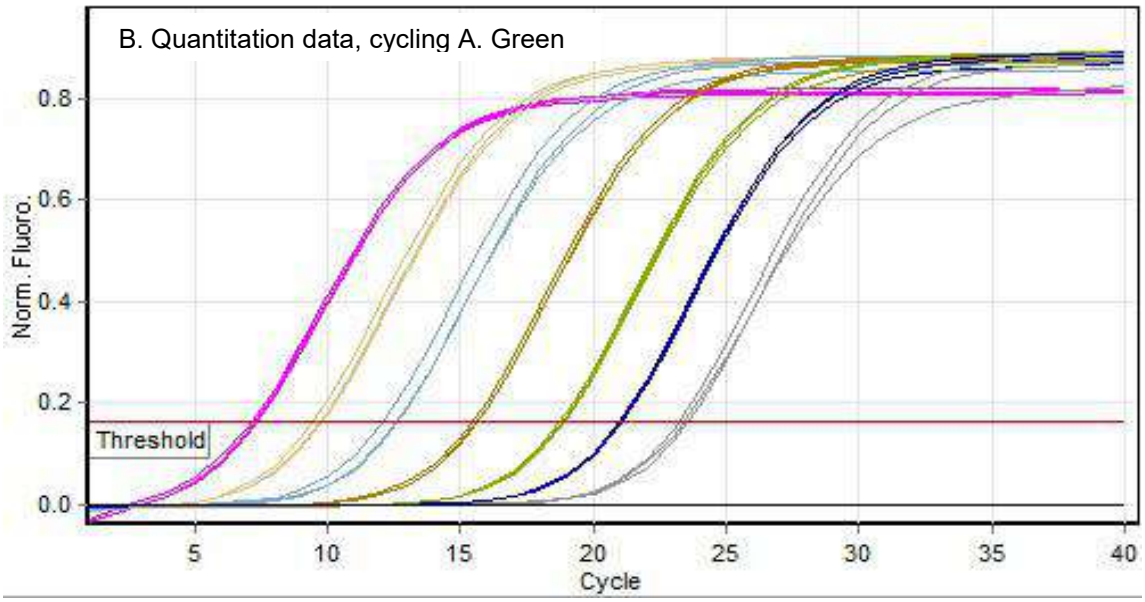
Notes: Different Fluorescent Proteins (FP) inserted in the constructed plasmids. Dasher Green FP: Yukon Orange FP; Cayenne Red FP; Match Yellow FP.

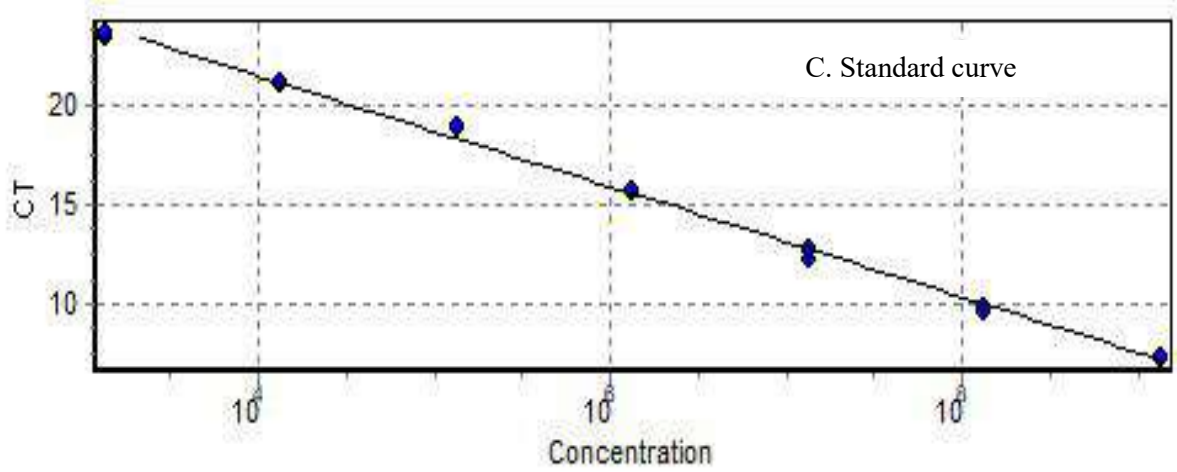
Appendix 7

RT-qPCR Standard Curve for *Chequa iflavirus*

Threshold	0.164
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	conc= 10^(-0.361*CT + 11.724)
Standard Curve (2)	CT = -2.770*log(conc) + 32.479
Reaction efficiency (*)	(* = 10^(-1/m) - 1) 1.296
M	-2.7703
B	32.47898
R Value	0.99818
R^2 Value	0.99637
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	% 5
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis ettings	


A. Raw data, cycling A. Green
Fluorescence
10
5
5
10
15
20
25
30
35
40
Cycle


B. Quantitation data, cycling A. Green
Norm. Fluoro.
0.8
0.6
0.4
0.2
0.0
Threshold
5
10
15
20
25
30
35
40
Cycle


C. Standard curve
CT
20
15
10
Concentration

Appendix 8

Presentations and Publications

Poster presentations

- Syahidah D., J. Elliman, C. Constantinoiu, L. Owens (2016). Mosquito Cells (C6/36) fail to Support the Complete Replication of Penaeus merguiensis hepandensovirus. The 6th International Conference of Aquaculture (6th ICAI), Denpasar, Indonesia
- Syahidah D., J. Elliman, L. Owens L (2017). HE staining fails to confirm the infection crustacean viruses in mammalian cell cultures. 2017 World Aquaculture Society (WAS) conference, Cape Town, Africa
- Syahidah D., J. Elliman and L. Owens L. (2019). PCR column contamination gave false positive result *for* Cherax quadricarinatus ambidensovirus (CqADV) in Sf 9 cell cultures. Corect-2019 Indonesian Japan Joint Scientific Symposium (IJJSS), Denpasar, Indonesia

Oral presentations

- Syahidah D., Elliman, J., Constantinoiu, C. and Owens, L. (2016). Development of cell cultures to propagate Decapod hepandensovirus1. 1st High Degree Research (1st HDR) Conference, JCU Townsville
- Syahidah D., Elliman, J., Constantinoiu, C. and Owens, L. (2017). Vero cell lines expressing nuclear location signals of Penaeus merguiensis hepandensovirus: An early study. 10th International Conference of Diseases in Asian Aquaculture (10th DAA), Kuta, Indonesia
- Syahidah D., Elliman, J., Constantinoiu, C. and Owens, L. (2019). Assessment of cell cultures to propagate crustacean viruses. The 10th International Conference of Biosciences and Biotechnology (10th ICBB), University of Udayana, Denpasar, Indonesia
- Syahidah D., Elliman, J., Constantinoiu, C. and Owens, L. (2019). Experimental infection of Raw-blue™ ISG cell cultures with *Chequa iflavirus* and athtabvirus: The first report. The 3rd International Conference of Life Sciences and Biotechnology (3rd ICOLIB), Jember, Indonesia
- Syahidah D. and Owens, L. (2019). Hepandensoviruses: an overview of what have been reported. International Conference on Science and Engineering (ICSE III), Yogyakarta, Indonesia
- Syahidah D. and Owens, L. (2019). G418 selective antibiotic dose response of Vero cell line. International Conference on Science and Engineering (ICSE III), Yogyakarta, Indonesia

Journal Publications

- Syahidah D., Elliman, J., Constantinoiu, C. and Owens, L. (2017). Mosquito cells (C6/36) fail to support the complete replication of Penaeus merguiensis hepandensovirus. Journal of Invertebrate Pathology 145 (2017) 31–38
- Syahidah D., Elliman, J., Constantinoiu, C. and Owens, L. (2018). Vero cell lines expressing nuclear location signals of Penaeus merguiensis hepandensovirus: An early study. Asian Journal of Advanced Research 1(1):1-10

Appendix 9

Awards and achievements

- Three modules of JCU Professional College cultural competency, community engage module and leadership, 2013-2014
- JCU Student Sustainable Awards 2014, Townsville, 2014
- On awards support: SAMUDRA-Skills and Awareness in Marine Understanding–Discoveries, Resources and Achievements, Adelaide, 2015
- Hadi Soesastro Prize Awards recipient, 2016
- Bronze membership awards from the Indonesian Australian Awards, 2016
- Higher Degree by Research Enhancement Scheme (HDRES) JCU conference travel grants round 2, 2016
- Join the research communication skills training of Women in Science Technology Engineering and Mathematics (STEM), Brisbane, 2017
- Student Travel grant from Asian Fisheries Society (AFS) – Fish Health Section (FHS) 2027 DAA10, Bali 2017
- Selected as a member of ExeCom for AFS-FHS, 2018-2020
- Thesis Completion Grant from The Graduate Research School (GRS), 2017

Appendix 10

Manuscript of publications from this work

Mosquito cells (C6/36) fail to support the complete replication of Penaeus merguiensis hepandensovirus

Journal of Invertebrate Pathology 145 (2017) 31–38

Contents lists available at ScienceDirect

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journal homepage: www.elsevier.com/locate/jip

Mosquito cells (C6/36) fail to support the complete replication of *Penaeus merguiensis* hepandensovirus

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HPV

ABSTRACT

Mosquito cell lines (C6/36) were reported in the literature to support the propagation of *Penaeus monodon* hepandensovirus (PmoHDV). We aim to evaluate the susceptibility and viral propagation of *P. merguiensis* hepandensovirus (PmeHDV) which is ~22% different to PmoHDV in *Aedes albopictus* cell line (C6/36). Cellular changes in the infected cell culture were detected. Vacuole formation was seen in both infected and uninfected cell cultures. The average number of disrupted cellular membranes in the infected cells (presumptive dead cells) was significantly higher than that of uninfected cells at passage two ($F = 9.749$, d.f. 1, 22, $p < 0.05$). Using a proliferation assay, light absorption of infected cells peaked at 2 weeks post-infection (O.D. = 0.27) but was significantly lower than that of the uninfected groups (O.D. = 0.37) ($F = 6.879$, d.f. 1, 94, $p < 0.05$) suggesting hindered cell growth. PCR of the serial passages of the infected cell cultures indicated weak positive results for PmeHDV infection and TaqMan quantitative PCR confirmed that the average number of viral copies declined from 3.8×10^5 to 5.69×10^2 copies per μL and the mean of cycle times increased from 19.26 to 27.63. These results are interpreted to mean C6/36 allows the initial stage of PmeHDV replication, but the virus was incapable of using C6/36 for patent replication of its' virions.

1. Introduction

Viruses within the family *Parvoviridae* can infect vertebrates and invertebrates with the viruses of the subfamily *Densovirinae* infecting invertebrates especially arthropods (Cotmore et al., 2013). The *Parvoviridae* are described as isometric with a non-enveloped viral capsid and a non-segmented linear single-stranded DNA genome characterized by self-priming hairpins that are vital for the unique rolling-hairpin duplication (Tattersall, 2008; Safeena et al., 2012).

Parvoviral diseases are emerging as a constant threat to penaeid culture due to their ability to cause slow growth and mass mortality of infected prawns (Flegel et al., 1999; Safeena et al., 2012). *Penaeus merguiensis* hepandensovirus (PmeHDV) (GenBank accession No. DQ458781) is a shrimp hepatopancreatic parvovirus (HPV), an Australian strain of the species *Decapod hepandensovirus 1*, in the genus *Hepandensovirus*, subfamily *Densovirinae* (Bergoin and Tijssen, 2010; Cotmore et al., 2013). It has 20–25 nm, non-enveloped capsids, containing a linear ssDNA genome approximately 6 kb in size encoding one structural and two non-structural proteins (La Fauce et al., 2007a; Owens, 2013). Based on the analysis of the complete genome sequence among different strains of hepandensoviruses, the sequence variation between the Indian strain of *Decapod hepandensovirus 1* a.k.a. P. monodon hepandensovirus (PmoHDV4) and PmeHDV is 22.1%, which is unexpectedly high, showing that these *Hepandensovirus* strains are markedly genetically different (Safeena et al., 2012).

Control of the parvoviral diseases is crucial. However, the knowledge of the pathogens and their host defence responses is relatively poor. The major problem of understanding the replication dynamics of shrimp viruses is hampered by the lack of any continuous cell line.

Mosquito cell lines (C6/36) have been examined for *in vitro* propagation of shrimp viruses. Sudhakaran et al. (2007) reported that Macrobrachium rosenbergii nodavirus (MrNV) and extra small virus (XSV) were successfully propagated in C6/36 by serial passaging of the culture supernatant. Sriton et al. (2009) found weak positive results for PmoHDV in C6/36 cells by PCR three days post-viral inoculation and strong positive results on day 4 and 5 but the virus could not be further passaged. Sriton et al. (2009) demonstrated

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0022-2011/

that the C6/36 mosquito cells exposed to other shrimp viruses, including white spot syndrome virus (WSSV) and yellow head virus (YHV) followed by serial passaging could lead to insect cell cultures in which 100% of cells persistently expressed viral antigens. Sriton et al. (2009) determined that the supernatant of the infected cell cultures was free from virions, suggesting that the genomic material remained in the cultures and it transferred from mother to daughter cells during the cell's cultivation. Also, they *hypothesised* that the viral genomic material must have replicated to support antigen production as the infected cells remained 100% antigen positive for more than 100 passages.

Gangnonngiw et al. (2010) demonstrated that C6/36 persistently expressing YHV antigens could serve as YHV inoculum to infect shrimp. The authors determined that the fifth passage of the infected cell cultures caused mortality to exposed shrimp, while the 15th passage of the infected cells did not, even though immunohistochemically, the challenged shrimp were positively infected by YHV antigens. Arunrut et al. (2011) reported the successful production of C6/36 that persistently expressed Taura syndrome virus (TSV) antigens as previously described for WSSV and YHV. The authors also claimed the successful infection of *P. vannamei* with homogenates of TSV-infected cells. They obtained positive reverse transcriptase PCR (RT-PCR) results for TSV in the immunopositive mosquito cell cultures and the successful infection of *P. vannamei* with homogenates of TSV-immunopositive mosquito cell cultures from passage 15. None of these studies proved that the virus actually replicated to patency or that the shrimp in bioassays that died were not dying from the original inoculum placed in the cell cultures. Indeed, the results of Sriton et al. (2009) and Gangnonngiw et al. (2010) suggest this is exactly the scenario being observed. Partial translation could result in positive immunoassays.

Hayakijkosol and Owens (2012) observed mortality in virus-exposed mosquito cell lines but using quantitative PCR, found no patent replication of Australian isolate of MrNV, suggesting that C6/36 are non-permissive for this virus. Madan et al. (2013) reported that C6/36 cultures were susceptible to PmoHDV4 and the infected cultures showed cytopathic effect (CPE) in the form of vacuole formation and cell shrinkage. Madan et al. (2013) confirmed the infection by PmoHDV4 in C6/36 cultures based on cytology, PCR, reverse transcriptase PCR (RT-PCR) and Western blotting. The CPE in the virus-exposed cell cultures was continuously detected for five cell passages. The infected cell cultures were PCR positive for PmoHDV4, producing a 441 bp amplicon. The RT-PCR detected that the RNA specific to capsid gene of HPV was in infected C6/36 cells at different days post-infection. Further investigation to quantify the PmoHDV4-load was conducted using quantitative PCR (qPCR), ELISA and immunocytochemistry. qPCR and ELISA indicated an increase in viral nucleic acid and viral protein in the infected C6/36 cells within 12 days of exposure (Madan et al., 2013). In addition, immunofluorescence was observed in the PmoHDV4-exposed cells. Madan et al. (2013) determined that immersion of postlarval *P. monodon* into water containing cell culture fluids from after the twentieth passage of PmoHDV4 in C6/36 cell line caused very slow, total mortality of postlarvae by 7 weeks post-exposure.

This present study aims to investigate the susceptibility of the mosquito cell line (C6/36) to *P. merguiensis* hepandensovirus (PmeHDV) which has 22% nucleotide difference to PmoHDV4 and also examine the replication of the virus in the mosquito cells. Differential staining was undertaken to understand cellular events occurring during PmeHDV infection. Confirmation of the viral exposure was conducted by PCR. A quantitative, specific PCR assay (TaqMan qPCR) for PmeHDV (Owens et al., 2015) was used to detect any viral replication of the virus and calculate the viral copies in the infected cell cultures.

2. Materials and methods

2.1. Preparation of viral inoculum

PmeHDV inocula were prepared from hepatopancreata of frozen, infected (inclusion body positive) *P. merguiensis* stored at the College of Public Health, Medical and Veterinary Science laboratory, James Cook University (JCU) Townsville, Australia. The hepatopancreata were thawed and homogenized in 25% Tris-HCl, NaCl (TN) buffer (0.4 M NaCl, 0.02 M Tris-HCl, pH7.4). The homogenate was centrifuged twice for 10 min at 720*g* and 4500*g*. The final supernatant was subsequently filtered through 0.45 μm and 0.22 μm membranes. The presence of PmeHDV was confirmed by PCR using a diagnostic primer set (Table 1) (Owens et al., 2015). The filtrate was stored at −20 °C until used.

2.2. Cell culture and maintenance

The C6/36 mosquito cells, derived from frozen stocks at JCU Townsville, Australia were used to propagate the virus. The cell lines were grown in Roswell Park Memorial Institute-1640 (RPMI) and Minimum Essential Medium Eagle (MEM) media supplemented with 10% foetal bovine serum (FBS), penicillin (200 U/mL), streptomycin (200 μg/mL), kanamycin (80 μg/mL), polymyxin B (30 U/mL) and 1× amphotericin B (Sigma Aldrich, Australia). The cells were propagated at 28 °C with media changed when required.

The cells were sub-cultured when monolayers formed. For passaging, the old medium was aseptically decanted and the cells were washed with 1× phosphate buffer saline (PBS). The cells were detached by incubating them with 1 mL pre-warmed 0.25% trypsin/EDTA solution (Life Technologies, Australia) for 3–5 min or until the 75% of the cells detached (Life Technologies, Australia). The cells then were re-suspended in complete fresh medium and were equally distributed in two new culture flasks. Live cell images were taken using an inverted microscope (Olympus, Japan) equipped with a digital camera and Q Capture P software (Olympus, Japan).

2.3. Viral susceptibility

T25 mL flasks (Sarstedt, Germany) were used to culture C6/36 cells (approximately 10^5 cells/mL) at 28 °C to give a confluence of 70–80%. After removing the medium, 1 mL of viral inoculum per flask was inoculated on the surface of the C6/36 cells in 4 flasks, and allowed to adsorb for 1 h (Hirt, 1967). The 4 negative control flasks received 1 mL medium for each flask. After incubating for 1 h at 28 °C, 9 mL of fresh media were added into all flasks. The trial was conducted in triplicate. Passaging and sample collection from each flask was conducted when cells were in log phase (5 days after passage). In our study, C6/36 cells that were exposed to viral inoculum are considered as the infected cell culture groups while the uninfected C6/36 cells were considered as the negative control cell cultures.

Table 1
Primers and probe used in the present study.

Primer/probe	Nucleotides (5′-3′)	Annealing temp. (°C)
HPV140F	CTA CTC CAA TGG AAA CTT CTG AGC	63.5
HPV140R	GTG GCG TTG GAA GGC ACT TC	62.5
HPV4507R	GGC ACT TCC TGT ATT TTT CCC G	62.1
Probe	FAM TAC CGC CGC ACC GCA GCA GC TAMRA	66.7

Fluorogenic probe FAM (6-carboxyfluoresin); quenching dye TAMRA (6 carboxy-tetramethylrhodamine (La Fauce et al., 2007b).

At the time of passaging, 50% of each (uninfected and infected) cell culture was collected and subjected to various analyses for PmeHDV infection and replication. After sampling, the remaining collected cells from the control, non-infected cell cultures were added into each infected cell culture for on-growing of the virus. This ensured that the necessary dividing cells were available for replication of the parvovirus. Both infected and uninfected control cells were re-grown at 28 °C until the next passage. Four replicate samples of infected cells from each culture flask, serially passaged four times were used for the PCR analyses at each passage (see below). Negative control PCRs (including qPCR) were from four non-infected cell cultures, passaged 4 times.

2.4. Cytology

For each stain, a twenty-four well plate was used to culture C6/36. The cells were seeded (approximately 10^5 cells/mL) onto cover slips in 16 wells of a 24 well plate for maximum 24 h to give a confluence of 70–80%. Cells in the first 8 wells were exposed to the prepared inoculum PmeHDV (100 μL/well) while the uninfected groups received medium (100 μL/well). After the viral inoculation, the plates were incubated at 28 °C for 1 hr and following this, fresh medium was added (900 μL/well) into both infected and uninfected cell cultures.

The presence of cytopathic effect (CPE) was examined daily under an inverted microscope (Olympus DP21, Japan). Staining of both uninfected (negative control cells) and infected cells was conducted on days 2, 4, 7 and 14 post-inoculation with four replicate samples. Cells were washed and fixed with different fixatives according to each staining protocol.

2.4.1. Mayer's Haematoxylin & Eosin (H&E)

Cells were fixed in Bouin's solution and stained with H&E to observe cellular morphological changes (Drury and Wallington, 1980 in Hayakijkosol and Owens, 2012).

2.4.2. Giemsa stain

Cells were fixed with 100% methanol before staining with Giemsa stain overnight. After overnight staining, the cells were rinsed with distilled water containing 0.5% aqueous glacial acetic acid (Kiernan, 2010). Observations were conducted using a microscope (Olympus, Japan) equipped with a digital camera and Q Capture P software (Olympus, Japan).

2.4.3. Acridine orange

Acridine orange (AO) was used to observe nucleic acid proliferation in cells. Cells were fixed at 7 days with 100 μL of 95% ethanol for 30 min and rehydrated with 80%, 70% and 50% ethanol, respectively, for 10 s each and then rinsed with water. One percent acetic acid (100 μL) was added for 6 s and then removed. One hundred μL of 0.1% AO solution was used to stain the cells for 3 min before they were washed with McIlvain citric acid and M/10 calcium chloride (Culling et al., 1985). The C6/36 cells were examined under an epifluorescent microscopes (Olympus, Japan and Zeiss, Australia), Zeiss Imager Z1, (Zeiss, Australia) using 475/530 nm and 560/650 nm excitation/emission filters. Photographs of cells were taken using a digital camera equipped with AxioVision version 4.8.2 SP3 software.

2.5. Counting of disrupted cell membranes (presumptive dead cells)

Equal amounts of cells suspension and 0.14% trypan blue in 1x PBS were mixed, 20 μL of the mixture was loaded into the counting chamber of a haemocytometer (Neubauer, UK) and examined using a light microscope, Olympus CX31, (Olympus, Japan). The live (translucent) and presumptive dead (stained blue) cells were counted in the eight corners of the haemocytometer. The percentage of cell viability (%) was calculated by dividing the number viable cells/mL by the total number of cells/mL and the result was multiplied by 100.

2.6. Water Soluble Tetrazolium-1 (WST-1) assay for cell proliferation

The stable tetrazolium salt WST-1 (Sigma Aldrich, Australia) is cleaved to a soluble formazan by a complex cellular mechanism that occurs primarily at the cell surface. This bio-reduction is largely dependent on the glycolytic production of NAD(P)H in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture.

Twelve replicates of infected and control C6/36 cells were prepared in a 96 well plate. Fifty microliters of complete cell medium and 50 μL of viral inoculum were placed in each appropriate well producing 12 replicates. Fifty microliters of cell suspension were added into all wells. An extra 50 μL medium was added into the non-infected, control wells. The insect cells were grown at 28 °C and analyzed on day 2, 4, 7, and 14. On each examination day, ten microliters of WST-1 reagent (Roche, Germany) were added into the 24 wells and the plate was re-incubated at 28 °C for 4 h prior to the absorbance reading. Plates were shaken for 1 min and the absorbance of the cells was measured at 450 nm on a Varioskan flash reader version 2.4.3 (Thermoscientific, Australia).

2.7. Confirmation of PmeHDV exposure by PCR

At each passage, PCR was conducted to confirm the presence of PmeHDV in the C6/36 cells using diagnostic primer sets (Table 1). DNA was extracted from both infected and control cells with a Genomic Isolate-II kit (Bioline, Australia), following the manufacturer's instructions. Cells and supernatant were collected from the flasks and spun down at 583*g* for 5 min. The pellets were re-suspended in 1× PBS before being extracted. The concentration of DNA in each sample was estimated using a spectrophotometer (Eppendorf, Australia) by measuring the absorbance towards UV light at 260 nm (A_{260}).

Each PCR reaction using MyFi master mix (Bioline, Australia) consists of 5 μL of reaction buffer (5×), MyFi *Taq* polymerase (2μ/μL), 1 μL of each primer HPV140F and HPV140R, 2.5 μL of DNA template and nuclease-free water to a final volume of 25 μL. Amplification was performed in a thermal cycler (Applied Biosystems, USA). PCR profile consisted of an initial denaturation at 95 °C for 1 min, then 35 cycles of 95 °C denaturation for 15 s, 54 °C annealing for 20 s and extension at 72 °C for 5 min. Samples were polymerized for 7 min at 72 °C following the last cycle and held at 10 °C. The amplified products were run on a high speed gel electrophoresis system (Biokeyston Co. USA) and visualized on to 2% agarose gel containing 0.05 μL/mL GelRed (Biotium USA). Ten microliters of PCR reaction mixture of each sample was used for the electrophoresis. A UV transilluminating box (SynGene USA) linked to Gensnap software was used to view and photograph gel images.

2.8. Quantification of PmeHDV copy number by TaqMan real-time PCR

TaqMan qPCR based on the capsid gene was conducted to determine the copy number and C_T (cycle threshold) value of PmeHDV in the infected C6/36 cell cultures. Cells at each passage, every 5 days up to 20 days, were sampled for viral copy numbers by qPCR. Prior to the qPCR assay, a standard curve was generated using serial dilutions from 1×10^9 to 1×10^0 copies of a positive control plasmid to determine the sensitivity of the TaqMan assay according to the protocol described by La Fauce et al. (2007b). A forward primer (HPV140F) and a TaqMan probe used were those

of La Fauce et al. (2007b) whereas a newly designed reverse primer (HPV4507R) (Owens et al., 2015) was used in this assay (Table 1).

Each TaqMan qPCR reaction for generating standard curve and quantitation of viral copy number was carried out in a 20 μL volume containing 10 μL of 2× buffer (Applied Biosystems, Australia), 5 pmol of probe, 20 pmol of each primer 140F and HPV4507R (Macrogen, Korea), 1 μL of DNA template, and nuclease-free water to a final volume of 20 μL. The Taqman cycle pattern consisted of initial incubation at 95 °C for 3 min, followed by a two – step cycle pattern consisting of 40 cycles at 95 °C for 10 s, and 60 °C for 45 s. The data acquisition and analysis were performed using Corbett Rotor-Gene Q (Qiagen, Australia).

2.9. Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 19.

3. Results

3.1. Characteristics of the infected cells

Examination of live cell images (Fig. 1) showed only minor differences in characteristics between control and viral infected cells during the four passages, with infected cells appearing slightly more clumped than the cells of control groups.

3.2. Cytology

Vacuole formation was observed in both infected and control C6/36 cells stained with H&E and Giemsa. Even though enlarged nuclei (anisonucleosis) were observed in the infected cells stained with Giemsa, no viral inclusion bodies were detected in the infected cells (Figs. 2 and 3).

Acridine orange staining of both infected and control groups demonstrated nucleic acids (Fig. 4). No red/orange fluorescence was detected using 460/650 nm filters in either cell groups and they stained predominantly green under examination with 475/530 nm filters. The green fluorescence of infected cells was more intense and a brighter green colour in the cytoplasm than in the uninfected control cells.

3.3. Counting of cells with disrupted membranes (presumptive dead cells)

The number of cells stained by trypan blue indicating disrupted cell membranes (presumptively dead cells) of both virus infected and control cell groups fluctuated over the serial passages (Fig. 5). The highest percentage of dead cells (7.6%) was detected in the infected groups at the 2nd passage while the control groups had the lowest percentage of dead cells at the 2nd passage ($F = 9.749$, d.f. (1, 22), $p < 0.05$).

3.4. Cell proliferation

Different light absorbances between virus infected and control cells were recorded by the WST-1 assay (Fig. 6). Light absorbance of infected cells was significantly lower ($F = 6.879$, d.f. (1, 94), $p < 0.05$) than that of control groups during 2 weeks, suggesting less metabolically active cells i.e. cell death.

3.5. Molecular studies

3.5.1. PCR

Using a specific primer set for PmeHDV (Table 1), the PCR of the infected C6/36 samples showed weak positive 140 bp amplicons (not shown).

3.5.2. TaqMan qPCR

The TaqMan qPCR results in this study demonstrated a dropping copy number in each subsequent passage of the infected C6/36 cells. The average viral copy numbers declined from 3.8×10^5 to 5.69×10^2 copies mL^{-1} and the mean of cycle times increased from 19.26 to 27.63 during four serial passages (Table 2), suggesting that the PmeHDV could not use the C6/36 for complete replication. With a cut off Ct value of 38 cycles, uninfected control cells had no measurable Ct values i.e. any virus was below detection limits.

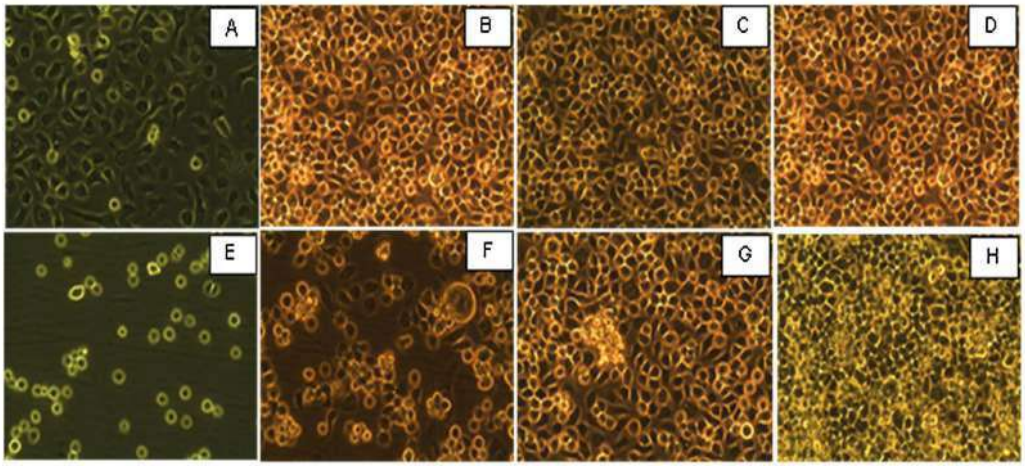

Fig. 1. (A–D) Uninfected C6/36 from the first to fourth passages, respectively. (E–H) PmeHDV infected C6/36 from the first to fourth passages, respectively. 20×.

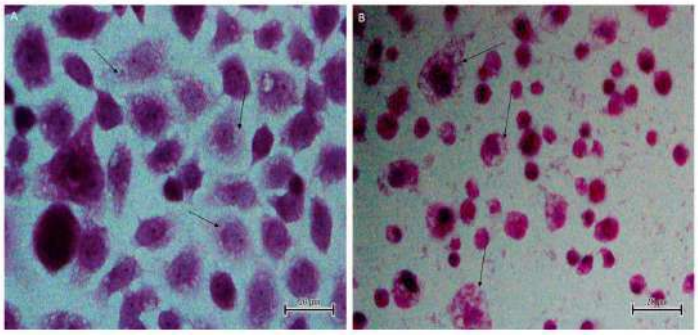

Fig. 2. H&E stain of C6/36 cell cultures 1 week post-infection. (A) Uninfected cell cultures; (B) PmeHDV-infected cells with multiple vacuoles (arrows). Bars 20 μm

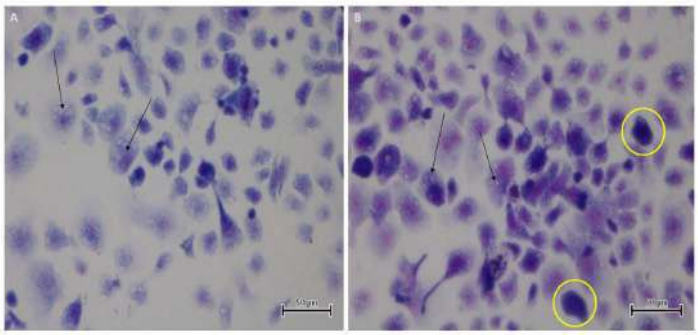

Fig. 3. Giemsa stain of vacuolation in C6/36 cells 1 week post-infection. (A) Control cells. (B) Infected cells show vacuolation (arrows) and possible anisonucleosis (circles). Bars 50 μm.

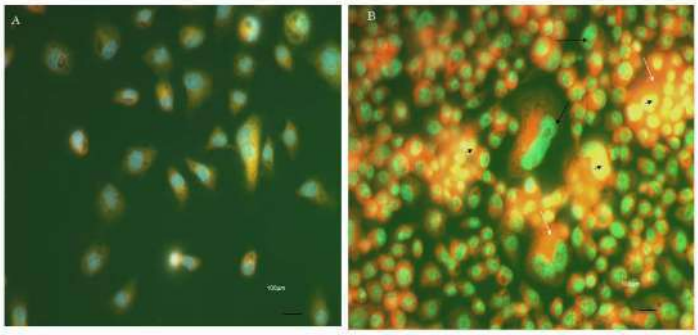

Fig. 4. Acridine Orange staining of C6/36 cell cultures at 1 week post infection. (A). Uninfected cell cultures. (B) Infected cell cultures. Red/orange fluorescence indicates the presence of single stranded nucleic acid (~cytoplasmic mRNA; white arrows); yellow fluorescence shows dsNA (probable hybrid ssDNA + mRNA transcription; short black arrows); green fluorescence shows nuclear dsDNA (long black arrows). Bars 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

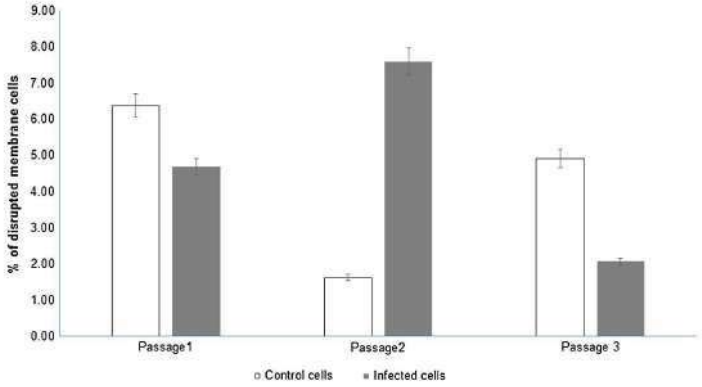

Fig. 5. Average number (±STDEV) of disrupted membranes (presumptive dead cells) between the PmeHDV-infected and control cell cultures.

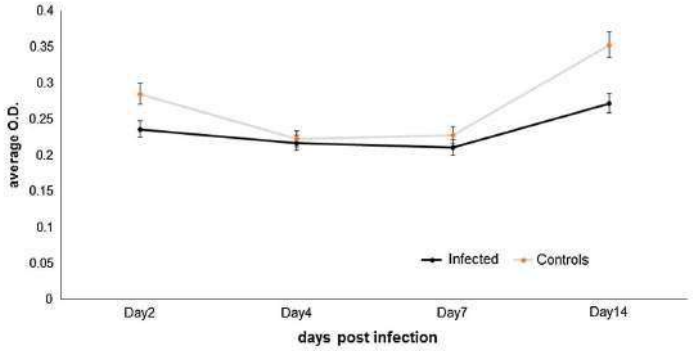

Fig. 6. WST-1 assay. Light absorbance of PmeHDV-infected and uninfected cell cultures observed over 2 weeks of viral infection.

4. Discussion

The lack of an established crustacean cell line is one reason why researchers have looked extensively for alternative systems to understand the interaction between host cells and shrimp viruses. As it was mentioned earlier, some research groups have published on the successful replication of different shrimp viruses in mosquito cell cultures (C6/36) which encourages future research on understanding the mechanism of the viral infection.

Our study demonstrated that the Australian strain of *Decapod hepandensovirus*1 (PmeHDV) cannot replicate to patency in C6/36 cell cultures. The cell cultures were affected by PmeHDV as demon-

Table 2
The calculated concentration (number of copies) and cycle times (C_T) of PmeHDV in four flasks with three replicate assays of C6/36 cell culture during serial passage.

Samples	Passage 1		Passage 2		Passage 3		Passage 4	
	Copies per µL	C_T	Copies per µL	C_T	Copies per µL	C_T	Copies per µL	C_T
1	4.70×10^5	18.92	4.55×10^4	21.92	4.64×10^3	24.86	8.62×10^2	27.02
2	4.94×10^5	18.86	4.53×10^4	21.93	5.68×10^3	24.60	7.13×10^2	27.27
3	4.88×10^5	18.87	4.32×10^4	21.99	5.01×10^3	24.76	9.10×10^2	26.95
4	3.46×10^5	19.31	9.61×10^4	20.96	8.21×10^3	24.12	4.50×10^2	27.86
5	3.79×10^5	19.20	9.35×10^4	21.00	7.65×10^3	24.21	4.49×10^2	27.86
6	3.52×10^5	19.29	8.19×10^4	21.17	8.36×10^3	24.10	3.32×10^2	28.25
7	2.32×10^5	19.82	5.22×10^4	21.74	5.87×10^3	24.55	6.61×10^2	27.36
8	2.42×10^5	19.77	5.53×10^4	21.67	6.81×10^3	24.36	6.65×10^2	27.35
9	2.26×10^5	19.86	5.00×10^4	21.80	5.37×10^3	24.67	6.02×10^2	27.48
10	4.44×10^5	19.18	8.13×10^4	21.18	5.89×10^3	24.55	3.49×10^2	28.19
11	4.55×10^5	19.04	1.55×10^4	20.34	7.59×10^3	24.22	3.84×10^2	28.06
12	4.35×10^5	19.02	8.79×10^4	21.07	5.66×10^3	24.60	4.53×10^2	27.85
Mean	3.80×10^5	19.26	7.39×10^4	21.40	6.39×10^3	24.47	5.69×10^2	27.63

strated by the H&E and Giemsa staining (Figs. 1–4). Examination of live C6/36 cells showed differences between control and PmeHDV-infected cell cultures (Fig. 1), in that cells were clumped upon viral inoculation but other changes in both infected and control cell cultures were similar. PCR results indicated that the cell cultures were exposed to virus. However, TaqMan qPCR clearly showed a dropping number of viral copies and increasing Ct values with serial passages indicating no replication to patency.

CPE such as vacuole formation was found in both infected and uninfected cell cultures as observed by HE and Giemsa staining (Figs. 2 and 3), suggesting the microscopical examinations were not a sensitive enough technique to determine the infection of PmeHDV. The presence of vacuoles in both infected and control cells was also described by Sudhakaran et al. (2007) and Hayakijkosol and Owens (2012).

Acridine orange was used to observe nucleic acid changes. AO is a metachromatic dye which differentially stains double-stranded (ds) and single-stranded (ss) nucleic acids. Under a fluorescent microscope, AO emits orange or red fluorescence when it interacts with single stranded nucleic acid upon excitation at wavelength 480–490 nm. An increasing yellow/green colour in infected C6/36 cells (Fig. 4) might suggest an increasing double stranded (ds) nucleic acid production, either dsDNA or a mRNA/DNA hybrid that occurs in the densovirus rolling cycle propagation. Notably the red/orange colours of ssDNA in the nucleus of cells from the patent densovirus were not observed. There was no indication that the inoculation of PmeHDV into C6/36 enhanced the viral ssDNA production as none was visualized by AO staining.

The positive results via PCR following gel electrophoresis is an indication that the cells were exposed to PmeHDV. However, quantification of viral copy numbers using TaqMan qPCR is required to confirm the viral replication. TaqMan qPCR results (Table 2) demonstrated dropping viral copy number from 3.85×10^5 to 3.32×10^2 copy μL^{-1} and the mean of cycle times increased from 18.86 to 27.36 during the four serial passages, suggesting PmeHDV was not able to use C6/36 cell cultures to multiply their viral DNA even though fresh, rapidly dividing cells (S phase) were added into the infected flasks at each subsequent passage. Uninfected cell cultures were negative proving the PCR primers did not react non-specifically with the C6/36 cells.

One interpretation of our data is that the initial stages of viral decapsidation, and transcription of early genes might be occurring as evidenced in our assays, but the late genes (VP) failed to produce the capsid and therefore, viable viruses. Similarly, observations of partial CPE and failure for complete virogenesis were seen with MrNV in the piscine SSN1 cells (Hernandez-Herrera et al., 2007) and with C6/36 cells (Hayakijkosol and Owens, 2012). Also note similar results in Sriton et al. (2009) and Gangnonngiw et al. (2010) with regard to WSSV and YHV, and YHV alone, respectively. This phenomenon of partial replication of parvoviruses is not new. E.g. in cells non-permissive for human parvovirus B19, there is a large build-up of NS1 and NS2 transcripts but low quantities of the capsid transcript (Liu et al., 1992). In cells permissive for B19, these transcripts are in equal amounts.

4.1. Differences in hepandensoviruses that caused contrary results between Madan et al. (2013) and the current study?

Differences in our study compared to that of the successful propagation of PmoHDV4 in C6/36 cell culture could be due to the 22.1% genetic variation between the PmoHDV4 and PmeHDV (Safeena et al., 2012). Of interest, the qPCR product in the capsid area of this current report completely incorporates the polyglycine motif. Only PmeHDV from Australia and New Caledonia, have a serine-free, polyglycine signal in the capsid protein (Owens, 2013). All other sequenced isolates have a serine in this polyglycine motif. Polyglycine motifs form capsid pores for nucleic acid entry on assembly and exit on decapsidation. The pore can also be used for cell ingress in some viruses. Does the lack of a serine affect the permissiveness of the C6/36 cell lines and could this be responsible for the different results between Madan et al. (2013) and the current report? This seems unlikely, as the qPCR indicating the falling copy number is based on the late gene for the capsid and it is not being replicated, so the block to replication appears to be occurring before this step in virogenesis not at the assembly stage or cell infection stage. This needs to be confirmed experimentally.

The ORF-1 (NS2) protein of PmoHDV4 is larger than that of PmeHDV recorded at 1281 and 1026 bp, respectively (Safeena et al., 2012). The NS2 protein of parvoviruses is important in viral assembly, in the production of viral ssDNA (Cotmore et al. 1997; Naeger et al., 1990, 1993) and it is involved in the production of other viral replicative forms (Cater and Pintel, 1992; Naeger et al., 1990, 1993). Equally important, it is suggested that NS2 protein enhances NS1-associated parvovirus-induced cell killing in some cell lines (Brandenburger et al., 1990; Caillet-Fauquet et al., 1990; Legrand et al., 1993). At present, we can find no data on why the size difference of the NS2s could cause differences in replication of these two strains.

5. Conclusion

This study examined the susceptibility of the mosquito cell lines (C6/36) to *P. merguiensis* hepandensovirus (PmeHDV) using cytological and molecular analyses (PCR). Even though cytological and PCR analyses showed that PmeHDV could cause changes in C6/36 *in vitro*, the TaqMan qPCR showed that the number of viral copies declined over four serial passages. Further research on trying different cell lines or adapting the PmeHDV to different clones of C6/36 may be necessary to successfully replicate PmeHDV. We suggest there should be more rigorous criteria applied to cell lines before the cell lines are classified as susceptible to viruses. qPCR for late genes for enumerating viral copies with a subsequent specificity check on amplicons should be the minimal acceptable evidence.

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VERO CELL LINES EXPRESSING NUCLEAR LOCATION SIGNALS OF *Penaeus merguiensis* HEPANDENSOVIRUS: AN EARLY STUDY

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. Author DS principal investigator, analyzed the data and wrote the report. Authors DS and CC designed and performed the experiments. Author JE provided supervision during preparation of materials and methods and checked the writing. Author LO gave assistance in the experimental design, data analyzing and writing. All authors read and approved the final manuscript.

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ABSTRACT

Aims: *Penaeus merguiensis* hepandensovirus (PmeHDV) (GenBank No. DQ458781) is a shrimp hepatopancreatic parvovirus (HPV), belonging to subfamily *Densovirinae*. Transportation of *Densovirinae* into and out of nucleus is allowed by the binding of nuclear location signals (NLSs) to importins (Imp). PmeHDV has putative NLSs that need to be experimentally tested. The aims of this study is to determine if the three putative NLSs of PmeHDV are functioning by transfecting NLS-inserted-plasmid DNAs into Vero cell lines using a transfection reagent.

Place and Duration of the Study: Data for this study was collected from the Veterinary and Biomedical Sciences Laboratories at James Cook University (JCU) during the duration from May 2015 to December 2016.

Methodology: Each plasmid has been synthetically inserted with each sequence of the putative NLSs and a fluorescent protein. The presence of the NLS in the cell nucleus and cytoplasm was screened. The overlay of visualization of transfected plasmids is presented.

Results: It appears the NLSs are not functioning well as that the proteins are blocked at the nuclear membrane, probably linked to importin beta-1 and not frequently entering the nucleus. Our study demonstrated small noticeable differences in the outer nuclei within transfected-Vero cells with the experimental NLSs genes.

Conclusion: In conclusion, our fluorescent study was not sufficiently sensitive to be confident of the detection in NLS-transfected cells under different filters. The study of crustacean virus-host interactions using proxy cell cultures as models remains a major challenge.

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