Novel RNA viruses causing muscle lesions in red claw crayfish (*Cherax quadricarinatus*)

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ABSTRACT

In 2014, a new syndrome was recognized in redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Queensland. Crayfish started to die with mortality reaching 40% in approximately three weeks after transportation and translocation stress. In trying to identify the cause of the disease, sequence dependent and independent PCRs failed to identify any aetiology. However, using next gen sequencing of RNA, two novel RNA viruses, namely chequa iflavirus and bunya-like virus were found in the stressed, dying crayfish. Chequa iflavirus is a positive sense, single stranded RNA virus in the order *Picornavirales*, marginally in the genus *Iflavirus*. This is the first iflavirus identified from crustracea. The bunya-like virus found is related to Whenzhou Shrimp Virus 2, a bi-segmented, unaligned, negative sense, single strand RNA virus. Efficient and sensitive detection methods for these viruses based on RNA dependent RNA polymerase (RdRp) was developed for large scale screening of crayfish on farm. Due to a lack of virus-free crayfish, house cricket, *Acheta domesticus* was trialled as a bioassay animal to prove River's postulates for chequa iflavirus and bunya-like virus. However, it was unsuccessful as the immune system of cricket might able to destroy and leave no viable virus or enough traces of RNA to be detected by reverse transcription polymerase chain reaction (RT-PCR). To solve the viral infection problem with chequa iflavirus infection on farm, some possible viral reduction strategies for farmers were trialled. The polyphenol flavonoid quercetin, inhabiting water temperature at 32℃ all had statistically significant effects reducing viral copy numbers ($P<0.018$; $P<0.05$ respectively). While RNA interference based on RdRp sequence appeared to drop viral copy number 80% at four weeks, it was not statistical significant ($P>0.05$). For the crayfish farmers, quercetin is the most favoured treatment as it is abundant, cheap and readily incorporated into the diet and should ameliorate the original, transport-stress mortality which started this study. Broodstock could be warmed to 32℃ in the hatchery to reduce viral dose. Although RNAi treatment was most expensive to implement, it could be delivered with limited amounts of RNAi at the hatchery.

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LIST OF ABBREVIATIONS

AHPNS	Acute hepatopancreatic necrosis disease
AIM2	Absent in Melanoma 2
ASC	Apoptosis-associated, speck-like protein containing a carboxy-terminal CARD
bp	Base pair
℃	Degree Celsius
cDNA	Copy DNA
CGV	Cherax giardiavirus
CMNV	Convert mortality nodavirus
cm^3	Cubic centimeter
CPE	Cytopathic effects
C. quadricarinatus	*Cherax quadricarinatus*
cm	Centimeter
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
EMS	Early mortality disease
ESTs	Expressed sequence tags
FAO	Food and Agriculture Organization of the United Nations
FBS	Foetal bovine serum
FHV	Flock House virus
fg	Femtogram
g	Gravitational force
GAV	Gill-associated virus
GEM	Group epitope mapping
GLV	Giardia lamblia virus

HPLC	High-performance liquid chromatography
HSP	Heat shock proteins
ICTV	International Committee on Taxonomy of Viruses
IHHN	Infectious hypodermal and haematopoietic necrosis
IMN	Infectious myonecrosis
IMNV	Infectious myonecrosis virus
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JCU	James Cook University
kb	Kilobases
kDa	KiloDalton
kg	Kilogram
Lamr	Laminin-receptor protein
LB	Luria Bertani
L. vannamei	*Litopenaeus vannamei*
MD	Molecular dynamics
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
mg	Milligram
MHV-MI	Murine hepatitis virus-MI stain
miRNA	microRNAs
mRNA	Messenger RNA
M. rosenbergii	*Macrobrachium rosenbergii*
MrNV	Macrobrachium rosenbergii nodavirus
MyD88	Myeloid differentiation primary response protein 88
NHP	Necrotising hepatopancreatitis
NGS	Next-generation sequencing
NLRP3	Nucleotide-binding domain-like receptor protein 3

nm	Nanometer
nt	Nucleotide
ORFs	Open reading frames
OIE	World Organization for Animal Health
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PmeHDV	Penaeus merguiensis hepandensovirus
PmRab7	Penaeus monodon Rab7
PRRs	Pattern recognition receptors
PvNV	Penaeus vannamei nodavirus
RdRp	RNA-dependent RNA polymerase
RISC	RNA-inducing silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease
RNAi	RNA interference
ROS	Reactive oxygen species
RT	Reverse-transcriptase
RT-PCR	Reverse-transcriptase polymerase chain reaction
shRNAs	Short hairpin RNAs
siRNA	Short double-stranded RNAs
ssDNA	Single-stranded DNA
SSH	Suppression subtractive hybridization
ssRNA	Single-stranded RNA
TBS	Tryptose phosphate broth
TEM	Transmission electron microscope
TRIF	Toll/IL-1 receptor domain-containing adapter-inducing interferon-β
TLR	Toll-like receptor
TS	Taura syndrome
UTR	Untranslated region

VDR	Vitamin D receptor
WTD	White tail disease
WSD	White spot disease
WZSV2	Whenzhou Shimp Virus 2
XSV	Extra small virus
YHV	Yellow head virus

LIST OF PUBLICATIONS

- Sakuna, K., Elliman, J., Owens, L. 2017. Discovery of a novel *Picornavirales*, Chequa iflavirus, from stressed redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Queensland, Australia. *Virus Research*, 238, 148-155.

- Sakuna, K., Elliman, J., Owens, L. 2018. Comparison of molecular detection PCR methods for chequa iflavirus in freshwater crayfish, *Cherax quadricarinatus*. *Journal of Virological Methods*, 251, 139-144.

- Sakuna, K., Elliman, J., Owens, L. 2017. Assessment of a cricket, *Acheta domesticus*, bioassay for Chequa Iflavirus and bunya-like virus from redclaw crayfish *Cherax quadricarinatus*. *Journal of Invertebrate Pathology*, 150, 41-44.

- Sakuna, K., Elliman, J. Tzamouzaki, A., Owens, L. 2018. A novel virus (order *Bunyavirales*) from stressed redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Australia. *Virus Research*, 250, 7-12.

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CHAPTER 1
GENERAL INTRODUCTION

In 2014, a new syndrome manifested in redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Queensland, Australia. Crayfish that were stressed by predominantly transportation and translocation stress, started to die with mortality reaching 20–30% after about three weeks and then mortalities waned. After transportation, crayfish from another farm had heavier mortalities reaching 40% within three weeks and 65% within 11 weeks. This problem has critical effect on the farms' economy. Generally, there are two main transportation and translocation events that induce this problem.

Firstly, when farmers want to sell live redclaw to restaurants and secondly, when they want to move redclaw from their pond to their hatchery or other hatcheries. To sell it to restaurants, redclaw has a premium price only when it is alive before being served. Llive redclaw costs around US$30 per kg, however, it will cost only US$10 per kg if dead. To be used as a broodstock, crayfish will have no value if the crayfish die and clearly these animals are of no use for breeding. Therefore, there are significant economic losses if the crayfish die after transportation and translocation events.

To solve this problem, proper consideration and fulfilment of following issues is needed. 1) An effective technique to identify or discover the aetiological agent causing muscle and nerve lesions. 2) Sensitive and specific detection systems to be used for large scale screening of crayfish. Assuming that a virus is responsible; 3) An animal model to complete River's postulates for viruses. 4) Possible viral reduction strategies to reduce the problem on farm. This thesis aims to address these four issues with the hope that the thesis outputs will contribute towards better strategies for sustainable aquaculture particularly, redclaw crayfish culture.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

2.1.1 Aquaculture

Fisheries and aquaculture production constitute an important source of the cheapest form of animal protein. However, total global fisheries production during 2006-2012 was stagnant at 90 million tonnes per year (FAO, 2012). For this reason, aquaculture, which has grown approximately 6% per year since 2003 (FAO, 2012), is considered the only way to meet the growing demand for aquatic animals.

2.1.2 Crustacean aquaculture

Crustacean aquaculture includes the production of freshwater and marine species such as crab, lobster, shrimp, and crayfish. Global crustacean aquaculture production in 2012 was 6,446,818 tonnes, twice the level reported to the Food and Agriculture Organization of the United Nations (FAO) in 2003 (FAO, 2012). Penaeidae is the principal family of farmed crustacean worldwide. Production in 2012 of the Pacific white shrimp (*Litopenaeus vannamei*) and the black tiger prawn (*Penaeus monodon*) was 3,178,721 tonnes and 855,055 tonnes respectively (FAO, 2012). Other cultured crustaceans of significance include freshwater species such as the giant freshwater prawn (*Macrobrachium rosenbergii*) and the red claw crayfish (*Cherax quadricarinatus*). In 2013, world production of *M. rosenbergii* and *C. quadricarinatus* was 203,299.55 tonnes and 142.75 tonnes with a value of USD 1.24 billion and USD 1.38 million respectively (FAO, 2015). However, as farming has expanded, disease issues have begun to emerge with increasing frequency.

2.1.3 Aquatic crustacean disease

White tail disease (WTD), Taura syndrome (TS), yellowhead virus (YHV), infectious myonecrosis (IMN), infectious hypodermal and haematopoietic necrosis (IHHN), necrotising hepatopancreatitis (NHP), crayfish plague (*Aphanomyces astaci*), and white spot disease (WSD) are listed as notifiable crustacean diseases by the World Organization for Animal Health (OIE). Almost all the important pathogens are viruses due to their pathogenicity, lethality and infectiousness (Pillai and Bonami, 2012). Moreover, viruses, particularly RNA viruses, which exhibit high mutation rates due to the lack of proofreading activity of viral RNA-dependent RNA polymerase (Yang, 2009), are considered as important pathogens in aquatic crustacean farms. Crustaceans lack an adaptive immune response against protein antigens; they cannot be vaccinated as a preventative method against viral disease (La Fauce and Owens, 2012). To solve the problem of viral disease in crustaceans, RNA interference (RNAi) technology for selective viral gene silencing (La Fauce and Owens, 2012), hyperthermia for viral reduction (Vidal et al., 2001, Claydon et al., 2004, Clarissa et al., 2006) and polyphenol flavonoids such as quercetin for down-regulating the inflammasome (Roopchand et al. 2015, Lee at al. 2016, Magrone et al. 2017) are considered as the alternative strategies to overcome viral disease in crustaceans.

This review will cover three possible viral reduction strategies including RNA interference (RNAi) technology, raising water temperature and quercetin. Moreover, the characterization, isolation, identification, protection and treatment for the RNA viruses, Macrobrachium rosenbergii nodavirus

(MrNV), convert mortality nodavirus (CMNV), Taura syndrome virus (TSV), yellowhead virus (YHV), infectious myonecrosis virus (IMNV), and Cherax giardiavirus (CGV) were reviewed.

2.2 RNA interference (RNAi) technology

RNAi is an RNA-dependent gene silencing process that is found in most eukaryotic species such as animals and plants (Doran and Helliwell, 2009). The initiator stage of the RNAi pathway, long double-stranded RNA (dsRNA) molecule or an endogenous hairpin precursor is cleaved by the enzyme Dicer, which is RNase III like-protein, into short double-stranded RNAs (siRNA) (21-23 nucleotides) or microRNAs (miRNA) (19-25 nucleotides), respectively (Dykxhoorn et al., 2003, Tijsterman and Plasterk, 2004, La Fauce and Owens, 2012, Wilson and Doudna, 2013). Characteristic of siRNA products from Dicer cleavage are double-stranded with 5' phosphorylated ends and 2 nucleotides unpaired and unphosphorylated 3' ends (Figure 2.1a) whilst miRNA products from Dicer cleavage are single-stranded with 5' phosphorylated ends and unphosphorylated 3' ends (Figure 2.1c) (Dykxhoorn et al., 2003). These siRNAs assemble with Argonaute proteins and incorporate into a RNA-inducing silencing complex (RISC) such that single-stranded, antisense strand is selected and used to guide RISC to its homologous target messenger RNA (mRNA) for endonucleolytic cleavage (Dykxhoorn et al., 2003, Wilson and Doudna, 2013). Endogenously expressed siRNAs have not been seen in mammals but the related miRNAs have been found in various organisms and cell types (Dykxhoorn et al., 2003). These miRNAs bind to sites that have partial sequence complementarity in 3' untranslated region (UTR) of their target mRNA, causing translation repression and protein synthesis inhibition (Dykxhoorn et al., 2003).

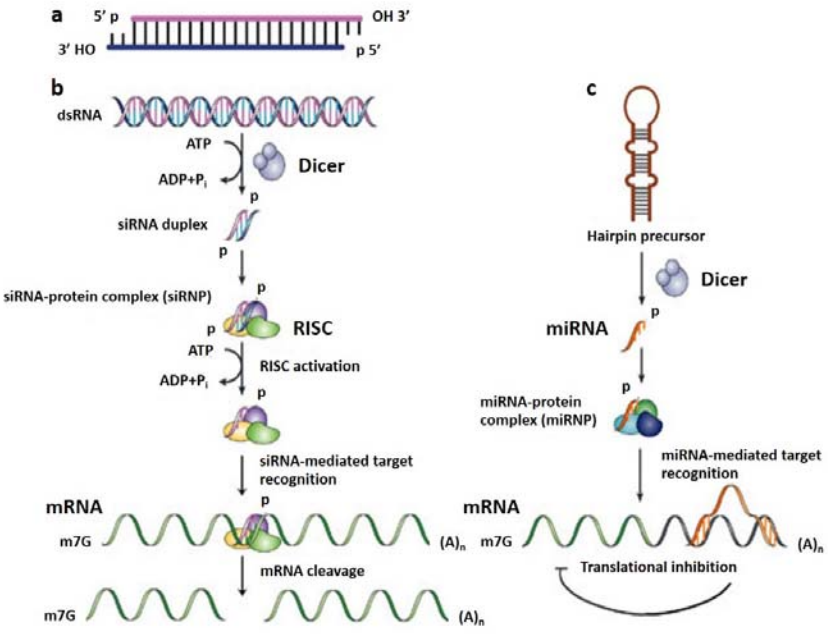

Figure 2.1 The RNA interference pathway (Dykxhoorn et al., 2003).

Six primary types of dsRNA that trigger RNAi have been identified, namely, Short interfering RNAs (siRNAs), MicroRNAs (miRNAs), Short hairpin RNAs (shRNAs), Long double stranded RNA (dsRNA), Short interfering RNA (siRNA)-based hairpin RNA, and MicroRNA (miRNA)-based hairpin-RNA (Dykxhoorn et al., 2003, La Fauce and Owens, 2012). However, the use of siRNAs have been proven

to produce the greatest gene silencing often with the least toxicity (La Fauce and Owens, 2012). Three methods can be employed to generate siRNAs including chemical synthesis, *in vitro* enzymatic synthesis, and *in vivo* (DNA plasmid vector) (Dykxhoorn et al., 2003, La Fauce and Owens, 2012). Chemical siRNAs can be synthesised at high amount and HPLC-purified by Integrated DNA Technologies or by oligo synthesis (Kim et al., 2005). The use of siRNAs prepared by chemical synthesis has been proven as the most efficient means of inducing RNAi but can be expensive when multiple siRNA are involved (La Fauce and Owens, 2012). The enzymatic synthesis using T7 phage RNA polymerase mediated *in vitro* transcription is the quickest and most cost effective method for siRNA synthesis (La Fauce and Owens, 2012). This synthesis involves three primary steps (Donzé and Picard, 2002) (Figure 2.2):

Step 1. The sense and antisense siRNA oligonucleotides single-stranded template (DNA) with encoding the siRNA and 8 nucleotides complementary to the T7 Promoter Primer at the 5' end are synthesized.

Step 2. The two template oligonucleotides are hybridized to a T7 promoter primer in separate reactions and are extended to create double-stranded templates.

Step 3. The sense and antisense templates are transcribed by T7 RNA polymerase and the resulting RNA transcripts are hybridized to create siRNA.

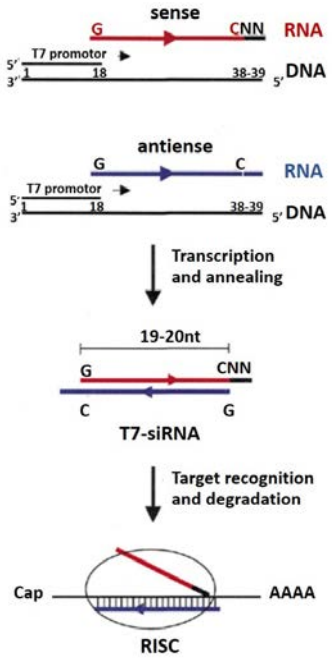

Figure 2.2 Strategy to generate T7 siRNAs (Donzé and Picard, 2002)

DNA vectors such as pSUPER (*in vivo*) can be used to express siRNAs as short-hairpin RNAs (shRNAs) (Brummelkamp et al., 2002, Sui et al., 2002, Bannister et al., 2007). Generating of shRNA involves design of a unique 19-29 nucleotides sequence derived from the target transcript, separated by a short spacer from the reverse complement of the same sequence and then a thermal cycler is employed to fold the sequence to form a pair stem-loop structure, which is cleaved by Dicer into active siRNAs (Brummelkamp et al., 2002, Sui et al., 2002). Although the use of plasmids is more economical for multiple sequences, the synthesis of siRNA using a DNA vector is labour intensive and success can be transfection-dependent (La Fauce and Owens, 2012).

2.3 Hyperthermia

The effect of experimentally induced hyperthermia on viral reduction has been previously reported in aquatic crustacean diseases (Vidal et al., 2001, Claydon et al., 2004, Clarissa et al., 2006, Montgomery-Brock et al., 2007).The hyperthermia-associated viral reduction might reflect either an improvement in the host antiviral response, or a direct negative effect on viral replication, or both (Clarissa et al., 2006). However, the signalling pathways and cell types involved in the response remain poorly defined. In an innate immune response, pathogen-associated molecular patterns (PAMPs) presented in microbial proteins, nucleic acids, lipids, and carbohydrates act as ligands to trigger pattern recognition receptors (PRRs)-dependent intracellular signaling pathways that ultimately induce the expression of pro-inflammatory and antiviral cytokines (Figure 2.3).

A key trigger of innate immune signaling for the production of pro-inflammatory and cytokines is the interactions predominantly mediated by two independent pathways for Toll-like receptor (TLR) signaling: 1) Myeloid differentiation primary response protein 88 (MyD88)-dependent pathway (Olson et al., 2015) and 2) MyD88-independent or Toll/IL-1 receptor domain-containing adapter inducing interferon-β (TRIF)-dependent pathway (Mitchell et al., 2010). All most TLRs except TLR3 signal through the MyD88-dependent pathway (Mitchell et al., 2010). According to previous hyperthermia studies in aquatic crustacean viruses both dsDNA (white spot syndrome virus (Vidal et al., 2001, Clarissa et al., 2006), intranuclear bacilliform virus (Claydon et al., 2004) and ssDNA (infectious hypodermal and hematopoietic necrosis virus (Montgomery-Brock et al., 2007)), the MyD88-dependent pathway might be referred as temperature-dependent pathway.

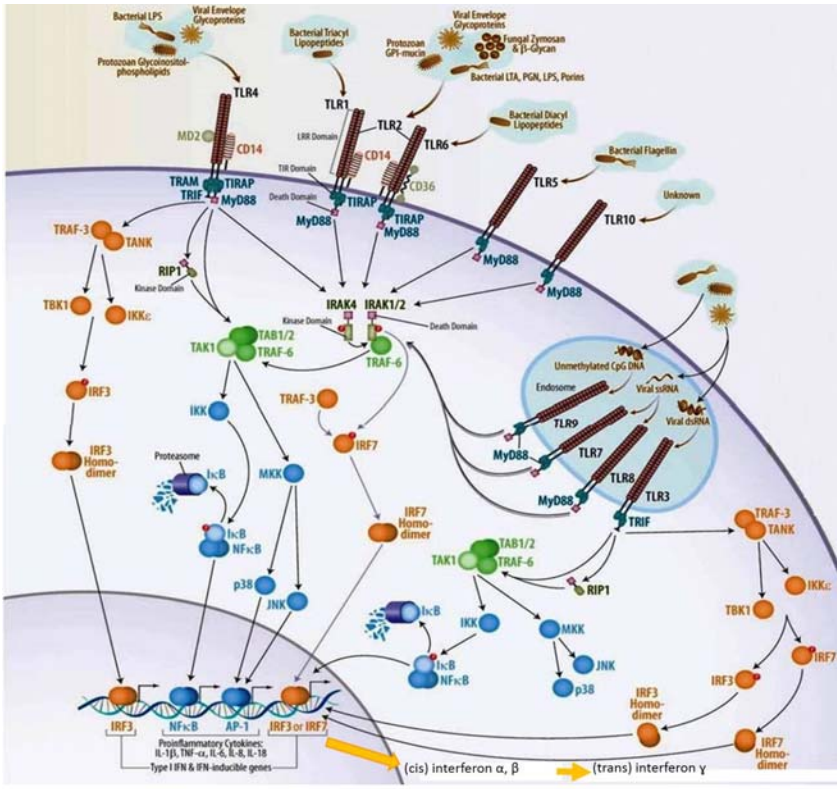

Figure 2.3 Pattern recognition receptors and the innate immune response (R&Dsystems, 2009).

2.4 Polyphenol flavonoids

Polyphenol flavonoids such as quercetin have been reported as the anti-inflammasome (Jiang et al., 2016, Domiciano et al., 2017) and antiviral effects (Cheng et al., 2015, Wong et al., 2017). The mechanism of antiviral action remains unclear, the inhibition might be associated with decreased heat shock proteins (HSP) and viral transcription levels (Cheng et al., 2015). The mechanism underlying inflammasome inhibition by quercetin might be that quercetin is a scaffold for the development of vitamin D receptor (VDR) modulators with selective biological activities resulting in the downregulation of inflammasome. The VDR-quercetin complex has been demonstrated using docking and molecular dynamics (MD) simulation (Lee et al., 2016). Moreover, the mechanism might be related to the inhibition of both Nucleotide-binding domain-like receptor protein 3 (NLRP3) (Jiang et al., 2016, Domiciano et al., 2017, Xue et al., 2017) and Absent in Melanoma 2 (AIM2) inflammasome activation by preventing Apoptosis-associated, speck-like protein containing a carboxy-terminal CARD (ASC) oligomerisation (Domiciano et al., 2017) and also inhibiting the release of mitochondrial reactive oxygen species (ROS) (Xue et al., 2017). Figure 2.5 and 2.6 show the proposed model for the inhibitory effect of quercetin. In summary, quercetin may be a potential therapeutic candidate against inflammation caused by viral infection or may have a benefit by viral reduction.

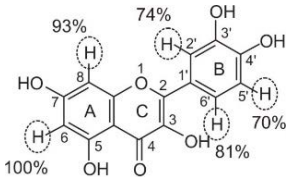

Figure 2.4 Group epitope mapping (GEM) of quercetin (Lee et al., 2016)

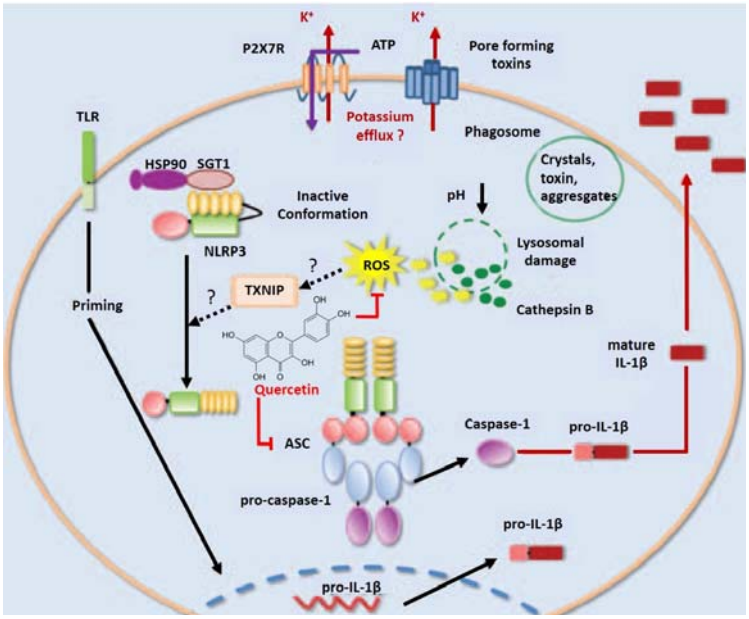

Figure 2.5 Schematic representation (Nucleotide-binding domain-like receptor protein 3) NLRP3 inflammasome activation and ROS production (Dowling and O'Neill, 2012) and inhibitory effect of quercetin.

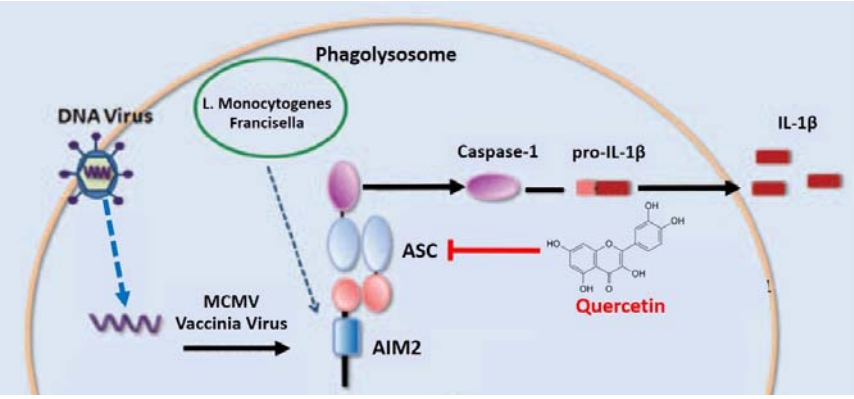

Figure 2.6 Schematic representation of Absent in Melanoma 2 (AIM2) inflammasome activation (Dowling and O'Neill, 2012) and inhibitory effect of quercetin.

2.5 Macrobrachium rosenbergii nodavirus (MrNV)

White tail disease (WTD) is defined as a viral infection caused by Macrobrachium rosenbergii nodavirus (MrNV). MrNV is the most serious problem in both hatchery and grow-out phases of the giant river prawn (*Macrobrachium rosenbergii*) (Pillai and Bonami, 2012). Mortality reaches 100% within 2 or 3 days with muscles exhibiting a milky whitish appearance particularly in the tail and abdominal regions, resulting in huge economic losses (Hameed et al., 2004). The disease has been reported in the West Indies (Pillai and Bonami, 2012), China (Qian et al., 2003), India (Hameed et al., 2004), Thailand (Yoganandhan et al., 2005), Taiwan (Wang et al., 2008), Australia (Owens et al., 2009) and Malaysia (Saedi et al., 2012).

2.5.1 Genome and Taxonomy of MrNV

MrNV is a small, icosahedral, non-enveloped virus 26 - 27nm in diameter, observed in the cytoplasm of connective tissue cells in infected prawns (Wang et al., 2008). The viral genome contains two positive-sense single-stranded RNA (ssRNA), RNA1 (2.9 kb) and RNA2 (1.26 kb) (Owens et al., 2009, Hameed and Bonami, 2012). RNA1 contains two open reading frames (ORFs), one (Hameed and Bonami, 2012) encoding an RNA-dependent RNA polymerase (RdRp) and another a B2 protein, which is encoded by the 3' region of RNA1 (Hameed and Bonami, 2012, Naveen et al., 2013b), whereas RNA2 encodes the capsid protein (Sudhakaran et al., 2008). The viral capsids exhibit a single polypeptide of 43 kDa; therefore, it has been placed in the family *Nodaviridae* (Bonami et al., 2005). Moreover, using phylogenetic analysis based on the amino acid sequence of the capsid protein, this virus was suggested to be placed in a new genus Gammanodavirus (Naveen et al., 2013b).

2.5.2 MrNV isolation and identification

2.5.2.1 Cell culture/ artificial media

The snakehead fish (*Ophicephalus striatus*) cell line (SSN-1), maintained in Leibowitz L-15 medium with glutamax supplemented with 10% foetal bovine serum and 1% antibiotic antimycotic solution (Invitrogen) at 26°C, has been reported as partially permissive to MrNV infection (Hernandez-Herrera et al., 2007). However, key elements for viral infection are lacking, such as regulatory factors for gene replication or post-translational modifications (Hernandez-Herrera et al., 2007). Mosquito

Aedes albopictus cell line (C6/36), cultured in Leibovitz L-15 medium containing 100 International Units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 2.5 µg ml^{-1} fungizone supplemented with 15% foetal bovine serum at 28°C, has been used for multiplication and observation of the cytopathic effects (CPE) of MrNV infection (Sudhakaran et al., 2007). However, the C6/36 cell line were reported as limited for patent replication of the Australian isolate of MrNV (Hayakijkosol and Owens, 2013).

2.5.2.2 Reverse-transcriptase polymerase chain reaction (RT-PCR)

The polymerase chain reaction is a genome-based application, which can amplify the small and undetectable quantities of target DNA by using specific oligonucleotide primers designed to the target DNA sequence. The World Organization for Animal Health (OIE) recommend reverse-transcriptase polymerase chain reaction (RT-PCR) as the best method for MrNV targeted surveillance, and both presumptive and confirmatory diagnosis for reasons of diagnostic specificity and sensitivity. The MrNV RT-PCR methods can be divided into two groups, namely, one-step RT-PCR, and RT nested PCR assay.

2.5.2.2.1 One-step RT-PCR assay

One-step RT-PCR can be used for confirmation of MrNV in larvae, postlarvae and adult prawns during suspected MrNV outbreaks (Sri Widada et al., 2003, Hameed et al., 2004, Owens et al., 2009) . The samples should be washed in sterile saline, transferred to sterile tubes, and stored at 20°C, or stored in 75% ethanol at 4°C (Sri Widada et al., 2003) until analysed. Homogenising of whole larvae or postlarvae, or pieces of adult prawn heads, or of organ pieces (gill tissue, hepatopancreas, heart, stomach, eyestalk, head muscle, abdominal muscle, tail muscle, ovary, intestine and pleopods) or haemolymph of adult prawns may be assayed for MrNV using RT-PCR (Sri Widada et al., 2003, Hameed et al., 2004, Owens et al., 2009). Table 2.1 shows pairs of primers that have been used to detect MrNV by the one-step RT-PCR technique.

Table 2.1. Pairs of primers used in one-step RT-PCR technique for the detection of MrNV

Target	Name	Designed from GenBank accession no	Size (bp)	Sequence (5′ to 3′)	Orientation	Reference
RNA1	1A775	AY222839 and AY222840	850	CCACGTTCTTAGTGGATCCT	Forward	(Sri Widada et al., 2003)
	1B690			CGTCCGCCTGGTAGTTCC	Reverse	
RNA1	1A729	AY222839 and AY222840	800	GTTCTTTACGCGCATCTTCC	Forward	
	1B633			GGTGGTAGGTGGCAACATAA	Reverse	
RNA1	MrNV1F	AY222839	590	TCCAACACCTCGCATAGC	Forward	(Hameed et al., 2004)
	MrNV1R			CACTCTTAACCCCCACTCC	Reverse	
RNA1	Forward	FJ379530	682	GATACAGATCCACTAGAT GACC	Forward	(Owens et al., 2009)
	Reverse			GACGATAGCTCTGATAATCC	Reverse	
RNA2	2A480	AY222839 and AY222840	650	AGGCAGGCTACGTCACAAGT	Forward	(Sri Widada et al., 2003)
	capC			ACAACCTAATTATTGCCGAC	Reverse	
RNA2	capS	AY222839 and AY222840	1,140	ATGGCTAGAGGTAAACAAAATTC	Forward	
	capC			ACAACCTAATTATTGCCGAC	Reverse	
RNA2	MrNV2F	AY222840	681	GATACAGATCCACTAGATGACC	Forward	(Hameed et al., 2004)
	MrNV2R			GACGATAGCTCTGATAATCC	Reverse	
RNA2	MrNV2aF	AY222840	425	GCGTTATAGATGGCACAAGG	Forward	
	MrNV2aR			AGCTGTGAAACTTCAACTGG	Reverse	

2.5.2.2.2 The RT nested PCR assay

The detection sensitivity of RT nested PCR is greater than the one-step RT PCR and suitable for MrNV screening in seed and broodstock (Sudhakaran et al., 2007). In general, nested PCR reaction involves two sets of primers, used in two successive runs of PCR, the second set targeted to amplify a secondary target within the first run product. The first step, the RT-PCR was performed with MrNV external primers using the Reverse-ITTM 1 step RT-PCR kit (ABgene), allowing RT and amplification to be performed in a single reaction tube. Table 2.2 shows pairs of primers that have been used in RT nested PCR technique for the detection of MrNV.

Table 2.2. Pairs of primers used in RT nested PCR technique for the detection of MrNV

Target	Name	Designed from GenBank accession no	Size (bp)	Sequence (5′ to 3′)	Orientation	Reference
RNA1	MrNV (External)	AY222840	425	GCGTTATAGATGGCACAAGG	Forward	(Hameed et al., 2004)
				AGCTGTGAAACTTCAACTGG	Reverse	
	MrNV (Internal)	AY222840	205	GATGACCCCAACGTTATCCT	Forward	(Sudhakaran et al., 2007)
				GTGTAGTCACTTGCAAGAGG	Reverse	

2.5.2.3 Quantitative RT-PCR assay

Quantitative PCR or real time PCR is a technique to amplify and simultaneously detect or quantify a targeted DNA molecule. Quantitative RT-PCR methods have the advantages of speed, specificity and sensitivity making them a useful tool for diagnostic, epidemiological and genetic studies in shrimp aquaculture. Quantitative RT-PCR have been performed to quantify the MrNV in the infected prawns using SYBR Green dye (Hernandez-Herrera et al., 2007) and Taqman probe (Zhang et al., 2006, Hayakijkosol et al., 2011, Hayakijkosol and Owens, 2012) (Table 2.3).

Table 2.3. Primers and probe used in quantitative RT-PCR technique for the detection of MrNV

Target	Designed from GenBank accession no	Size (bp)	Sequence (5′ to 3′)	Primers and probe	Reference
RNA1	AY231436	75	CAACTCGGTATGGAACTCAAGGT	Forward	(Zhang et al., 2006)
			AGGAAATACACGAGCAAGAAAAGTC	Reverse	
			[6FAM]ACCCTTCGACCCCAGCAATGGTG[6TAMARA]	Probe	
RNA1	AY222839	211	AGGATCCACTAAGAACGTGG	Forward	(Hernandez-Herrera et al., 2007, OIE, 2014b)
			CACGGTCACAATCCTTGCG	Reverse	
RNA1	AY222839, AY231436, and FJ751226	198	GAC CCA AAA GTA GCG AAG GA	Forward	(Hayakijkosol et al., 2011, Hayakijkosol and Owens, 2012)
			TTGTGATTTCCCTCTCCGG	Reverse	
			[6FAM] AAGCAACCGCCTTCAATGCC [TAM]	Probe	

2.5.3 Protection and treatment: using RNA interference

RNA interference (RNAi) against MrNV was first studied by Hayakijkosol and Owens (2012). This study demonstrated the protective effect of a triggered RNAi targeting MrNV protein B2, which is produced to inhibit the degradation of sequence-specific viral RNA in host cells. The results showed that injection of the specific dsRNA against protein B2 can prevent and reduce mortality in infected

C. quadricarinatus. However, average viral titres were not different between the RNAi treatment group and control group. Intramuscular injection of *in vitro* transcribed double-stranded RNA (dsRNA) targeting RNA dependent RNA polymerase (RdRp) and capsid genes of MrNV have been studied in the infected *M. rosenbergii* female brooder (Jitrakorn et al., 2014) The results showed that approximately forty percent of MrNV inhibition was found in the MrNV-specific dsRNA treatment group. Moreover, the researchers also suggested repeating the MrNV-specific dsRNA injection every seven days until breeding to reduce risk of MrNV re-infection.

Oral administration of bacterial expressed and encapsulated dsRNA for MrNV protection has been studied (Naveen et al., 2013a). In this study, not only were targeted genes of MrNV explored but also those of extra small virus (XSV), which is probably a satellite virus. Double-stranded RNA (dsRNA) of three different genes, namely, MrNV protein B2, MrNV capsid and XSV capsid either individually or in combination, were expressed in a bacterial expression system, inactivated, fed orally and the efficacy in controlling white tail disease was tested. *M. rosenbergii* fed with a combination of dsRNA of MrNV and XSV capsid genes represented higher relative percent survival (RPS) than fed with dsRNA of MrNV alone. Moreover, RNAi developed against protein B2 can be universally applied against different geographical isolates of MrNV due to the target region being highly conserved (Naveen et al., 2013a) (Table 2.4).

Table 2.4. Some of the current available RNAi therapies for MrNV

Target molecules	Administration route	Detail	References
MrNV protein B2	Intramuscular injection	Stealth RNAi CACCGACAACCUACUUUCAAAGCCA	(Hayakijkosol and Owens, 2012)
MrNV RdRp	Intramuscular injection	*In vitro* transcribed dsRNA	(Jitrakorn et al., 2014)
MrNV capsid			
MrNV protein B2	Oral feeding	Inactivated HT115(DE3) *E. coli* cells carrying LITMUS38i-B2 (MrNV) and induced with IPTG	(Naveen et al., 2013a)
MrNV capsid		Inactivated HT115(DE3) *E. coli* cells carrying LITMUS38i-capsid (MrNV) and induced with IPTG	
XSV capsid		Inactivated HT115(DE3) *E. coli* cells carrying LITMUS38i-capsid (XSV) and induced with IPTG	
MrNV protein B2 & MrNV capsid		Inactivated HT115(DE3) *E. coli* cells carrying LITMUS38i-B2 (MrNV), LITMUS38i-capsid (MrNV) and induced with IPTG	
MrNV capsid & XSV capsid		Inactivated HT115(DE3) *E. coli* cells carrying LITMUS38i-capsid (MrNV), LITMUS38i-capsid (XSV) and induced with IPTG	
MrNV protein B2 & XSV capsid		Inactivated HT115(DE3) *E. coli* cells carrying LITMUS38i-B2 (MrNV), LITMUS38i-capsid (XSV) and induced with IPTG	
MrNV protein B2, MrNV capsid & XSV capsid		Inactivated HT115(DE3) *E. coli* cells carrying LITMUS38i-B2 (MrNV), LITMUS38i-capsid (MrNV), LITMUS38i-capsid (XSV) and induced with IPTG	

2.6 Covert mortality nodavirus (CMNV)

Covert mortality nodavirus (CMNV) caused most moribund shrimp to hide on the bottom in deep water. CMNV has been associated with serious loss in China since 2009, particularly in *Litopenaeus vannamei* (Zhang et al., 2014). The researchers also reported clinical signs and mortality from both farmed and experimental challenged shrimp. Farmed shrimp showed hepatopancreatic atrophy, empty stomach and guts, soft shell, slow growth, and slightly whitish muscle lesion area in the abdominal segments with a cumulative mortality up to 80% in *L. vannamei* with CMNV infection. Experimental challenge caused cumulative mortality of *L. vannamei* in the 0.22 µm filtered extract, unfiltered extract and *per os* infection groups was 100, 100 and 84.85±2.14%, respectively, by day 10 post-injection.

2.6.1 Genome and Taxonomy of CMNV

CMNV is a spherical, non-enveloped virus with a mean size 32nm in diameter (Zhang et al., 2014). Using phylogenetic analysis based on RNA-dependent RNA polymerase sequences, this virus was placed in the genus *Alphanodavirus*, which includes Macrobrachium rosenbergii nodavirus (MrNV), Penaeus vannamei nodavirus (PvNV), Nodamura virus, Boolarra virus, Flock House virus (FHV), Black beetle virus (BBV), Drosophila melanogaster American nodavirus and Pariacoto virus (Zhang et al., 2014).

2.6.2 CMNV identification

2.6.2.1 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Nested reverse transcriptase polymerase chain reaction (nested RT-PCR) has been developed by Zhang et al. (2014) to detect CMNV infection using extracted RNA from haemolymph and cephalothorax. The reaction involves two sets of primers, namely CMNV-7F1/CMNV-7R1 and CMNV-7F2/CMNV-7R2 with a size of PCR product 619 bp and 165 bp respectively (Zhang et al., 2014) (Table 2.5).

Table 2.5. Primers used in RT nested PCR technique for the detection of CMNV

PCR	Name	Size (bp)	Sequence (5' to 3')	Orientation	Reference
1st run	CMNV-7F1	619	AAATACGGCGATGACG	Forward	(Zhang et al., 2014)
	CMNV-7R1		ACGAAGTGCCCACAGAC	Reverse	
2nd run	CMNV-7F2	165	CACAACCGAGTCAAACC	Forward	
	CMNV-7R2		GCGTAAACAGCGAAGG	Reverse	

2.6.3 Protection and treatment

No protective or treatment methods have been developed.

2.7 Taura syndrome virus (TSV)

Taura syndrome virus (TSV) is the causative agent of Taura syndrome, one of the most detrimental diseases in the penaeid shrimp culture industry, particularly in *Litopenaeus vannamei* (Navarro et al., 2009). Typical cumulative mortalities range from 40 to over 90% in cultured populations of postlarvae, juvenile and subadult life stages of *L. vannamei* (OIE, 2014a). TSV infections occur in two phases of gross pathology, the acute and recovery phases. The acute phase is characterized by reddening of the tail fan and by visible necrosis of the epithelium, whereas the recovery phase includes black cuticular lesions in the regions where the acute phase necrosis occurred (Flegel, 2006). The disease was first recognized along the Taura River in Ecuador in 1992 and later spread to many countries, including those in the Americas, Chinese Taipei, China, Thailand, Malaysia, Indonesia and Saudi Arabia (OIE, 2014a).

2.7.1 Genome and Taxonomy of TSV

TSV is a small, icosahedral, non-enveloped virus 32 nm in diameter, and which is observed in the cytoplasm of infected cells (Flegel, 2006). Its genome consists of a linear, positive-sense single-stranded RNA (ssRNA) 10,205 nucleotides in length, and it contains two large open reading frames (ORF), ORF1, that encodes the nonstructural proteins including, helicase, a protease and an RNA-dependent RNA polymerase (RdRp), and ORF2 that encodes three major capsid proteins, VP1 (55 kDa), VP2 (40 kDa) and VP3 (24 kDa), and one minor capsid protein (58 kDa) (Boube et al., 2014, Chaivisuthangkura et al., 2014). TSV has been assigned to the genus *Aparavirus* in a new family *Dicistroviridae* (in the order *Picornavirales*) by the International Committee on Taxonomy of Viruses (ICTV) (Chaivisuthangkura et al., 2014).

2.7.2 TSV isolation and identification

2.7.2.1 Cell culture/ artificial media

C6/36, the mosquito *Aedes albopictus* cell line, has been reported to persistently express TSV antigens, confirmed by positive RT-PCR results and the successful infection of *L. vannamei* with homogenates of TSV immunopositive insect cells from passage 15 (Arunrut et al., 2011). For persistent infections with TSV, C6/36 mosquito cells (a single cell-type clone obtained from the American Type Culture Collection, catalogue number CRL-1660) were maintained in supplemented Leibovitz L-15 medium (Gibco Invitrogen) containing 10% heat-inactivated foetal bovine serum (FBS) (Gibco Invitrogen), 10% tryptose phosphate broth (TBS) (Sigma) and 1.2% antibiotic (Penicilin G and Streptomycin) (Gibco Invitrogen) at 28℃ (Sriton et al., 2009).

2.7.2.2 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

For TSV targeted surveillance, presumptive and confirmatory diagnosis, RT-PCR and quantitative RT-PCR have been recommended as the best methods due to their diagnostic specificity and sensitivity (OIE, 2014a). Homogenising of gills, appendages, lymphoid organs (Phalitakul et al., 2006), haemolymph (Navarro et al., 2009), or whole prawns with carapace removed (Nunan et al., 1998) have been used for the RT-PCR assay of TSV detection in postlarvae, juvenile and adult prawns. Table 2.6 shows pairs of primers which have been used to detect TSV by the RT-PCR technique.

Table 2.6. Pairs of primers used in RT-PCR technique for the detection of TSV

Name	Designed from GenBank accession no	Size (bp)	Sequence (5' to 3')	Orientation	Reference
TSVF1	-	220	TCAATGAGAGCTTGGTCC	Forward	(Dhar et al., 2002)
TSVR1			AGTAGACAGCCGCGCTTG	Reverse	
9992F	AF277675	231	AAGTAGACAGCCGCGCTT	Forward	(Nunan et al., 1998, Phalitakul et al., 2006, Navarro et al., 2009, OIE, 2014a)
9195R			TCAATGAGAGCTTGGTCC	Reverse	
7171 F	-	341	CGA CAG TTG GAC ATC TAG TG	Forward	(Navarro et al., 2009)
7511 R			GAG CTT CAG ACT GCA AGT TC	Reverse	

The pair of 7171F/7511R primers have been designed to improve the sensitivity of RT-PCR detection. Its' detection has been determined to be 100 times more sensitive than the TSV primers 9992F/9195R (Navarro et al., 2009). Moreover, the same study also reported that primers 7171F/7511R were specific to TSV and did not react to either Penaeus stylirostris densovirus (PstDNV) (also known as Infectious hypodermal and hematopoietic necrosis virus (IHHNV)), White spot syndrome virus (WSSV), Yellow head virus (YHV), or Infectious myonecrosis virus (IMNV). Therefore, 7171F/7511R primers were highly recommended for TSV surveillance and diagnostic applications.

2.7.2.3 Quantitative RT- PCR

Quantitative RT-PCR methods have been developed to detect and quantify TSV in penaeid shrimp using SYBR Green dye (Dhar et al., 2002) or Taqman probe (Nunan et al., 2004, Tang et al., 2004) (Table 2.7). A quantitative RT-PCR assay has been used to quantify TSV in both acute and chronic infections in the tissues of the lymphoid organ, pleopods and gills of infected shrimp; however, there are no differences in TSV levels between the two stages of infection (Tang et al., 2004). For quantitation of TSV in chronically infected shrimp, the lymphoid organ is highly recommended for RNA extraction rather than either gills or pleopods (Tang et al., 2004).

Table 2.7. Primers and probe used in quantitative RT-PCR technique for the detection of TSV

Name	Size (bp)	Sequence (5' to 3')	Orientation	Reference
112F	50	CTGTTTGTAACACTACCTCCTGGAATT	Forward	(Dhar et al., 2002)
162R		TGATACAACAACCAGTGGAGGACTAA	Reverse	
TSV1004F	72	TTGGGCACCAAACGACATT	Forward	(Nunan et al., 2004, Tang et al., 2004, OIE, 2014a)
TSV1075R		GGGAGCTTAAACTGGACACAC-TGT	Reverse	
TSV-P1		[5FAM]CAGCACTGACGCACAATATTCGAGCATC[6TAMRA]	Probe	

2.7.3 Protection and treatment: using RNA interference

RNA interference (RNAi) technology using dsRNA targeting the endogenous gene *Penaeus monodon* Rab7 (PmRab7), which functions as a crucial regulator of intracellular trafficking of several viruses, has been developed for inhibition of TSV replication in *L. vannamei* (Ongvarrasopone et al., 2011). The results showed that injection with dsRNA-PmRab7 can effect a silencing of TSV genome expression; moreover, the dsRNA-PmRab7 group resulted in a significant reduction (84%) of the endogenous gene *L. vannamei* Rab7 (LvRab7) mRNA expression when compared with the control

group. Taken together, the results suggest that LvRab7 is involved in the endosomal trafficking pathway of TSV infecting penaeid shrimp (Ongvarrasopone et al., 2011).

Finding potential viral receptors in shrimp might lead to novel disease prevention methods. From this theory, a laminin-receptor protein (Lamr), which is identified as a binding protein for TSV has been studied in *L. vannamei* (Senapin et al., 2010). The scientists showed that 100% mortality occurred within 9 days in the *L. vannamei* group injected with dsRNA fragment covering the region coding the palindromic laminin-binding domain. Knockdown of Lamr is lethal to shrimp therefore, Lamr cannot be used as a direct TSV disease prevention method. However, indirect investigation such as, injection with an anti-Lamr antibody or with recombinant Lamr protein should be tested for efficacy in blocking TSV infection (Senapin et al., 2010) (Table 2.8).

Table 2.8. Some of the current available RNAi therapies for TSV

Target molecules	Administration route	Detail	References
PmRab7	Haemolymph injection	*In vitro* transcribed dsRNA	(Ongvarrasopone et al., 2011)
Lamr	Injection	dsRNA fragment covering the region coding the palindromic laminin-binding domain	(Senapin et al., 2010)

2.8 Yellow head complex virus

Yellow head virus (YHV) is a highly virulent pathogen that can cause total crop loss within 3 to 5 days of the first appearance. cClinical signs include a yellowish cephalothorax and a very pale overall coloration of moribund, infected shrimp, especially in the black tiger prawn (*Penaeus monodon*) (Chantanachookin et al., 1993, Flegel, 1997). Moreover, the host range for YHV also covers *Cherax quadricarinatus* (Soowannayan et al., 2015). However, in the yellow head complex, only genotype 1 and 7 of YHV and gill-associated virus (GAV) are associated with disease.

YHV genotype 1 was first reported as the cause of mass mortalities of *P. monodon* farmed in Thailand in 1990 (Wijegoonawardane et al., 2009). In 2012, YHV genotype 7 was detected in diseased *P. monodon* in Australia (Mohr et al., 2015). The negative impact of gill-associated virus (GAV) production of *P. monodon* farmed in Australian was reported in 2011 (Munro et al., 2011) . Four other genotypes (genotype 3-6) of YHV commonly occur in healthy *P. monodon* (Wijegoonawardane et al., 2008). Recently, non-pathogenic YHV genotype 8 was found in farmed shrimp *Fenneropenaeus chinensis* suffering from early mortality disease/acute hepatopancreatic necrosis disease (EMS/AHPNS) in China (Liu et al., 2014).

2.8.1 Genome and Taxonomy of YHV and GAV

YHV is rod-shaped, enveloped, and measures 150-170 x 40–50 nm, and is observed in the cytoplasm adjacent to the nuclei of infected cells from various tissues (Chantanachookin et al., 1993, Wongteerasupaya et al., 1995). Yellow head virions comprise a positive-sense, single-stranded RNA genome (Wongteerasupaya et al., 1995), three major structural proteins with molecular masses of 116 kDa (gp116), 64 kDa (gp64), and 20 kDa (p20) (Jitrapakdee et al., 2003). The gp116 and gp64 enveloped glycoproteins are encoded by ORF3, whereas the p20 nucleoprotein is encoded by ORF2

(Jitrapakdee et al., 2003, Sittidilokratna et al., 2006). Moreover, YHV also contains a large replicase gene (ORF1b), which encodes sequence motifs for polymerase, metal ion-binding and helicase domains (Sittidilokratna et al., 2002). According to molecular comparison based on 3 regions of the large ORFlb gene, GAV and YHV are closely related but distinct viruses (Cowley et al., 1999). Both YHV (Chaivisuthangkura et al., 2014) and GAV (Mayo, 2002) are classified in the new virus genus *Okavirus*, the new family *Roniviridae* and the order *Nidovirales* with GAV being the type species.

2.8.2 YHV and GAV isolation and identification

2.8.2.1 Cell culture/ artificial media

One hundred percent of C6/36 and Sf9 cells have been reported to persistently express YHV antigens (Sriton et al., 2009). The same study demonstrated that the insect cells remained 100% antigen positive for more than 100 passages. For persistent YHV infections, C6/36 mosquito cells (a single cell-type clone obtained from the American Type Culture Collection, catalogue number CRL-1660) were maintained in supplemented Leibovitz (L-15) medium (Gibco Invitrogen) containing 10% heat-inactivated foetal bovine serum (FBS) (Gibco Invitrogen), 10% tryptose phosphate broth (TBS) (Sigma) and 1.2% antibiotic (Penicillin G and Streptomycin) (Gibco Invitrogen) at 28℃, whereas, Sf9 cells, a clonal isolate of *Spodoptera frugiperda* IPLB-Sf21-AE (fall armyworm) were maintained in Sf-900 II SFM serum free medium complete 1X (Gibco Invitrogen) containing 1% antibiotic-antimycotic mix 100X (Gibco Invitrogen) at 28℃ (Sriton et al., 2009).

2.8.2.2 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR for the detection of YHV has advantages over direct dot blot nucleic acid hybridization tests in terms of a higher sensitivity and shorter detection time; therefore, this method would allow early detection of the virus (Wongteerasupaya et al., 1997). Homogenising of whole head, gill, lymphoid tissue or haemolymph have been used for RNA extraction (Cowley et al., 2004). RT-PCR method of YHV can be divided into two groups, namely, one-step RT-PCR, and RT nested PCR assays.

2.8.2.2.1 One-step RT-PCR assay

One-step RT-PCR using 10F/144R primers has been developed to detect YHV in infected shrimp (Wongteerasupaya et al., 1997). The protocol was retested by Wijegoonawardane et al. (2008), using 1 µg input RNA, and it utilized 10-fold more cDNA than the tests using random-primed cDNA. The suspected 135 bp amplicon only amplified the highly virulent genotype (genotype 1) of YHV, which was first detected in Thailand in association with yellow head disease but not gill-associated virus (GAV) or other genotypes of yellow head virus (OIE, 2014c). Whereas RT-PCR with primer pair GY1F/GY4R, described by Cowley et al. (2004), has been employed to amplify a 794 bp product for either YHV or GAV; therefore, this protocol cannot separate GAV from YHV.

RT-PCR assay has been demonstrated as more sensitive than other diagnostic methods. RT-PCR assay targeting the RdRp gene of YHV was determined as only one method that gave positive result of the experimentally exposed *C. quadricarinatus* while histopathology and immunoreactions using monoclonal antibodies against all 3 structural proteins of YHV gave negative results (Soowannayan

et al., 2015). This study also employed RT-RCR using primers targeting a house keeping gene, β-actin to verify RNA integrity before the RT-PCR assays for YHV infection (Table 2.9).

Table 2.9. Pairs of primers used in one-step RT-PCR technique for the detection of yellow head complex

Name	Size (bp)	Sequence (5′ to 3′)	Orientation	Reference
10F	135	CCGCTAATTTCAAAAACTACG	Forward	(Wongteerasupaya et al., 1997, OIE, 2014c)
144R		AAGGTGTTATGTCGAGGAAGT	Reverse	
GY1	794	GACATCACTCCAGACAACATCTG	Forward	(Cowley et al., 2004)
GY4		GTGAAGTCCATGTGTGTGAGACG	Reverse	
YHV pol F	164	CAATCAAGTGCCCGAAGAAT	Forward	(Soowannayan et al., 2015)
YHV pol R		GATTGATGATGGCGAGTGTG	Reverse	

2.8.2.2.2 RT nested PCR assays

Multiplex RT-nested PCR assays have been developed by Cowley et al. (2004) to detect and differentiate the closely related prawn viruses, GAV and YHV (genotype 1) in diseased prawn or for screening healthy carriers. This reaction involves two sets of primers, used in two successive runs of PCR, the second set amplified a secondary target within the first run product. As a first step, the conserved primer pair GY1F/GY4R was used to amplify a 794 bp product from both the GAV and YHV infected shrimp. The second step involved using, the conserved primer GY2F in combination with both Y3R and G3R to amplify a 277 bp YHV-specific product or a 406 bp GAV-specific product. The detection sensitivity of the second-step PCR enhanced approximately 1000-fold from the first-step PCR; moreover, GAV or YHV RNA extraction from lymphoid organ could be detected to a limit of 10 fg (Cowley et al., 2004). However, it still cannot detect six (two new genotypes not tested) of the eight known genotypes when retested by Wijegoonawardane et al. (2008).

For detection of the six of eight distinct genotypes in the yellow head complex or for screening healthy shrimp, a sensitive multiplex RT nested PCR protocol developed by Wijegoonawardane et al. (2008) was used. The first step amplified a 359 bp cDNA for genotypes 1-6 of yellow head complex using a combination of YC-F1a and YC-F1b for forward primers and combination of YC-R1a and YC-R1b for reverse primers.

In the RT nested PCR, a 147 bp product was detected with most genotypes (two genotypes not tested) of yellow head complex using a combination of YC-F2a and YC-F2b for forward primers and a combination of YC-R2a and YC-R2b for reverse primers. The sensitivity limits of RT-PCR and RT nested PCR were estimated to be approximately 1,250 and 1.25 RNA copies, respectively. However, this protocol cannot distinguish between genotypes. Therefore, nucleotide sequence analysis of the RT-PCR product is recommend to obtain assignment of genotype.

Table 2.10. Primers used in RT nested PCR technique for the detection of yellow head complex

PCR	Name	Size (bp)	Sequence (5' to 3')	Orientation	Virus	Reference
1st run	GY1	794	GACATCACTCCAGACAACATCTG	Forward	YHV and GAV	(Cowley et al., 2004)
	GY4		GTGAAGTCCATGTGTGTGAGACG	Reverse		
2nd run	GY2	227	CATCTGTCCAGAAGGCGTCTATGA	Forward	YHV	
	Y3		ACGCTCTGTGACAAGCATGAAGTT	Reverse		
	GY2	406	CATCTGTCCAGAAGGCGTCTATGA	Forward	GAV	
	G3		GCGTTCCTTTGTGAGCATAAATGA	Reverse		
1st run	YC-F1a	359	ATCGTCGTCAGCTACCGCAATACTGC	Forward	YHV, GAV and genotypes 3-6	(Wijegoonawardane et al., 2008)
	YC-F1b		ATCGTCGTCAGYTAYCGTAACACCGC			
	YC-R1a		TCTTCRCGTGTGAACACYTTCTTRGC	Reverse		
	YC-R1b		TCTGCGTGGGTGAACACCTTCTTGGC			
2nd run	YC-F2a	147	CGCTTCCAATGTATCTGYATGCACCA	Forward	YHV, GAV and genotypes 3-6	
	YC-F2b		CGCTTYCARTGTATCTGCATGCACCA			
	YC-R2a		RTCDGTGTACATGTTTGAGAGTTTGTT	Reverse		
	YC-R2b		GTCAGTGTACATATTGGAGAGTTTRTT			
1st run	Targeting β-actin	NA	CCCCATTGAGCACGGTATCA	Forward	Host	(Soowannayan et al., 2015)
			ACGCTCAGGAGGAGCAATGA	Reverse		
2nd run	YHV pol F	NA	CAATCAAGTGCCCGAAGAAT	Forward		
	YHV pol R		GATTGATGATGGCGAGTGTG	Reverse		

Note: Mixed nucleotides: R(A/G), Y(C/T), D(A/G/T) are nucleotides differing between the a and b primers

2.8.2.3 Quantitative RT-PCR

Well-documented advantages of quantitative RT- PCR include the ability to quantify infection loads accurately, high sample throughput and reduced opportunities for sample cross-contamination. The sensitivity and specificity of SYBR Green RT-PCR to determine the YHV load in shrimp have been developed in several studies (Dhar et al., 2002, Mouillesseaux et al., 2003, Wijegoonawardane et al., 2010). Dhar et al. (2002) developed a method to amplify a 65 bp product for YHV using SYBR Green as a fluorescence dye in the PCR mixture. However, the small size of the product, and the ability of SYBR Green I dye to bind to a dsDNA indiscriminately may occasionally result in non-specificity in the RT-PCR assay. By increasing the amplicon length, which not only increases the specificity of the amplification, but also enhances the detection limit for YHV was developed by Mouilesseaux et al. (2003), and can address this limitation. However, these two methods were only designed based on the sequence of the cloned segment of YHV while other genotypes of yellow head complex were not involved. RT nested PCR primers developed by Wijegoonawardane et al. (2008) have been modified to develop a SYBR-Green quantitative RT-PCR (Wijegoonawardane et al., 2010). This protocol has been employed to detect six yellow head genotypes but not tested on the new yellow head genotypes 7 and 8.

Table 2.11. Primers used in quantitative RT-PCR technique for the detection of yellow head complex

Name	Size (bp)	Sequence (5′ to 3′)	Orientation	Reference
141F	65	CGTCCCGGCAATTGTGAT	Forward	(Dhar et al., 2002)
206R		CCAGTGACGTTCGATGCAATA	Reverse	
399F	98	ATCGGCACAGGAGCAGACA	Forward	(Mouillesseaux et al., 2003)
496R		GTAACCCCGGCCATGACTT	Reverse	
912F	50	TCAATGAGTTCAATGACGTCGAA	Forward	
962R		GAATGGTATCACCGTTCAGTGTCTT	Reverse	
YHc-F2	147	CGCTTYCARTGTATCTGYATGCACCA	Forward	(Wijegoonawardane et al., 2010)
YHc-R2		RTCAGTGTACATGTTKGAGAGTTTRTT	Reverse	

2.8.3 Protection and treatment: using RNA interference

Double-stranded RNA (dsRNA) targeting the protease motif of YHV has been studied in the YHV infected *P. monodon* (Yodmuang et al., 2006, Tirasophon et al., 2007). YHV-protease dsRNA given to shrimp prior to a YHV challenge, a complete inhibition of YHV replication and no mortality were found (Yodmuang et al., 2006). YHV-protease dsRNA given post-viral infection, showed a strong inhibition of YHV replication up to 12 hours post-inoculation with YHV and mortality could be prevented by the dsRNA at 3 hours post-infection with YHV (Tirasophon et al., 2007). Taken together, YHV-protease dsRNA should be used to protect and treat for YHV infection. The endogenous gene *P. monodon* Rab7 (PmRab7), which is involved in endosomal trafficking of several viruses, has been developed as dsRNA for inhibition of YHV replication in *P. monodon* (Ongvarrasopone et al., 2008); PmRab7 mRNA specifically decreased at 48 hours after dsRNA-PmRab7 injection. The silencing of PmRab7 can prevent YHV replication; therefore, PmRab7 could be a novel method to preventing YHV infection in shrimp.

In the case of GAV, dsRNAs targeted to the RNA-dependent RNA polymerase (RdRp) and helicase gene regions was studied in *P. monodon* (Oanh et al., 2011). Both dsRNAs reduced GAV genetic loads, delayed the onset of mortalities and improved survival following a GAV challenge. Inhibiting GAV replication and disease by intramuscular injection and oral delivery were studied by Sellars et al. (2011). The study explored the possibility of inhibiting GAV replication and disease using dsRNA targeted open reading frame 1a/b (ORF1a/b) expressed in bacteria. Intramuscular injection of dsRNA-ORF1a/b can be highly effective at slowing GAV replication and protecting shrimp against acute disease and mortalities. In contrast, feeding with dsRNA-ORF1a/b cannot reduce elevated in GAV loads and mortality (Sellars et al., 2011). Therefore, injection-based delivery of dsRNA is recommended, particularly in breeding programs.

Table 2.12. Some of the current available RNAi therapies for yellow head complex

Target molecules	Administration route	Detail	References
YHV-protease	Hemolymph injection	*In vitro* transcribed dsRNA, dsRNA given prior to viral challenge	(Yodmuang et al., 2006)
		In vitro transcribed dsRNA, dsRNA given post-viral challenge	
PmRab7		*In vitro* transcribed dsRNA, dsRNA given prior to viral challenge	(Ongvarrasopone et al., 2008)
GAV RdRp	Intramuscular injection	*In vitro* transcribed dsRNA, dsRNA given immediately after viral challenge	(Oanh et al., 2011)
GAV helicase gene			
GAV ORF1a/b	Intramuscular injection	*In vitro* transcribed dsRNA, dsRNA given prior to viral challenge	(Sellars et al., 2011)
	Oral feeding	Inactivated HT115(DE3) *E. coli* cells carrying pL4440- ORF1a/b and induced with IPTG, dsRNA given prior to viral challenge	

2.9 Infectious myonecrosis virus (IMNV)

Infectious myonecrosis virus (IMNV) causes a crucial emerging disease in cultured penaeid shrimp, especially in the Pacific white shrimp *(Litopenaeus vannamei)*, a species of great economic importance (Andrade et al., 2007). Cumulative mortalities due to IMV infection in shrimp ponds have reached 70%, with striated muscle exhibiting white necrotic areas, mainly in the distal abdominal segments and tail fan. These lesions are accompanied by liquefying of the necrotic fibrotic muscles in the chronic stage, giving the appearance of cooked shrimps (Poulos et al., 2006, Chaivisuthangkura et al., 2014). The first reported outbreak of IMNV was in Brazil in 2004 and has since been reported in Indonesia (Senapin et al., 2007).

2.9.1 Genome and Taxonomy of IMNV

IMNV is an icosahedral, non-enveloped virus and approximately 40 nm in diameter, and is seen in the cytoplasm of muscle cells, haemocytes and connective tissue cells of infected shrimp (Poulos et al., 2006, Melo et al., 2014). The genome comprises a double-stranded RNA containing two open reading frames ORFs, ORF1, which encodes an RNA-binding motif, and a capsid protein and ORF2, which encodes an RNA-dependent RNA polymerase (RdRp) (Poulos et al., 2006, Melo et al., 2014). Based on phylogenic analysis of the RdRp, IMNV clustered with Giardia lamblia virus (GLV), in the family *Totiviridae* (Poulos et al., 2006).

2.9.2 IMNV isolation and identification

2.9.2.1 Cell culture/ artificial media

No cell culture for IMNV has been reported to date. However, C6/36, Sf9, and HzAM1 cell lines have been used to isolate a novel double-stranded RNA virus, a tentative member of the family *Totiviridae* which has been shown to be related to IMNV (Zhai et al., 2010, Yang et al., 2012). The Sf9 and HzAM1 cell lines were cultured in Grace's insect medium, the C6/36 cell lines in 60% Dulbecco's

modified Eagle medium (DMEM) and 30% RPMI medium 1640, moreover, all media were supplemented with 10% FBS at 28℃ in a humidified 5% CO_2 atmosphere (Yang et al., 2012).

2.9.2.2 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

A RT-nested PCR method has been developed to detect IMNV using RNA extracted from pleopods or tail muscle (Poulos et al., 2006). A two steps reaction with two primer sets was employed to amplify a 328 bp amplicon in the first step and a 139 bp amplicon in the nested reaction. The first step reaction can detect 100 copies of the IMNV viral genome whereas the nested reaction can detect 10 copies (Poulos and Lightner, 2006). However, this protocol, retested by Andrade et al. (2007), showed that this nested RT-PCR is only capable of detecting IMNV when the number of viruses is over 1500.

Table 2.13. Primers used in RT-PCR technique for the detection of IMNV

PCR	Name	Size (bp)	Sequence (5′ to 3′)	Orientation	Reference
1st run	4587F	328	CGACGCTGCTAACCATACAA	Forward	(Poulos et al., 2006, Andrade et al., 2007, OIE, 2014d)
	4914R		ACTCGGCTGTTCGATCAAGT	Reverse	
2nd run	4725 NF	139	GGCACATGCTCAGAGACA	Forward	
	4863 NR		AGCGCTGAGTCCAGTCTTG	Reverse	

2.9.2.3 Quantitative RT-PCR

A quantitative RT-PCR method using TaqMan assay has been developed by Andrate et al. (2007) to detect and quantify IMNV. This protocol can detect as few as 10 IMNV copies/µl RNA, whereas, the nested RT-PCR developed by Poulos and Lightner (2006), can detect fewer than 1000 IMNV copies/µl RNA. These results demonstrate that this quantitative RT-PCR assay is a sensitivity diagnostic method for IMNV.

Table 2.14. Primers and probe used in quantitative RT-PCR assay for IMNV

Name	Size (bp)	Sequence (5′ to 3′)	Orientation	Reference
IMNV412F	134	GGACCTATCATACATAGCGTTTGCA	Forward	(Andrade et al., 2007, OIE, 2014d)
IMNV545R		AACCCATATCTATTGTCGCTGGAT	Reverse	
IMNVp1		[6FAM] CCACCTTTACTTTCAATACTACATCATCCCCGG [TAMRA]	Probe	

2.9.3 Protection and treatment: using RNA interference (RNAi)

Double-stranded RNA (dsRNA) targeting three regions of the IMNV genome (GenBank accession no. EF061744), namely dsRNA95-475, dsRNA3764-4805 and dsRNA5518-6391 have been studied in the IMNV infected *L. vannamei* (Loy et al., 2012). Administration of the dsRNA95-475, which targets the protein 1 (ORF1) and, dsRNA3764-4805 (ORF1) which targets the major capsid protein improved survival by 30 days following an IMNV challenge. In contrast, no significant difference was evident between survival in the dsRNA5518-6391 group, which targets the non-structural RNA-dependent RNA polymerase (RdRp, ORF2) and the controls. Therefore, the targeted region for dsRNA is a crucial factor in maximizing the degree of IMNV protection in shrimp.

Additionally, dsRNA194-475 that targets the 5' end of the IMNV genome, and previously showed outstanding antiviral protection when administered prior to infection (Loy et al., 2012), has been tested by Loy et al. (2013) for therapeutic efficacy when administered post-infection.
The administration of dsRNA194-475 resulted in a significant reduction of the viral load. Moreover, the group that was given dsRNA194-475 at 48 hours post-infection, demonstrated 50% mean survival for 20 days, whereas the infectious control groups showed 100% mortality. These data indicate that this optimized RNAi antiviral molecule holds promise for use as a protection and treatment for IMNV.

In addition, Feijó et al. (2015) used the same types of dsRNA as Loy et al. (2012) but based on a different GenBank accession number to investigate its ability against IMNV in *L. vannamei*. The ORF1a (197-789) gene dsRNA, the ORF1b (4239-4839) gene dsRNA and the ORF2 (5939-6536) gene dsRNA targeted protein 1, the major capsid protein and the RdRp, respectively. Injection of dsRNA-ORF1a and dsRNA-ORF1b, but not dsRNA-ORF2, strongly inhibited IMNV replication over 3 weeks following an IMNV challenge and resulted in 90% and 83% survival, respectively. These results concur with the previous study by Loy et al. (2012).

Table 2.15. Some of the current available RNAi therapies for IMNV

Target molecules	GenBank accession no	Administration route	Detail	References
IMNV 95-475 (Protein 1)	EF061744	Intramuscular injection	*In vitro* transcribed dsRNA, dsRNA given prior to viral challenge	(Loy et al., 2012)
IMNV 3764-4805 (Major capsid protein)				
IMNV 5518-6391 (RdRp)				
IMNV 194-475 (Protein 1)		Intramuscular injection	*In vitro* transcribed dsRNA, dsRNA given post-viral challenge	(Loy et al., 2013)
IMNV ORF1a (Protein 1)	AY570982	Intramuscular injection	*In vitro* transcribed dsRNA, dsRNA given post-viral challenge	(Feijó et al., 2015)
IMNV ORF1b (Major capsid protein)				
IMNV ORF2 (RdRp)				

2.10 Cherax giardiavirus (CGV)

Cherax giardiavirus (CGV) has been identified as a cause of serious mortalities in *Cherax quadricarinatus*, with the highest mortality being 85% by week 8 (Edgerton et al., 1994). CGV infected *C. quadricarinatus* are characterized by patches of opaque musculature and easily collected by hand (Edgerton et al., 1997).

2.10.1 Genome and Taxonomy of CGV

CGV is an icosahedral, non-enveloped virus and approximately 25 nm in diameter, containing double-stranded RNA, which accumulate in the nucleus (Edgerton et al., 1994). Based on morphology and intranuclear position suggest that CGV is most closely related to Giardia lamblia virus (GLV), therefore CGV is tentatively placed in the family *Totiviridae* (Edgerton et al., 1994).

2.10.2 CGV identification

2.10.2.1 The classical H&E staining

The diagnosis of infection by CGV has been based on the demonstration of the key histological features of this disease, especially the variably hypertrophic with marginated and clumped chromatin. The most common of lesion is seen only in the hepatopancreocytes, particularly resorptive (R) cells whilst less commonly within fibrillary (F) and blister (B) cells whilst infection of embryonic (E) cells was not detected (Edgerton et al., 1994). Well-developed intranuclear inclusions are intensely purple-red whilst less-developed inclusion are eosinophilic (Edgerton et al., 1994).

2.10.3 Protection and treatment

No protective or treatment methods have been developed.

2.11 Conclusion

The important, known RNA viruses of concern to aquatic crustacea are Macrobrachium rosenbergii nodavirus (MrNV), covert mortality nodavirus (CMNV), Taura syndrome virus (TSV), yellow head virus (YHV), infectious myonecrosis virus (IMNV), and Cherax giardiavirus (CGV). *Mr*NV and CMNV are placed in the family *Nodaviridae* whilst TSV, YHV, IMNV and CGV are placed in the family *Dicistroviridae, Roniviridae* and the last two in family *Totiviridae,* respectively. Currently, reverse transcriptase, polymerase chain reaction (RT-PCR) and quantitative, reverse transcriptase polymerase chain reaction (quantitative RT-PCR) have been developed as the most rapid and sensitive methods for viral detection.

RNA interference (RNAi), which is a specific post-transcriptional regulatory pathway resulting in silencing gene functions, has been demonstrated as a potential treatment for viral diseases. Based on effect of the host antiviral response increasing and decreasing of viral replication, hyperthermia might be simple technique to solve the RNA viral infection. Quercetin to down-regulate the inflammasome might be also an alternative treatment to relieve inflammation caused by viral diseases. Based on understanding and data of the known RNA viruses, this could be applied for dealing with the emergence of pathogenic unknown RNA viruses in aquatic crustacea.

CHAPTER 3
GENERAL MATERIAL AND METHODS

This chapter contains methods that were used more than once across the research chapters.

3.1 RNA extraction

Total RNA was extracted from crayfish sample (muscle or haemolymph) or cricket tissue (flight muscle, gut and reproductive tissue) using total RNA Purification Kit (NorgenBiotek®, CA) according to the manufacturer's instructions.

3.1.1 Preparation from animal tissues

Approximately 10 mg of tissue was transferred into an RNase-free microcentrifuge tube containing approximately 500 µl liquid nitrogen. The tissue was ground thoroughly using a pestle. After the liquid nitrogen evaporated, the sample was homogenized in 600 µl Lysis Buffer RL from the above kit and further homogenized by passing the lysate 5-10 times through a 25 gauge needle attached to a 1ml syringe. Then the lysate was transferred into an RNase-free microcentrifuge tube and centrifuged for 2 minutes at 2,000 x *g* to pellet any cell debris. An equal volume of 70% ethanol was added to the lysate volume collected and mixed vigorously. The lysate with the ethanol was transferred into the reservoir of a spin column with its collection tube and centrifuged for 1 minute at 3500 x *g*. After the column was emptied, it was washed with 400 µl Wash Solution A and dried by spinning at 14,000 x *g* for 1 minute to remove the ethanol residue. The spin column was assembled to a new RNase-free microcentrifuge tube, and added with 50µl Elution Solution A. After 2 minutes centrifugation at 2,000 x *g*, followed by 1 minute at 14,000 x *g*, the flow-through containing total RNA was stored at -80℃ until used.

3.1.2 Preparation from haemolymph

Approximately 100 µl of haemolymph was homogenized in 350 µl Lysis Buffer RL by vortexing for 15 seconds. The transparent mixture was added with 200 µl of 100% ethanol and mixed by vortexing for 10 seconds. The lysate with the ethanol was processed in the same manner of RNA extraction from animal tissues.

3.2 cDNA synthesis

cDNA was produced using Tetro cDNA Synthesis Kit (Bioline, UK) following the manufacturer's protocol. Briefly, cDNA mixture containing 1x RT Buffer, 10U Reverse Transcriptase, 0.5U RNase Inhibitor, 0.5 µM dNTP Mix, 1 µl Random Hexamer Primer Mix, RNA template and DEPC-treated water to a final volume of 20 µl was prepared. The cDNA was performed under the thermal profile consisting 25℃ for of 10 minutes at, 45℃ for 30 minutes and 85℃ for 5 minutes, and hold at 4℃ using C1000 Touch thermal cycler (BIO-RAD, US).

3.3 Reverse transcription polymerase chain reaction (RT-PCR) and gel electrophoresis

DNA amplifications were under undertaken in a C1000 Touch thermal cycler (BIO-RAD, US) using MyFi™ Mix (Bioline, UK) following the manufacturer's protocol. Briefly, PCR mixture containing 1x MyFi Mix, 0.4 µM each forward and reverse primer, cDNA template and DEPC-treated water to a final volume of 25 µl was prepared. The PCR profile was 95℃ for 1 minute followed by 30 or 35 cycles of 95℃ for 15 seconds, 59℃ for 15 seconds and 72℃ for 15 seconds. Samples were polymerised for an additional 5 minutes at 72℃ following the last cycle. The obtained PCR products were electrophoresed on 1.8% agarose gel with GelRed™ (Biotium, CA) to visualize the products on a UV transilluminator.

3.4 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The amplification was carried out in 20 µl of reaction volume containing 1 x SensiFast SYBR No-ROX Mix (Bioline, UK), 0.4 µM each forward and reverse primer, cDNA template and RNase-free water. The thermal profile was 95℃ for 10 minutes followed by 40 cycles of 95℃ for 5 seconds, 59℃ for 10 seconds and 72℃ for 10 seconds. The data acquisition and analysis were carried out with Rotor-Gene Q Series Software 2.3.1 (QIAGEN, GE).

3.5 DNA extraction from agarose gel

The PCR products were extracted and purified from agarose gels using ISOLATE II PCR and Gel Kit (Bioline, UK) according to the manufacturer's instructions. Briefly, approximately 100 mg of DNA fragment from 1.8% agarose gel was cut and transferred into a clean microcentrifuge tube containing 200 µl of Binding Buffer CB. After the sample was incubated for 5-10 minutes at 50℃ to dissolve the gel slice, the dissolved gel mixture was transferred into reservoir of a spin column with its collection tube and centrifuged for 30 seconds at 11,000 x *g*. After the column was emptied, it was washed with 700 µl of Wash Buffer CW and dried by spinning at 11,000 x *g* for 1 minute to remove ethanol residue. The spin column was assembled to a new microcentrifuge tube and added with 20 µl of Elution Buffer C direly onto silica membrane. After incubation 1 minute at room temperature, it was centrifuged at 11,000 x *g* for 1 minute. The flow-through containing DNA was stored at -20℃ until used.

3.6 Cloning and sequencing

Extracted PCR products from agarose gels were transformed into *Escherichia coli* JM 109 High Efficiency cells using pGEM®-T Easy Vector System (Promega, US) according to the manufacturer's instructions. Briefly, ligation reactions containing 1x Rapid Ligation Buffer, T4 DNA ligase, 50 ng pGEM-T Easy Vector, 3 Weiss units T4 DNA Ligase, PCR product and Nuclease-free water to a final volume of 10µl were set up. After 4℃ overnight incubation, the ligation reaction was spread plated onto duplicated Luria Bertani (LB) agar plates containing ampicillin (100 µg/ml), Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM) and (5-Bromo-4-Chloro-3-Indolyl -D-Galactopyranoside) (X-Gal) (80 µg/ml) and incubated at 37℃ overnight. Blue and white bacterial colonies were screened for recombinant plasmids.

Two white colonies were screened for recombinant plasmids, putatively containing a DNA insert, were inoculated to universal vials with 15 ml LB broth containing ampicillin (100 µg/ml) and incubated at 37°C overnight. LB broths were purified using Isolate II plasmid Mini kit (Bioline, UK) according the manufacturer's instructions. Briefly, 5 ml of saturated *E. coli* LB culture was centrifuged for 30 seconds at 11,000 x *g* to pellet cells. After the supernatant was discarded, the cell pellet was resuspended with 250 µl Resuspension Buffer P1 by vortexing. The solution was further added with 250 µl Lysis Buffer P2 and mixed gently by inverting tube 6-8 times. After 5 minutes at room temperature incubation, the clear lysate was added with 300 µl Neutralization Buffer P3 and Mixed thoroughly by inverting tube 6-8 times. The mixture solution was centrifuged for 5 minutes at 11,000 x *g* for clarification of lysate. The clarified sample supernatant was placed into reservoir of a spin column with its collection tube and centrifuged for 1 minute at 11,000 x *g*. After the column was emptied, it was washed with 600 µl of Wash Buffer PW2 and dried by spinning at 11,000 x *g* for 2 minute to remove ethanol residue. The spin column was assembled to a new microcentrifuge tube and added with 50 µl of Elution Buffer P direly onto silica membrane. After incubation 1 minute at room temperature, it was centrifuged at 11,000 x *g* for 1 minute. The flow-through containing DNA plasmid was stored at -20°C until used. Plasmids with DNA inserts of the PCR product were sent to Macrogen Inc (Seoul, Korea) for sequencing using M13F-pUC and M13R-pUC primers to confirm the sequences. All obtained sequences were analysed using Geneious software (version 9.1.8).

3.7 Crayfish

Redclaw crayfish (14-20 g) were obtained from an population isolated since ~1995 and held at James Cook University (JCU) and from a crayfish farms in northern Queensland to make up sufficient numbers. Crayfish were housed individually in plastic cages 20 cm in diameter and 18 cm in height. Crayfish were assigned to treatments randomly. Fifteen cages were placed in large aquaria 110 cm in width, 215 cm in length and 50 cm in height (Figure 3.1). Crayfish were fed a commercial chicken diet once a day. Water exchanges were performed weekly to maintain appropriate water quality.

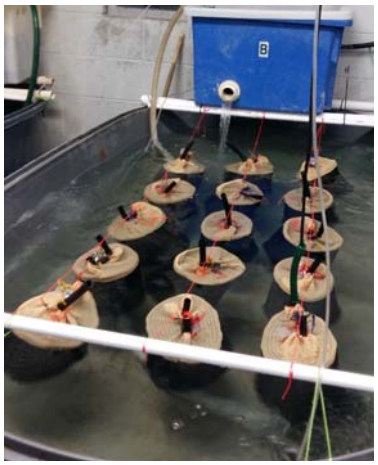

Figure 3.1 Crayfish housing

CHAPTER 4

Discovery of a novel *Picornavirales*, Chequa iflavirus, from stressed redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Queensland, Australia

In 2014, crayfish farmers reported annoyingly high mortalities in crayfish being moved to hatcheries for breeding and when being transported to research institutions for feeding trials. To identify the cause of the transport-related, stress mortalities in redclaw crayfish, the study started with examination of external characteristics. But no obvious clinical signs were found. However, during tissue preparation for routine histology, the muscle was brittle and friable and often shattered when cut with a scalpel. With histological examination, fractured muscle fibres with haemocytic infiltration were found in broodstock and juveniles crayfish. These lesion tend to be a reminiscent of viral infection. Moreover, the nerve cords and ganglionic nerve masses with pyknotic nuclei and necrosis were seen in young metamorph crayfish (craylings). Viruses causing muscle and nerve lesions in crustacea and already recorded present in Australia, like Macrobrachium rosenbergii nodavirus (MrNV) were the first target. MrNV is endemic in Australia and it can cause muscle lesions in experimental crayfish (Hayakijkosol etal., 2011). Moreover, *Macrobrachium rosenbergii* was found as a pest in some crayfish farms. It might be possible that MrNV was in northern Queensland crayfish and caused the problem. Therefore, reverse transcriptase polymerase chain reaction (RT-PCR) using MrNV primers was performed. However, size and sequencing of the RT-PCR products were different from the MrNV positive control. Therefore, it seemed likely that MrNV was not the cause of this problem. Multiple attempts of sequence-dependent and independent PCR with both consensus and degenerate primers were inconsistent in producing amplicons related to any viruses or pathogens. However, under transmission electron microscope (TEM), icosahedral/sphere-shaped presumptive capsomeres of approximately 14 nm diameter were identified with a density of 1.10 g/cm^3.
Due to conventional techniques not identifying the presumed virus, a whole transcriptome (all active RNA) approach based on RNA next generation sequencing was trialled using a case crayfish from a farm and unaffected crayfish from an isolated population. This study discovered and therefore mainly focused on chequa iflavirus (GenBank: KY659604.1). More detail of this study can found in the following peer-reviewed papers:

- Sakuna, K., Elliman, J., Owens, L. 2017. Discovery of a novel *Picornavirales*, Chequa iflavirus, from stressed redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Queensland, Australia. *Virus Research*, 238, 148-155. (Appendix C1)
- Sakuna, K., Elliman, J. Tzamouzaki, A., Owens, L. 2018. A novel virus (order *Bunyavirales*) from stressed redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Australia. *Virus Research*, 250, 7-12. (Appendix C4)

CHAPTER 5

Comparison of molecular detection PCR methods for chequa iflavirus in freshwater crayfish, *Cherax quadricarinatus* and description of Athtab Bunya-like virus

After chequa iflavirus (order *Picornavirales*) and bunya-like virus (order *Bunyavirales*) were discovered, efficient and sensitive detection methods to identify the infection status of these viruses were needed. RNA dependent RNA polymerase (RdRp) had been reported as a suitable gene to diagnose and classify members of the order *Picornavirales* (Baker and Schroeder, 2008). Therefore, detection methods based on RdRp sequence were developed and were then compared. The most comprehensive study mainly focused on chequa iflavirus detection whilst the bunya-like virus was examined less intensively. More detail of these studies can found in the following papers:

- Sakuna, K., Elliman, J., Owens, L. 2018. Comparison of molecular detection PCR methods for chequa iflavirus in freshwater crayfish, *Cherax quadricarinatus*. *Journal of Virological Methods*, 251, 139-144. (Appendix C2)
- Sakuna, K., Elliman, J. Tzamouzaki, A., Owens, L. 2018. A novel virus (order *Bunyavirales*) from stressed redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Australia. *Virus Research*, 250, 7-12. (Appendix C4)

CHAPTER 6

Assessment of a cricket, *Acheta domesticus*, bioassay for chequa iflavirus and bunya-like virus from redclaw crayfish *Cherax quadricarinatus*

To confirm River's postulates on viral cause and effect, virus-free animals especially, a species-specific model are required. Based on preliminary testing, more than 85% of redclaw crayfish samples were infected with chequa iflavirus and bunya-like virus. The lack of chequa iflavirus and bunya-like virus-free crayfish and with no cell lines available, an alternative bioassay was needed. House crickets (*Acheta domesticus*) had been reported as an animal model to study the crustracean virus, Penaeus merguiensis hepandensovirus (PmeHDV) (La Fauce and Owens, 2008), and were subsequently used for testing a gene silencing via RNAi against PmeHDV (La Fauce and Owens, 2013) as well as being used extensively for other biomedical studies. Therefore, house crickets were tested to use as an alternative bioassay for chequa iflavirus and bunya-like virus. Detail of the study can found in the following paper:

- Sakuna, K., Elliman, J., Owens, L. 2017. Assessment of a cricket, *Acheta domesticus*, bioassay for Chequa Iflavirus and bunya-like virus from redclaw crayfish *Cherax quadricarinatus*. *Journal of Invertebrate Pathology*, 150, 41-44. (Appendix C3)

CHAPTER 7
Therapeutic trials against pre-existing Chequa iflavirus redclaw crayfish (*Cherax quadricarinatus*)

To help solve the viral infection problem, direct and/or indirect therapeutic strategies for viral reduction are required to eradicate virus or reduce the impact of virus. For viral disease eradication, enhancing specific protection via vaccines is a proven strategy. However, convincing evidence of enhancing specific protection via putative protein vaccines is currently lacking in invertebrates. Therefore, other possible viral reduction strategies for crayfish farmers to follow in the future were required. This study focused only for chequa iflavirus treatments, while bunya-like virus might be tested in the future. More detail of this study can found in the following paper:

- Sakuna, K., Elliman, J., Owens, L. 2018. Therapeutic trials against pre-existing Chequa iflavirus in redclaw crayfish (*Cherax quadricarinatus*). *Aquaculture,* 492, 9-14. (Appendix C5)

CHAPTER 8
GENERAL DISCUSSION

Conventional techniques including histological examination, sequence dependent and independent PCRs and the bioinformatics searches failed to identify any aetiology causing a new syndrome of mortalities associated with stress in redclaw crayfish (*Cherax quadricarinatus*). However, presumptive capsomeres found under transmission electron microscope (TEM) provided strong evidence for viral infection. In a previous study, PCR methods using target degenerate primers were also unsuccessful to identify an unknown RNA virus infection caused by murine hepatitis virus-MI stain (MHV-MI) which required alternate methods to identify (Islam et al., 2015).

For identification of unknown viruses which couldn't be identified by a serological or molecular methods, suppression subtractive hybridization (SSH) has been used (Islam et al., 2015). Briefly, SSH can be used to subtract viral transcripts (mRNAs) produced in the infected host cells from non-infected control cells by the conversion of both mRNA populations to cDNAs, hybridization of the cDNAs to each other, removal of the hybridized cDNAs and then amplification of the unhybridized or subtracted cDNAs by suppression PCR (Islam et al., 2016). However, remarkable advancement has been achieved in the next-generation sequencing (NGS) field, and it compares favourably with SSH as the limited number of expressed sequence tags (ESTs) obtained after SSH is one the disadvantages compared to NGS technologies (Sahebi et al., 2015).

Using next-generation sequencing, the whole transcriptomes of a dying crayfish and a tank-reared, unaffected crayfish were assembled producing 507,970 contigs. This high amount of data obtained after NGS is one of the challenges compared to SSH (Sahebi et al., 2015). Automatic and manual computational pipeline have been reported as a robust technique to analysis the massive data (Zhao et al., 2013, Li et al., 2016). However, the previous computational pipelines were poor viral specific pipelines. Based on our new computational pipeline, a positive sense, single stranded RNA virus (9,933 nucleotides) and the large (6,868 nucleotides) and medium segments (3,537 nucleotides) of bunya-like virus were determined. The 9,933 nucleotide sequence (GenBank: KY659604) has the best identity to Hubei myriapod virus 1 (37%) and Dinocampus coccinellae paralysis virus (36%) which are unclassified RNA viruses and placed in genus *Iflavirus,* order *Picornavirales*. An identity of more than 32% is acceptable to be considered the same genus of virus (Fauquet et al., 2005). Therefore, the 9933 nucleotide presumptive viral sequence, marginally in the genus *Iflavirus* in a clade of Chinese and Northern American terrestrial arthropod viruses, is named chequa iflavirus. Its low identity matching is probably why the sequence dependent and independent PCRs and the bioinformatics searches gave such poor results; this virus is very different to other known viruses and will probably be in a new viral genus in the fullness of time. The near complete sequences of the large (6,868 nucleotides, GenBank: MG654468.) and medium (3,537 nucleotides, GenBank: MG674172) segments of bunya-like virus found in diseased redclaw crayfish is closely related to Whenzhou Shimp Virus 2 (WZSV 2). The Small segment has not been found as with the WZSV2 study (Li et al., 2015) suggesting this is a bi-segmented virus. With the information at present, both bunya-like virus and WZSV2 belong to the unaligned, negative-sense, single stranded RNA virus, order *Bunyavirales*. This virus is named athtabvirus (Atherton Tableland Crayfish Virus 2).

This thesis concentrated on chequa iflavirus as it was most closely related to other disease causing viruses of the order *Picornavirales*. Detection assays based on RdRp sequence (nt 8383-9873) are highly accurate and efficient to detect chequa iflavirus. The RT-qPCR is the preferred diagnostic

assay as it gave a low limit of detection, high diagnostic sensitivity and it has the melt curve as a confirmation step. Based on the measurement of uncertainty, this RT-qPCR is equivalent to PCRs of other researchers measuring crustacean viruses such as Decapod penstyldensovirus 1, white spot syndrome virus (Dhar et al., 2001), Taura syndrome virus, yellow head virus (Dhar et al., 2002), Cherax quadricarinatus Ambidensovirus (Bochow, 2016) and unpublished values for Penaeus merguiensis hepandensovirus (Owens, unpublished).

Due to a lack of chequa iflavirus and bunya-like virus-free crayfish, house crickets (*Acheta domesticus*) were used to complete River's postulates for viruses. 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 in several studies. Attempts to use viral-free crickets to prove River's postulates for both chequa iflavirus and bunya-like virus was unsuccessful. No trace of the inoculum of either RNA virus after 30 days was found with RT-PCR for chequa iflavirus and bunya-like virus. The results suggest recognition of patterns of the virus enough to allow the immune system of these crickets to destroy and leave no viable virus or enough traces of RNA to be detected by RT-PCR. This is a similar result to a study of Penaeus merguiensis hepandensovirus (PmeHDV) in mealworms (La Fauce and Owens, 2008) and a study of Bohle Ranavirus in redclaw crayfish (Field, 1993). Clearly, not all invertebrates are passive carriers of viruses due to active removal of viral sequence.

With increasing viral copy number, a significantly lower weight gain was found at ~16% reduction in growth. To reduce the impact of this virus, the inflammasome suppressant, quercetin was most favoured as it is abundant, cheap and readily incorporated into the diet and should ameliorate the original, transport-stress mortality which started this study. Temperature at 32℃ reduced viral copy numbers by ~82-85% at about 2 weeks onwards, and RNA interference based on RdRp sequence appeared to drop viral copy number to about 80% at four weeks. Broodstock could be warmed to 32℃ in the hatchery, via thermocouple-controlled solar hot water systems, relatively efficiently. RNAi treatment was not statistical significant in the drop of RNA copy numbers and most expensive to implement so it should remain as the last option. However, it could be delivered at the hatchery which would limit the amount of RNAi to be manufactured, injected or fed relative to treating the ponds.

There may also be other agents that could be used to depress or eliminate viral numbers. Ribavirin has been approved as broad-spectrum agent with activity against several viruses including positive-sense, ssRNA viruses such as hepatitis C virus (Yang et al., 2017). To remove chequa iflavirus from crayfish stocks, ribavirin should be tested either alone or in combination with any of the above methods.

Next generation sequencing of the transcriptome, with an appropriately sensitive pipeline for analysis of data, has been shown to effectively identify two new viruses, where the novelty of the sequence meant that other protocols were not effective. This allowed for development of quantitative measurement of the viruses in a small population. With a measure of viral load it was possible to identify therapeutics for use by the industry. This overall methodology, starting with a protocol more usually used to study the host animal, shows promise to provide solutions at the farm for syndromes of unknown aetiology.

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APPENDIX A

The genome sequence of Chequa iflavirus reported in this thesis (GenBank No. KY659604.1)

GGGGTGTATCCCCGCGATCTTTTCCTCTATTCTCCTTATCAGGCTTGCTTGTTAGTTAGTCTCGTTTGGA
GCTTAGAGTTTCTTGCTAGGTTTTGATGTATCTTGCCTTCGTTTCGTTTGTGACGTATCGATTTTCCTAG
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GGAGTAAACAGATGTAATGTTCTGGTAGCTTTGTGTTGCTGTTAGGGTCCCCTACGGGGGAT

APPENDIX B
List of oral presentation and poster

2017 “A novel virus causing muscle and nerve lesion in redclaw crayfish (*Cherax quadricarinatus*)”, the International Conference on Marine Science & Aquaculture 2017 (ICOMSA), 14-15 March, Kota Kinabalu, Malaysia

2017 “Sequencing of a novel (Chequa virus) from the Picornavirales in redclaw crayfish (Cherax quadricarinatus)” the 10th Symposium on Diseases in Asian Aquaculture (DAA10), 28 August- 1 September, Kuta, Bali, Indonesia

APPENDIX C

C1: Discovery of a novel Picornavirales, Chequa iflavirus, from stressed redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Queensland, Australia.

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Discovery of a novel *Picornavirales*, Chequa iflavirus, from stressed redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Queensland, Australia

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Chequa iflavirus
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ABSTRACT

In 2014, northern Queensland crayfish from farms affected by particularly transportation and translocation stress, started to die with mortality reaching 20–40% after about three weeks and then mortalities subsided. Crayfish from 1 farm had 65% mortalities within 11 weeks. With histological examination of broodstock and juveniles, the muscle fibres were fractured with haemocytic infiltration reminiscent of viral infection or vitamin E/selenium deficiencies. Sequence dependent and independent PCRs failed to identify a viral aetiology. However, the whole transcriptomes of a case crayfish and an unaffected crayfish from a different population were assembled producing over 500,000 contigs. The complete sequence of a positive sense, single stranded RNA virus (+ve ssRNA virus; 9933 bp) and the large and medium segments of a bunya-like virus were detected. Transcript back-mapping and newly developed PCRs indicated that the viruses were in the case crayfish but not the control crayfish. The +ve ssRNA virus is clearly in the order *Picornavirales*, marginally in the genus *Iflavirus* in a clade of Chinese and Northern American terrestrial arthropod viruses. The internal *Picornavirales* motifs; RNA-dependent RNA polymerase, helicase (P-loop) and 2 viral capsids genes were easily identified. This is the first iflavirus identified from crustacea and is named Chequa iflavirus. Whether these viruses are responsible for the stress-related mortalities is unproven but the Chequa virus' role seems limited as it appears it has been present in crayfish from at least the early 1990s; unless low-grade, chronic mortalities have been largely unnoticed.

1. Introduction

In 2014, a new syndrome manifested in redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Queensland, Australia. Crayfish that were stressed in any way but particularly transportation and translocation stress, started to die with mortality reaching 20–30% after about three weeks and then mortalities subsided. After transportation, crayfish from another farm had heavier mortalities reaching 40% within three weeks and 65% within 11 weeks. Histological examination of broodstock and juveniles failed to detect heavy viral or bacterial pathogens normally associated with crayfish deaths. However, it was noticed that the muscles were brittle and friable on dissection. With histological examination, the muscle fibres were fractured with haemocytic infiltration reminiscent of infection with Macrobrachium rosenbergii nodavirus (MrNV) in *Macrobrachium* or perhaps vitamin E/selenium deficiencies. Therefore, a reverse transcriptase-PCR using the OIE diagnostic primers of MrNV was conducted which produced amplicons. However, the sequence of the nucleotides in the amplicons was not MrNV nor any other identifiable virus.

This paper reports the investigation of these samples and the discovery of a unique iflavirus (order *Picornavirales*), and the large and medium segments of a bunya-like virus. Since the sequence of the bunya-like virus is incomplete with the small (S) segment missing and for the sake of simplicity and the incomplete knowledge on the bunya-like virus, this paper will concentrate on the iflavirus, named Chequa iflavirus, whilst the search for the S segment of the bunya-like virus continues.

2. Materials and methods

2.1. Crayfish

Juvenile and broodstock moribund crayfish after stress were collected from farms between 2014 and 2016 and either frozen (−20 °C)

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or fixed with injections of Davidson's fixative (case crayfish). In addition, samples of eye-staged, embryonated eggs through to day 6 metamorphs were collected from the hatchery incubators and fixed in Davidson's fixative. Crayfish not displaying the sensitivity to stress (negative control crayfish) were collected from an isolated population held at JCU since ~1995 and subject to most of the same assays described below.

2.2. Histopathology

After 48 h in Davidson's fixative, the cephalothorax, gills and muscles of redclaw crayfish were transferred to 70% ethanol, then dehydrated though a series of alcohols to xylene and then embedded in paraffin wax. Tissue sections were cut at 5 μm and stained with Mayer's haematoxylin and eosin (H & E). The sections were screened under light microscopy for lesions and viral inclusion bodies. Digital photographs were taken with a MicroPublisher 5.0 RTV.

2.3. Sequence dependent PCR

Muscle tissue collected from the red claw crayfish (*Cherax quadricarinatus*) that had high cumulative mortality with nuclear pyknosis of muscle cells was subjected to nucleic acid extraction using High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer's instructions. cDNA was produced using Tetro cDNA Synthesis Kit (Bioline) following the manufacturer's protocol before DNA amplification (MyFi™ Mix, Bioline). The cDNA was used as a template for PCR assay, using primers for Macrobrachium rosenbergii nodavirus (MrNV) (Table 1) and newly designed primers based on the previously obtained sequence (Table 2) with a PCR profile consisting of initial denaturation at 95 °C for 5 min, 40 cycles at 95 °C for 30 s denaturation, 56 °C annealing for 30 s and polymerisation at 72 °C for 30 s. Samples were polymerised for an additional 5 min at 72 °C following the last cycle. The obtained PCR products were electrophoresed on agarose gels.

The PCR products were purified using ISOLATE II PCR and Gel Kit (Bioline) and transformed into *Escherichia coli* JM 109 High Efficiency cells using pGEM®-T Easy Vector System (Promega) according to the manufacturer's instructions. The ligation reaction was spread plated onto duplicated Luria Bertani (LB) agar plates containing ampicillin (100 μg/ml), IPTG (0.5 mM) and X-Gal (80 μg/ml) and incubated at 37 °C ovemight. Blue and white bacterial colonies were screened for recombinant plasmids. Two white colonies were screened for recombinant plasmids, putatively containing a DNA insert, were inoculated to universal vials with 15 ml LB broth containing ampicillin (100 μg/ml) and incubated at 37 °C overnight. LB broths were purified using Isolate II plasmid Mini kit (Bioline) according the manufacturer's instructions. Plasmids with DNA inserts of the PCR product were sent to Macrogen Inc (Seoul, Korea) for sequencing using M13F-pUC and M13R-pUC primers to confirm the sequences. All obtained sequences were assembled into one continuous sequence using Geneious software (version 9.1.8) and compared with both single-stranded and double-stranded RNA and DNA viruses published in NCBI GenBank using bioinformatic tools (BLAST).

Table 1
Primers used to detect *Macrobrachium rosenbergii* nodavirus (*Mr*NV) using RT-PCR.

	Primer name	Sequences (5'-3')	References
1	MrNV-external Forward	GCGTTATAGATGGCACAAGG	Sahul Hameed et al., 2004
2	MrNV-external Reverse	AGCTGTGAAACTTCCACTGG	
3	MrNV B2 Forward	GACCCAAAAGTAGCGAAGGA	Hayakijkosol et al., 2011

2.4. Transmission electron microscope (TEM)

Crayfish muscle was homogenised in sterile Sf-900™ III SFM (Life Technologies) using a sterilized mortar and pestle. The homogenate was then sequentially centrifuged at 4 °C at 300 rpm for 30 min and 10,000 rpm for 15 min. The resulting supernatant was filtered through a 0.45 μm membrane filter (Sarstedt) to remove any cellular debris and bacteria.

The resulting suspension was then deposited onto a discontinuous sucrose gradient 66%, 800 μl/30%, 3.5 ml sucrose in Sf-900™ III SFM and ultracentrifuged in a Beckman Coulter Optima™ L-90 K Ultracentrifuge (SW 55 Ti rotor) at 130,000g (32 800 rpm) for 2 h at 4 °C. As no obvious viral band was found, the resulting solution was harvested in eight separate layers using a micropipette. 100 μl of each of the eight layers and density of fractions was determined. Each fraction was diluted in 1 M PBS and ultracentrifuged (L-90 K Ultracentrifuge, 50.2 Ti rotor) at 202,601g (41,000 rpm) for 4 h at 4 °C. The resulting pellet in each tube was resuspended in 100 μl PBS, stained with 1% aqueous uranyl acetate and observed under transmission electron microscopy (Centre for Microscopy and Microanalysis, University of Queensland) for the presence of viral structures.

2.5. RNA next-generation sequencing (NGS)

2.5.1. Sample preparation

The muscle from case crayfish was taken from −20 °C and deep frozen to −80 °C for at least three hours before further processing. The presumptive negative control muscle was prepared from a freshly killed crayfish and treated the same. The case and control muscles were homogenised separately with sterilized pestles in an RNase-free microcentrifuge tube containing an appropriate amount of liquid nitrogen to cover the sample.

The homogenised samples were subjected to total RNA extraction using RNA Purification Kit (NorgenBiotek®) according to the manufacturer's instructions. Quantitation of RNA was determined using QuantiFluor® RNA System (Promega) and Nano Drop®. The RNA Integrity Numbers (RIN) were determined by the entire electrophoretic trace of the RNA sample using Agilent 2200 TapeStation System (Agilent Technologies) and was used to estimate quality. As the crustacean ribosomes disassociate in processing, the RIN number is not as reliable an estimate of RNA quality as for other animals. Both RNA samples were subjected to RNA sequencing using Illumina TruSeq stranded total RNA library preparation with Ribozero treatment followed by Illumina Hiseq™ 2 × 125 bp paired-end reads (NZGL, Otago Genomics Facility, University of Otago, New Zealand). The results were checked for sequence quality by scanning the output from FastQC software.

2.5.2. De Novo assembly of reads

Case and control crayfish sequences were pooled and assembled using Trinityrnaseq (version r20140413p1) at the High Performance Computing facility at James Cook University. All contigs were imported into Geneious for analysis. All contigs greater than 5000 bp and their translated proteins were further compared with both single-stranded and double-stranded RNA and DNA viruses published in NCBI GenBank using Bioinformatic tools (BLAST) by Geneious according to the computation pipeline shown in Fig. 1. Identified putative viral sequences were used to interrogate unpooled case and control sequence databases to identify the source (or sources) of the sequences. The potential sequence and the sequences they hit in Blast were then used to create phylogenetic trees using Bayesian analysis in Geneious.

2.6. Functional analysis of the assemblies

To annotate function of predicted genes from the resulting assemblies, the Conserved Domain Database (CDD v3.16-50369 PSSMS,

Table 2
Primers designed based on putative viral sequence.

No.	Primer name	Orientation	Sequences	Location	Predicted size (bp)
1	864 F	Forward	CTCCTTCTGGGTGCGCTTTA	9304 ≥ 9323	104
	967 R	Reverse	ATACTCTGGCGCATGCTCTC	9407 ≥ 9388	
2	864 F	Forward	CTCCTTCTGGGTGCGCTTTA	9304 ≥ 9323	618
	1481 R	Reverse	GGGACCCTAACAGCAACACA	9921 ≥ 9902	

NCBI), which consists of a collection of well-annotated multiple sequence alignment models for ancient domains and full-length proteins was applied with an E value cut-off of 10^{-5}. Relationship trees were built with Geneious Tree Builder using Jakes-Cantor genetic distance model with neighbour-joining which gives emphasis to site specific identities in nucleotides.

2.7. RT-PCR assay to detect the new virus

A set of PCR primers (Table 2) was designed from potential sequence using in Geneious 9.1.8. The case and the presumptive negative control muscle were used as a positive and negative control, respectively. Both samples subjected for total RNA extraction using RNA Purification Kit (NorgenBiotek®) according to the manufacturer's instructions. cDNA was produced using Tetro cDNA Synthesis Kit (Bioline) following the manufacturer's protocol before DNA amplification (MyFi™ Mix, Bioline) with a PCR profile consisting of initial denaturation at 94 °C for 7 min, 3 cycles at 94 °C for 45 s denaturation, 53 °C annealing for 45 s, polymerisation at 72 °C for 2 min and 30 cycles at 94 °C for 45 s denaturation, 55 °C annealing for 45s, polymerisation at 72 °C for 2 min. Samples were polymerised for an additional 7 min at 72 °C following the last cycle. The obtained RT-PCR products were electrophoresed on agarose gels. Bands were sequenced using the same protocol as for sequence-dependant PCR. To check the accuracy, the obtained sequences were imported into Geneious 9.1.8 and mapped to the original sequence of the virus.

3. Results

3.1. Histopathology

In the juvenile and broodstock crayfish, the pathogens normally associated with moribund crayfish were not in high prevalence or in abundance. *Psorospermium* was widespread in tissues (Herbert, 1987) but at a low intensity; ~5% prevalence of ciliates (c.f. *Tetrahymena*, Edgerton et al., 1996) in the haemolymph and a single inclusion body of cherax bacilliform virus in the hepatopancreas (Anderson and Prior, 1992) were seen. These pathogens were not deemed to be highly pathogenic or abundant enough to be aetiological agents of most of the mortalities but were considered indicators of ill health. The only tissue changes that were consistently noted across the crayfish were in the striated muscle. Grossly the muscle was brittle and friable and often shattered when cut with a scalpel. With histopathology, the long muscle fibres showed fragmentation, loss of striations, hyperchromatosis and had a focal haemocytic infiltrate (Fig. 2A).

Some larval craylings from embryos to hatchling and young metamorph crayfish were examined. Lesions were seen in the nerve cords (Fig. 2B) and ganglionic nerve masses with pyknotic nuclei and necrosis. A meconium (Fig. 2C) was seen in the fully formed crayling's gut until day 4 after which the gut was patent. At day 5, Gram-negative bacteria invaded the gut (Fig. 2D) which became a fulminating infection and a bacteraemia by day 6. These craylings all died or were destroyed. An investigation of the role of these bacteria in larval crayfish mortalities was published by Hayakijkosol et al. (2017).

3.2. Sequence-dependent PCR

Published *Macrobrachium rosenbergii* nodavirus (*Mr*NV) primers were used for reverse transcriptase polymerase chain reaction (RT-PCR) assay, with results being positive for 2 pairs of *Mr*NV primers, namely, MrNV-external F/R and B2F/MrNV-external 1R (Table 1). However, size and sequencing of the RT-PCR products were different from *Mr*NV positive control (Fig. 3). Sequences were submitted to bioinformatical analysis (BLAST) to retrieve homologous sequences published in NCBI GenBank. The homologies with ssRNA viruses had E^{-10} probability of occurring by accident but they had short coverage. Multiple attempts of sequence-dependent and independent PCR with both consensus and degenerate primers were inconsistent in producing amplicons related to RNA viruses.

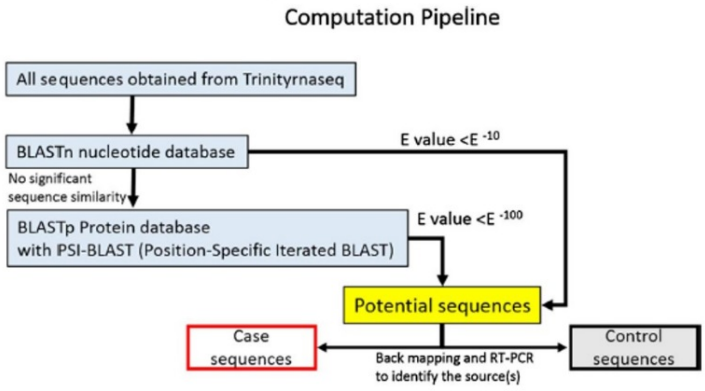

Fig. 1. Computation pipeline to determine potential sequences.

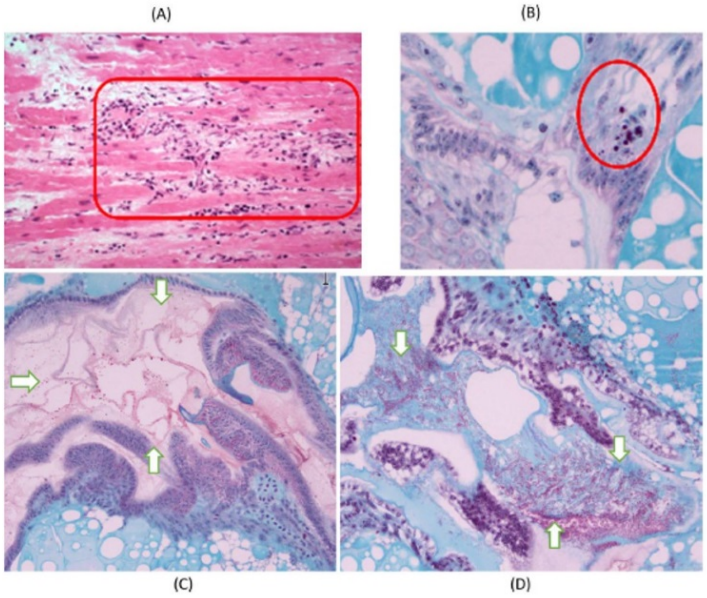

Fig. 2. Light microscopy of crayfish with H & E (A) and Gmm stains (B, C, D) respectively. (A) degenerating muscle fibres with a haemocytic infiltrate (B) nuclear pyknosis of nerve cells. (C) Meconium (white arrows) filling the lumen of the gut of a day 4 crayling. (D) Gram-negative bacterial cells (white arrows) filling the lumen of the gut of a day 6 crayling.

3.3. Transmission electron microscope (TEM)

Based on transmission electron microscope (TEM), icosahedral/sphere-shaped presumptive capsomeres of approximately 14 nm diameter (n = 20) were identified in the pellet of layer 2 with a density of 1.10 g/cm^3 (Fig. 4). Extracted RNA from this pellet was RT-PCR positive using the 104 bp primers for the iflavirus (Table 2).

3.4. RNA next-generation sequencing (NGS)

3.4.1. Quality control of next generation sequencing

Initial quality and quantity analysis (Table 3) indicated that the fresh control sample was of better quality overall than the frozen case sample. Sequencing of both samples produced very similar quality and quantity results (Table 4), with high quality scores, but also a high level of duplication and adaptor contamination. All sequences were 125 bp in length. Graphs are available on request.

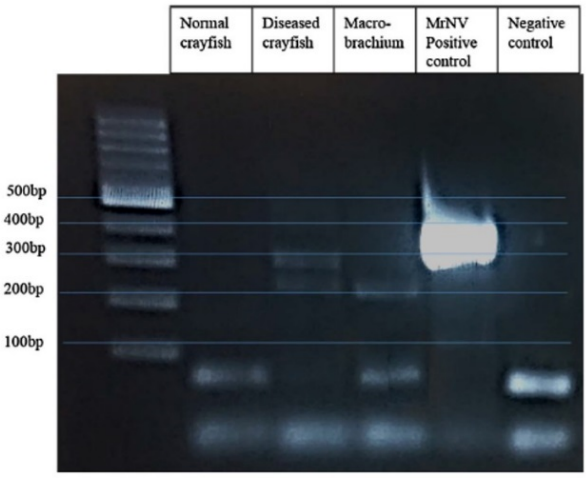

Fig. 3. Weak amplicons in gel electrophoresis of RT-PCR products using MrNV OIE F/R primers from a case crayfish and a cohabiting macrobrachium shrimp.

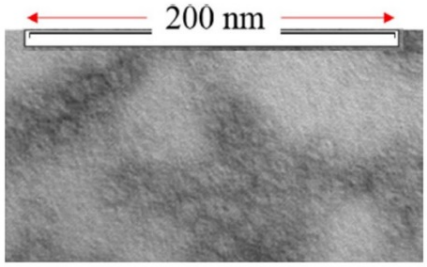

Fig. 4. Transmission electron microscope (TEM). Icosahedral/sphere approximately 14 nm diameter.

Table 3
Quality and quantity analysis of samples before RNA sequencing.

Sample	Conc. (ng/uL)	RIN	260 nm/280 nm ratio	260 nm/230 nm ratio
Case	12	4.9	1.85	0.78
Control	71.1	7.6	1.87	0.84

Table 4
Read Quality of Next Generation sequencing, based on FastQ report and number of reads.

Sample	# sequences (million)	#poor quality sequences	% GC	% sequence duplication	Position at which > 10% of sequences include adaptor
Case-A	122.5	0	47	83.4	104
Case-B	122.5	0	47	80	104
Control-A	114.0	0	47	88.4	105
Control-B	114.0	0	47	85.4	105

3.4.2. De Novo assembly of reads

507,970 pooled sequences were assembled from Trinityrnaseq. The average depth of reads was 18 with a range from 15 to 44. A 9933 bp sequence which translated to a polyprotein of 2983 amino acids in length had highly significant similarity (E ≤ 100) with various +ve ssRNA viruses (Table 5). The 9933 bp sequence was present in the case crayfish sequences but not for the negative control crayfish sequences which was confirmed by sequence back-mapping and two new, specific PCRs (see below). The sequence with the 9933 bp presumptive viral sequence is named Chequa (pronounced checka) virus derived from *Cherax quadricarinatus*. This nucleotide sequence has been deposited in the NCBI GenBank as KY659604.

From the phylogenetic trees (Fig. 5), almost all sequences (35/36) were identified as +ve ssRNA viruses with only one sequence (JAT85648.1) identified as possibly non-viral, most likely a mistake. Twenty-one of the RNA viral sequences were placed in order *Picornavirales* (+ve ssRNA viruses), with twenty sequences placed in family *Iflaviridae*, and one sequence in the family *Picornaviridae*-like. Therefore, it is highly likely that Chequa virus is an *Iflavirus* in order *Picornavirales* as it has the correct organisation of functional motifs as well as its' nucleotide similarity to its near iflaviral neighbours. Chequa iflavirus joins lastly into a clade of mostly Chinese and Northern American terrestrial arthropod viruses.

3.5. Functional analysis of the assemblies

Following gene prediction using the Conserve Domain Database (CDD) (Fig. 6), from 2152 to 2529 nucleotides (nt) and from 2953 to 3345 nt matched to picornavirus capsid protein domain-like. From 5734 to 6051 nt and from 8383 to 9873 nt; these matched to RNA helicase (P-loop) and reverse transcriptase (RT, RNA-dependent RNA polymerase)-like superfamily, respectively. In terms of amino acid positions from the extreme 5′ end, (glutamine/threonine) QT-picornain 3C protease cleavage sites were identified at 603, 918, 2171, 2212, 2305, 3097 and 3265 whilst (glutamine/serine) QS-picornain 3C protease cleavage sites were present at 1163, 1835, 2544 and 2552. If the polyprotein is cleaved at all sites, the resultant proteins would be 289, 315 (capsid), 245 (capsid), 672, 336 (RNA helicase), 41, 93, 239 (all NS polyproteins), 8, 545 (heterotrimer RdRp), 168 (heterotrimer RdRp), 30 (heterotrimer RdRp) aa in length.

3.6. RT-PCR assay to detect the new virus

Two pairs of primers namely, 864F/967R and 864F/1481R (Table 2) were successful in producing amplicons (Fig. 7) and the sequencing results of the PCR products were identical to the Chequa iflavirus original sequence. No specific bands were produced for negative control crayfish and no-template control samples. The non-specific bands were expected (Fig. 7) given the low annealing temperature selected to broaden the utility of the assay in early scoping runs. Using the correct annealing temperature of 59 °C removed all spurious amplicons.

4. Discussion

A number of positive sense (+ve), ssRNA viruses cause noticeable white lesions in the muscle of crustacea. Macrobrachium rosenbergii nodavirus (MrNV); Covert mortality nodavirus (CMNV); (both Order *Picornavirales*, Family *Nodaviridae*); Taura syndrome virus (TSV) (Order *Picornavirales*, Family *Dicistroviridae*, Genus *Aparavirus*); Infectious myonecrosis virus (IMNV) (Family *Totiviridae*) being the most well-known. Of these viruses, only MrNV is officially endemic in Australia in the same geographical region where these current farm cases came from (Owens et al., 2009) even though TSV has been detected in commodity prawns imported into the region (Ueda et al., 2008). Furthermore with experimental inoculation and feeding of MrNV to *C. quadricarinatus*, similar myonecrotic lesions could be reproduced (Hayakijkosol et al., 2011). In addition, *Macrobrachium* c.f. *rosenbergii* are common co-cultivars in the ponds where the crayfish came from. This is why we decided to start with shot-gun PCR and sequence for MrNV under the principle of Ockham's Razor that it is more likely to be simply one virus in circulation rather than many.

However, the viral sequences obtained were not related to any of the reported viruses from crustacea and constituted new viruses.

Interestingly, the hosts of these RNA viral sequences having E ≤ 100 were almost all placed in phylum Arthropoda with thirty one sequences having hosts in class Insecta, two sequences having hosts in class Arachnida (YP_009329861.1 and YP_145791.1) and one sequence having host in subphylum Myriapoda (YP_009330050.1). Notably, apart from our newly described sequence, there are no other iflaviral sequences recorded from crustacea as yet. Only one RNA viral sequence has a host in phylum Chordata from bat faeces (YP_009345906.1) (Table 5). Logically, this sequence is probably from an insect that was preyed upon by the bat and the insect virus was passed in its' faeces.

Chequa virus has very weak identity to other iflavirus with the best being Dinocampus coccinellae paralysis virus at 36% and slightly better identity at 37% with Hubei myriapod virus 1 which is an unclassified RNA virus at present. This level is just acceptable to be considered the same genus of virus, Iflavirus (> 32% identity) (Fauquet et al., 2005). This low identity is probably why the sequence dependent and independent PCRs and the bioinformatics searches gave such poor results; this virus is very different to other known viruses. Indeed, only with position-specific iterated protein BLAST (Fig. 1) did we make real progress. We speculate that in time, this virus will probably be shown to be an ancestral virus that went from marine crustacea through freshwater crayfish to terrestrial scorpions, myriapods, arachnids and lastly

Table 5
Sequences producing significant alignments (E ≤ 100) with Chequa Iflavirus. Those sequences in red are the nearest neighbours in the same clade of the phylogenetic tree (Fig. 5). The table background colour identifies the host group; green, Class Insecta; orange, Class Arachnida; yellow, subphylum Myriapoda; grey, "insect host sequence"; blue, Phylum Chordata.

No	Description	Query cover	E value	Identity	Accession
1	hypothetical protein [Hubei coleoptera virus 1]	73%	2E-141	28%	YP_009337127.1
2	protease/RNA-dependent RNA polymerase [Nasonia vitripennis virus]	24%	1E-129	35%	ACN94442.1
3-6	hypothetical protein [Shahe heteroptera virus 2]	73%	2E-127	27%	YP_009333199.1 APG77402.1 APG77409.1 APG77364.1
19-20		78%	5E-109 6E-109	26%	APG77357.1 YP_009336939.1
7	polyprotein [Nilaparvata lugens honeydew virus 1]	73%	6E-123	27%	BAN19725.1
8	polyprotein [Dinocampus coccinellae paralysis virus]	77%	2E-121	36%	YP_009111311.1
9-10	hypothetical protein [Hubei tetragnatha maxillosa virus 2]	83%	3E-121 1E-120	26%	APG76669.1 YP_009337271.1
11-12	polyprotein [Brevicoryne brassicae picorna-like virus]	78%	9E-119 4E-118	27%	YP_001285409.1 AKJ70949.1
13	polyprotein [Laodelphax striatellus picorna-like virus 2]	76%	2E-117	26%	YP_009110667.1
14	hypothetical protein [Wuhan spider virus 2]	70%	3E-117	27%	YP_009329861.1
15	hypothetical protein [Hubei picorna-like virus 28]	74%	2E-112	27%	YP_009337284.1
16	polyprotein [Heliconius erato iflavirus]	73%	2E-111	26%	YP_009026409.1
17	polyprotein [Bombyx mori iflavirus]	70%	7E-111	27%	YP_009162630.1
18	polyprotein [Formica exsecta virus 2]	79%	2E-110	25%	YP_008888537.1
21 23 26 28-29	polyprotein [Deformed wing virus]	 81% 80%	6E-108 2E-107 2E-106 3E-106 6E-106	25%	ADK55526.1 APP91308.1 ADK55525.1 AMK01489.1 AHL46850.1
22 24 27 30	polyprotein [Varroa destructor virus-1] VDV-1/DWV recombinant	80% 78% 80%	1E-107 4E-107 3E-106 9E-106	25%	YP_145791.1 AEM63700.1 APV34356.1 AGO86045.1
25	polyprotein [Moku virus]	73%	8E-107	26%	YP_009305421.1
31	hypothetical protein g.15935 [Pectinophora gossypiella]	70%	1E-104	26%	JAT85648.1
32	polyprotein [Diaphorina citri picorna-like virus]	62%	2E-104	36%	ALJ52073.1
33	polyprotein [Bat iflavirus]	80%	6E-104	25%	YP_009345906.1
34	hypothetical protein [Wuhan insect virus 13]	81%	3E-102	31%	YP_009342321.1
35	hypothetical protein [Hubei myriapoda virus 1]	68%	2E-100	37%	YP_009330050.1
36	hypothetical protein [Wuhan coneheads virus 1]	73%	4E-100	33%	YP_009342053.1

insects and will constitute a new viral genus. Chequa virus and Hubei myriapod virus 1 are almost like outgroups to their clade (Fig. 5). However at the moment, the new iflavirus has strongest similarity to the insect iflavirus and has been named Chequa iflavirus as a construct from the first three letters of the virus' host genus and species name, *Cherax quadricarinatus*.

When checking the specificity of the diagnostic PCRs, the sequences hit strongly (104 bp amplicon, 90% identity, e-30 and the 618 bp amplicon, 93% identity e-175) with a 418 bp mRNA clone_219 of *C. quadricarinatus* from Israel, NBCI entry DQ847740.1. of Yudkovski et al. (2007). When comparing the translated clone_219 with Chequa virus, there is only one amino acid change, a cysteine$_{9316}$ to an arginine but the clone_219 sequence has an extra A at position 9676 bp causing a frame shift and a stop codon at 9686 which if correct, would truncate the polyprotein and the RdRp by 67 amino acids. Despite truncation of the RdRp, all critical motifs would still be present (Fig. 6). This extra A

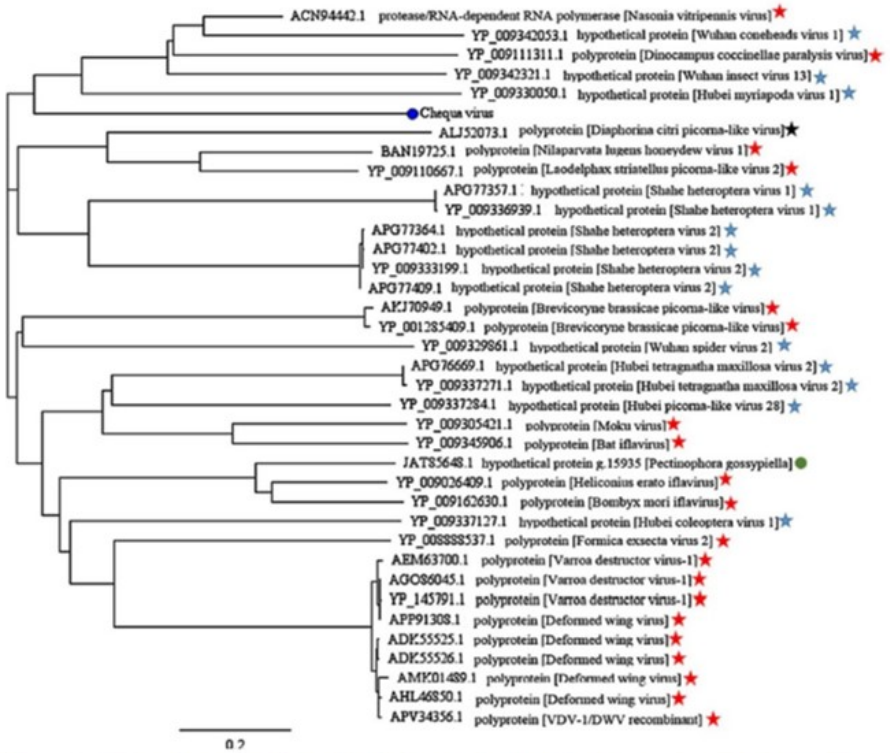

Fig. 5. Phylogenetic tree of protein translation of Chequa iflavirus and its related protein sequences (E ≤ 100). Red stars are +ve ssRNA Iflaviridae; black stars are +ve ssRNA Picomaviridae-like; blue stars are unclassified RNA viruses and the green dot was non-viral sequence (host). Trees were built with Geneious Tree Builder using Jukes-Cantor genetic distance model with neighbour-joining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

might be a PCR error or a sequencing error and it would be useful to check for this A in fresh Israeli infected crayfish. Yudkovski et al. (2007) identified their sequence as a RNA-dependent RNA polymerase but failed to realise that this might be a virus or a retrotransposon as they did not comment on this further. Clearly with only one amino acid change and a potential non-critical protein truncation, these two

nt Interval	Name	Description	Accession	E-value
2152-2529	Rhv-like superfamily	Picornavirus capsid protein domain-like	c113999	2.87E-08
2953-3345	Rhv-like	Picornavirus capsid protein domain-like	cd00205	1.45E-18
5734-6051	RNA helicase	RNA helicase P-loop	pfam00910	9.04E-24
8383-9873	RT-like superfamily	Reverse transcriptase (RT, RNA-dependent RNA polymerase)-like superfamily	c102808	8.70E-63

Fig. 6. Graphical summary for functional motifs identified in the Chequa virus sequence using the Conserved Domain Database (CDD v3.16-50369 PSSMS). nt = nucleotide.

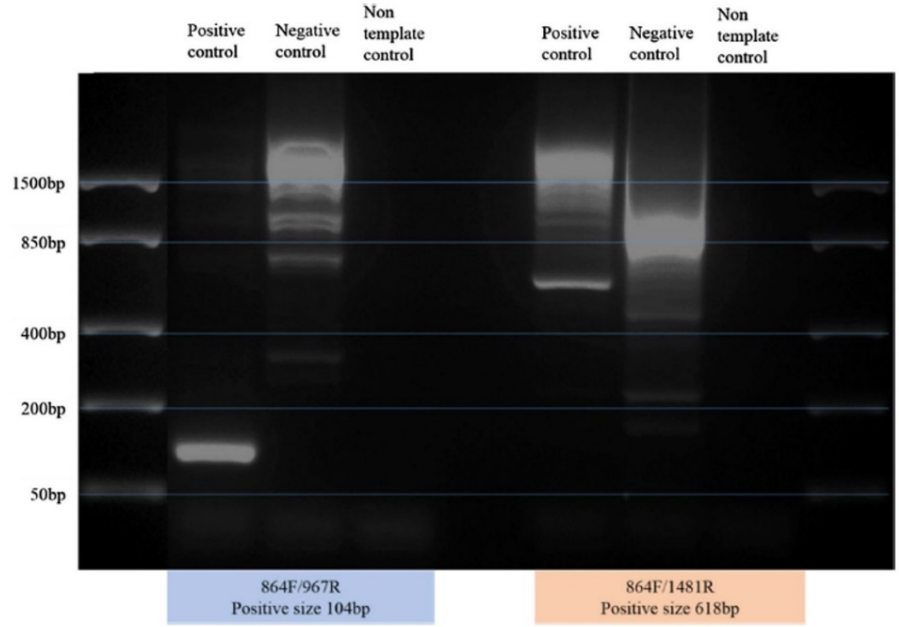

Fig. 7. Gel electrophoresis of RT-PCR products using two pairs of Chequa iflavirus primers.

isolates are from the same ancestral virus. As far as we can tell, *C. quadricarinatus* was transshipped to Israel from Australia in the early 1990's (< 1994) and the virus was probably present in the crayfish then. *C. quadricarinatus* have been shipped to Ecuador, Chile, USA, China, Thailand and Singapore where aquaculture populations have been established. One would hazard a guess that all these populations will have Chequa iflavirus when tested as cherax bacilliform virus was spread the same way (Groff et al., 1993).

From the results available at present, it is impossible to say if Chequa iflavirus is the cause of the poor stress response in crayfish as a second virus, a bunya-like virus was also present but neither virus was present in the negative control crayfish. However, both groups of related viruses are responsible for disease in insects (iflavirus) and in livestock, even some disease in humans (bunyavirus). Iflaviruses cause notable diseases like infectious flacherie in silkworms and the debilitating deformed wing, sacbrood, and slow paralysis in bees. Given that the Chequa virus has been spread to Israel in the 1990s without publications of mortalities over almost 3 decades, it seems likely Chequa virus will be widespread in Australia without commonly causing overt morbidities or mortalities. Indeed, both of these viruses might be orphan viruses and something else might be agents for the stress-related mortalities. Further studies are planned to try and complete River's postulates with Chequa iflavirus and to determine the farm populations of crayfish that are infected with both of the viruses, thus giving an approximate geographical spread and prevalence of the viruses.

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APPENDIX C

C2: Comparison of molecular detection PCR methods for chequa iflavirus in freshwater crayfish, *Cherax quadricarinatus*.

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Comparison of molecular detection PCR methods for chequa iflavirus in freshwater crayfish, *Cherax quadricarinatus*

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RT-LAMP

ABSTRACT

Chequa iflavirus (+ve sense ssRNA virus) infects redclaw crayfish (*Cherax quadricarinatus*) and it may cause mortality reaching 20–40% after about three weeks following stress. The sequence of the RNA-dependent RNA polymerase at nucleotide position 8383–9873 was used for developing and comparing PCR-based detection protocols. The reverse transcription, quantitative, polymerase chain reaction (RT-qPCR) was specific against nine *Picornavirales* and crustacean viruses and its' measurement of uncertainty (0.07–1.37) was similar to PCRs for other crustacean viruses. *In vitro*, the reverse transcription loop-mediated isothermal amplification (RT-LAMP) read at 60 min had poor repeatability for a linearized plasmid with an iflavirus insert when compared with RT-PCR visualised on an electrophoretic gel and RT-qPCR; both sensitive to 10^2 copies. In a limited, comparative sample of clinical crayfish haemolymph, the lowest, non-zero copies were 2.88×10^4 for RT-PCR and 4.60×10^1 for the RT-qPCR. In 68 further clinical crayfish haemolymph samples tested by RT-qPCR only, copy numbers ranged from 0 to 1.14×10^6. For RT-qPCR, the amplification plots, melt curves and the C_T values indicated that the C_T above 34.0 is a *potential* negative result but examination of the melt curve is necessary for an accurate interpretation. A suggested program of testing for crayfish farmers would consist of non-destructive bleeding, labelling of crayfish and screening with RT-qPCR. Only those crayfish nominally negative (below detectable limits) would be used for broodstock or selective breeding.

1. Introduction

Chequa iflavirus infects redclaw crayfish (*Cherax quadricarinatus*) and it may cause mortality reaching 20–40% after about three weeks subsequent to a stress event. Histological examination reveals fractured muscle with haemocytic infiltration in broodstock and juveniles and nerves with lesions in larval craylings. The syndrome was first reported in 2014 and the virus was first recognised in 2017 from redclaw crayfish farms in northern Queensland, Australia (Sakuna et al., 2017). The complete chequa iflavirus genome is a linear, positive-sense, single stranded RNA virus of 9933 nucleotides and is in the order *Picornavirales*, marginally in the genus *Iflavirus* in a clade of Chinese and Northern American terrestrial arthropod viruses (Sakuna et al., 2017). Domains with conserved amino acids include two capsid protein domains (nt 2152–2529, 2953–3345) and two non-structural protein domains, the RNA helicase (P-loop) domain (nt 5734–6051) and RNA-dependent RNA polymerase (RdRp) domain (nt 8383–9873) (Sakuna et al., 2017). According to Baker and Schroeder (2008), detection of the RdRp is suitable to diagnose and classify members of the order *Picornavirales*. Therefore, the sequence on RdRp domain was used for developing detection systems, which were then compared. In addition, sensitivity and specificity of detection systems were compared in order to make recommendations to farmers on the most efficient and sensitive detection method to use for large scale screening of crayfish.

2. Materials and methods

2.1. Positive and negative controls with chequa iflavirus

The muscle from an infected farmed crayfish that was positive for chequa iflavirus (positive control) and a James Cook University (JCU) tank-reared crayfish that was negative for chequa iflavirus (negative control) when sequenced via a RNA next-generation sequencing protocol (Sakuna et al., 2017) were selected for the following experiments.

2.2. RNA extraction and cDNA template preparation

Total RNA was extracted from muscle of chequa iflavirus positive

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Table 1
RT-PCR and RT-LAMP primers used in this study to detect chequa iflavirus.

Primers	Sequence (5′ to 3′)	Position	Source
RT-PCR and RT-qPCR			Sakuna et al. (2017)
F	CTCCTTCTGGGTGCGCTTTAA	9305–9324	
R	ATACTCTGGCGCATGCTCTC	9389–9408	
LAMP			This study
LAMP F3	GCTTCTGTAGAAGATTGTGTT	9664–9684	
LAMP B3	AGAACATTACATCTGTTTACTCC	9894–9872	
LAMP LF	ACGAGTGTGCTCTCACGAAT	9728–9709	
LAMP LB	ACGGGTATAAGAGAATTGGTGT	9806–9827	
FIP (LAMP F1c + LAMP F2)	CCTAGCATACGCTAACTCGCAAGGGTATGGAAGTCATCGGAT		
BIP (LAMP B1c + LAMP B2)	TTCGTATTGTTCAAAGCATTCGCATGTAGTTCCTGCCACGTAG		
LAMP F2	GGTATGGAAGTCATCGGAT	9690–9708	
LAMP F1c	CCTAGCATACGCTAACTCGCAAG	9765–9743	
LAMP B2	TGTAGTTCCTCCCAGGTAG	9860–9842	
LAMP B1c	TTCGTATTGTTCAAAGCATTCGCA	9782–9805	

and negative crayfish (see above) using the Total RNA Purification Kit (Norgen Biotek*, Canada) according to the manufacturer's instructions. cDNA was produced using Tetro cDNA Synthesis Kit (Bioline, UK) following the manufacturer's protocol and used as a template for reverse transcription polymerase chain reaction (RT-PCR) and reverse transcription loop-mediated isothermal amplification (RT-LAMP).

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

A set of PCR primers from Sakuna et al. (2017) (Table 1) for the chequa iflavirus detection was designed from the RdRP domain using Primer 3, Geneious software (version 9.1.8) (Fig. 1; position 1 of the plasmid insert corresponds to 9305 nt of the virus). DNA amplifications were undertaken in a C1000 Touch thermal cycler (BIO-RAD, US) using MyFi™ Mix (Bioline, UK) with chequa iflavirus forward and reverse primers (Fig. 1). The PCR profile was 95 °C for 1 min followed by 30 or 35 cycles of 95 °C 15 s, 59 °C 15 s and 72 °C 15 s. Samples were polymerised for an additional 5 min at 72 °C following the last cycle. The PCR products (618 and 104 nt) were electrophoresed on 1.8% agarose gel with GelRed™ (Biotium, CA) to visualize the products on a UV transilluminator.

2.4. Quantitative assay for measuring the load of chequa iflavirus by reverse transcription quantitative polymerase chain reaction (RT-qPCR)

A DNA plasmid containing a 618 bp chequa iflavirus insert (Fig. 1) was linearized by EcoRI (Promega, US) digestions. An aliquot of the digested plasmid was run in a 1.8% agarose gel to confirm the digestion before purifying the remaining digestion reactions by Isolate II PCR and Gel kit (Bioline, UK). DNA was quantified using a spectrophotometer NanoPhotometer™ Pearl (IMPLEN, GE) and standards were prepared by 10-fold serial dilutions in nuclease-free water to prepare stocks containing 1×10^7–10^1 copies/µl. Two microliters of template was used in RT-qPCR reaction with the same primers as the conventional RT-PCR. The amplification was carried out in 20 µl of reaction volume containing 10 µl of 2 x SensiFast SYBR No-ROX Mix (Bioline, UK), and 0.4 µM of chequa iflavirus forward and reverse primer. The thermal profile was 95 °C for 10 min followed by 40 cycles of 95 °C 5 s, 59 °C 10 s and 72 °C 10 s. The data acquisition and analysis were carried out with Rotor-Gene Q Series Software 2.3.1 (QIAGEN, GE).

2.5. Reverse transcription loop-mediated isothermal amplification (RT-LAMP)

The RdRp domain of chequa iflavirus was chosen as the target gene for RT-LAMP primer design. RT-LAMP primers (inner primer pair FIP/BIP, outer primer pair F3/B3 and loop primer pair LF/LB) were

Fig. 1. Sequence and location of PCR primers and RT-LAMP primers on the 618 bp chequa iflavirus insert in a plasmid.

designed using Primer Explorer version 5 (https://primerexplorer.jp/e/) (Table 1). The outer primer pair F3/B3 and loop primer pair LF/LB were synthesized by Macrogen Inc (Korea), while inner primer pair FIP/BIP were synthesized by Thermo Fisher Scientific (Australia). A 25 µl reaction mixture contained 12.5 µl WarmStart Colorimetric LAMP 2X Master Mix (NEB, US), 1.6 µM inner primers, 0.2 µM outer primers,0.4 µM loop primers, 8 µl nuclease free water and 2 µl template. The mixture was incubated at 65 °C for 30 min onwards. The result was examined by eye; positive reactions should turn yellow (solution becomes acid from released protons) while negative controls should remain pink. If an orange colour was visible, reaction was returned to 65 °C for an additional 10 min.

2.6. Analytical specificity of the RT-PCR, RT-qPCR and RT-LAMP assays

The ability of the RT-PCR, RT-qPCR and RT-LAMP assays to discriminate chequa iflavirus from other RNA viruses from crustacea and from viruses in the order *Picornavirales* was explored. The reactions containing cDNA prepared from heat inactivated gill-associated virus (GAV), Taura syndrome virus (TSV), cherax reovirus (RV), Macrobrachium rosenbergii nodavirus (MrNV), Murray Valley encephalitis virus (MVEV), dengue virus (DENV), Kokobera (KOK) virus, Kunjin virus (KUNV) and bovine enteroviruses (BEV) were carried out to ensure that non-specific products were not produced. The structure, taxonomy and the source of the viruses is shown in Table 2. All the cDNAs were produced using Tetro cDNA Synthesis Kit (Bioline, UK) following the manufacturer's protocol. Reactions with cDNA from the RNA extraction from crayfish negative for chequa iflavirus by next-generation sequencing; negative with RT-PCR and a no-cDNA template were also included as negative controls.

2.7. Limit of detection of the RT-PCR, RT-qPCR and RT-LAMP assays

Comparative molecular sensitivity of three replicates of each of the RT-PCR, RT-qPCR and RT-LAMP was performed using ten-fold serial dilutions (10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 copies) of linearized plasmid DNA containing 618 bp chequa iflavirus insert as templates (Fig. 1). The RT-LAMP was read at 30 min, 60 min (45–60 min recommended time), and the reactions pushed to 90 min and 120 min. The RT-qPCR was used as a standard reference instead of cell cultural methods as a dependable cell line for crayfish viral culture has not been developed.

Table 2
Nine RNA viruses used for specificity testing.

	Virus	Structure and Taxonomy	Source
1	Cherax Reovirus (RV)	dsRNA, Family *Reoviridae*	Infected *C. quadricarinatus*
2	Gill-associated virus (GAV)	+ ssRNA, Order *Nidovirales*	Infected *P. monodon*
3	Taura syndrome virus (TSV)	+ ssRNA, Order *Picornavirales*	Infected *P. vannamei*
4	Macrobrachium rosenbergii nodavirus (MrNV)	+ ssRNA, Family *Nodaviridae*	Infected C6/36 cells
5	Murray Valley encephalitis virus (MVEV)	+ ssRNA, Genus *Flavivirus*	TropBio (JCU, Townsville)
6	Dengue virus (DENV)		
7	Kokobera (KOK) virus		
8	Kunjin virus (KUNV)		
9	Bovine enteroviruses (BEV)	+ ssRNA, Order *Picornavirales*	

2.8. Reliability of the RT-PCR, RT-qPCR and RT-LAMP assays in clinical samples from crayfish

Screening of clinical crayfish of unknown chequa iflavirus status by the RT-PCR, RT-qPCR and RT-LAMP assays was conducted using 21 individual haemolymph samples obtained from 21 redclaw crayfish held at JCU, Townsville, Australia. All extracted RNA was assayed for purity using 260 nm/280 nm ratio (1.7–2.0) with NanoDrop. Haemolymph from a further 68 crayfish was tested by the RT-qPCR only. The haemolymph was collected by sterile Terumo (1 ml) syringes with a 26-gauge needle and mixed with 1 part sodium citrate (3.8%) per 9 parts haemolymph. These haemolymph were subjected to total RNA extraction using total RNA Purification Kit (Norgen Biotek®, Canada) and further cDNA synthesis using Tetro cDNA Synthesis Kit (Bioline, UK) following the manufacturer's protocol. Haemolymph was chosen as a non-destructive way of sampling crayfish for their later use after their iflavirus status was determined.

3. Results

3.1. Quantitative assay for measuring chequa iflavirus load by reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The assay by RT-qPCR was used for the detection of chequa iflavirus (Fig. 2A). The melt data indicated that the amplicons had a melting temperature at 79.93 °C (Fig. 2B). A strong linear correlation ($r^2 = 0.995$) was obtained between threshold cycles (C_T) and viral quantities over a 6-log range from 10^7 to 10^2 viral copy numbers per microliter (Fig. 2A and C). The amplification efficiency was 0.89. The coefficient of variation (CV) within each concentration was less than 1.9 (Table 3). The measurement of uncertainty, equivalent to 95% confidence limits ranged from 0.07–1.37.

3.2. Analytical specificity of the RT-PCR, real-time RT-PCR and RT-LAMP assays

Chequa iflavirus-positive crayfish produced specific amplification in the RT-PCR (Fig. 3A, B) which had 100% identity on sequencing to the viral genome, and with RT-qPCR (Fig. 3C, D), and RT-LAMP (Fig. 3G, H). Extracted RNA samples infected with GAV, TSV, RV, MrNV, MVEV, DEN, KOK, KUN, BEV and chequa iflavirus negative crayfish were negative by RT-PCR, RT-qPCR and RT-LAMP, indicating that the assays were specific for chequa iflavirus (Fig. 3C, D). However, crayfish positive by histopathology for RV from a JCU source gave positive result in all three assays, indicating that this sample was also infected with chequa iflavirus (Fig. 3C, E). This was repeated with the same results. Unfortunately, the MVEV gave a late amplification (Fig. 3D) that had a low amplitude (Fig. 3F) but the same melt curve as chequa iflavirus. The MVEV sample was repeated from extraction onwards and the new melt curve's major peak was at 83.8 °C not at 79.93 °C demonstrating this first result was likely a low grade contamination event. Note also that the MrNV sample had a reasonably low C_T at 34.6 (Fig. 3C) overlapping with the low copy numbers ($\sim 10^{2-3}$) of chequavirus but use of the melt curve separated the viruses easily (Fig. 3E). The amplification plots, melt curves and the C_T values indicated that the C_T above 34.0 is a potential negative result for this 104 bp amplicon and examination of the melt curve is necessary for an accurate interpretation. The RT-LAMP had clear colour changes (Fig. 3G, H) and again showed the RV sample had chequa iflavirus as well.

3.3. In vitro sensitivity of the RT-PCR, RT-qPCR and RT-LAMP assays

The repeatability of the RT-LAMP was poor with often two of three replicates negative at many dilutions (Table 4; Supplement Fig. S1). When the time of RT-LAMP was extended outside the recommended time (> 60 min), more samples became (falsely) positive including the

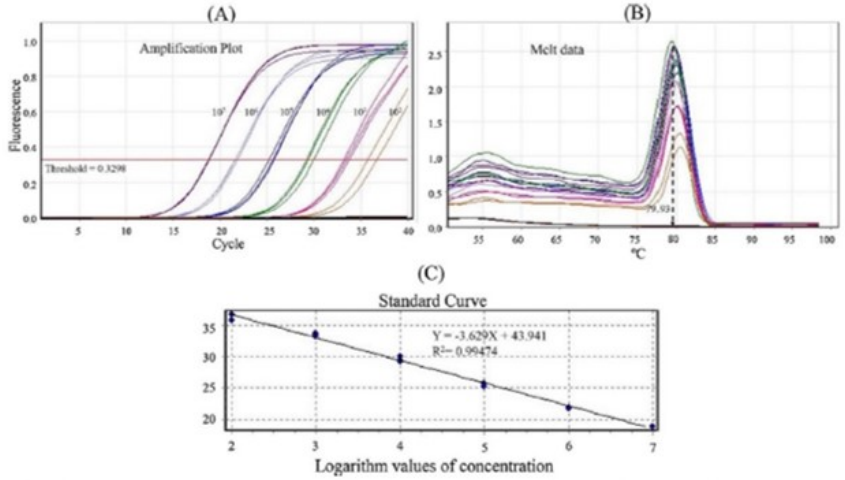

Fig. 2. Quantitative RT-PCR assay. (A) Amplification plot, (B) melt data (Mean 79.93, SD 0.32, CV 0.40%) and (C) standard curve from 10-fold serial dilutions of linearized plasmid DNA containing 618 bp chequa iflavirus insert.

Table 3
The cycle threshold (C_T) values of replicate assays for chequa iflavirus. nr = no result, below detection level.

Viral copy numbers/μl	C_T values			Mean	Measurement of Uncertainty	CV (%)
	1st	2nd	3th			
10^7	18.83	18.90	18.86	18.86	0.07	0.19
10^6	21.91	21.65	21.66	21.74	0.29	0.68
10^5	25.68	25.78	25.27	25.58	0.54	1.06
10^4	30.04	29.44	29.25	29.58	0.82	1.39
10^3	33.79	33.25	33.49	33.51	0.54	0.81
10^2	36.75	35.78	nr	36.27	1.37	1.89
10^1	–	–	–	–	–	–

no template control at 120 min (Fig. S1). For these reasons, the RT-LAMP was considered not fit-for-purpose and therefore the rest of the data pertaining to the RT-LAMP is in the Supplement.

The *in vitro* detection limits of RT-PCR at 35 cycles (Table 4) and RT-qPCR assays were both sensitive detecting 2×10^2 copies of linearized plasmid per reaction (Table 3) but some replicates were failing at this dilution indicating the likely end point of both PCRs (Table 4).

3.4. Clinical reliability of the RT-PCR and RT-qPCR assays in haemolymph from crayfish

Reliability of the RT-PCR and RT-qPCR assays was tested with 21 individual crayfish haemolymph samples of unknown status for chequa iflavirus (Supplement Table S1). In the RT-qPCR, Ct ranged from 22.00–32.28 and copy numbers from 1.14×10^6 to below detectable limits (nominal zero). The RT-PCR gave one negative mismatch when the RT-qPCR assay gave positive results with the RT-qPCR melting temperature at 80 °C and threshold cycles (C_T) at 30.64 (Supplement Table S1; crayfish #21). The statistical diagnostic performance (Abramson and Abramson, 2001) (Table 5) was similar but with the RT-PCR being a little less accurate. Another 68 crayfish haemolymph samples were tested by RT-qPCR which produced copy numbers from nominal 0 (below detectable levels, (two crayfish), 10^1 (15 crayfish), 10^2 (11 crayfish), 10^3 (29 crayfish), $\geq 10^4$ (11 crayfish), with the lowest positive copy number detected being 4.6×10^1 copies/μl.

4. Discussion

In the present study, the RT-PCR, RT-qPCR and RT-LAMP assays based on the RdRp domain were developed for detection of chequa iflavirus. Used correctly, all the assays showed high analytical specificity to discriminate chequa iflavirus from other RNA viruses from crustacea and from viruses of the order *Picornavirales*. Cherax reovirus-positive crayfish from a JCU population (confirmed by histopathology; Hayakijkosol and Owens, 2011) gave positive results in all three assays, indicating that this sample was not only infected with cherax reovirus but also was infected with chequa iflavirus. This is not surprising as this crayfish population is a multiple virus-infected population kept for the purpose of having stocks of virus for experimentation as crustacean cell culture does not exist for viral propagation.

The RT-qPCR is the preferred diagnostic assay as it gave a low limit of detection and high diagnostic sensitivity and it has the melt curve as a confirmation step. The measurement of uncertainty for RT-qPCR was 0.07–1.37 within the published ranges of qPCRs for Cherax quadricarinatus *Ambidensovirus* (Bochow, 2016); Taura syndrome virus, yellow head virus (Dhar et al., 2002); *Decapod penstyldensovirus* 1, white spot syndrome virus (Dhar et al., 2001) and unpublished values for Penaeus merguiensis hepandensovirus (Owens, unpublished) demonstrating the RT-qPCR is equivalent to PCRs of other researchers measuring crustacean viruses (cited above) and is fit-for-purpose.

There was no difference of sensitivity, specificity and accuracy in application to clinical samples when compared with the RT-qPCR assay as all the positive crayfish had a chequavirus copy number greater than 10^3, well above the limit of detection for all assays. Although the RT-LAMP had poor repeatability, it showed the lowest limit of detection (Table 4). Even though we tried mixing extensively, this problem with repeatability may be poor homogeneity of the samples in the RT-LAMP. Any future work with RT-LAMP should increase mixing dramatically and therefore the resultant homogeneity. Furthermore, if the RT-LAMP was deliberately pushed by increasing incubation time (120 mins), false positives came up which is always a risk for a diagnostic test. Without further optimisation, the RT-LAMP was considered not fit-for-purpose.

Testing has found crayfish without detectable levels of chequa

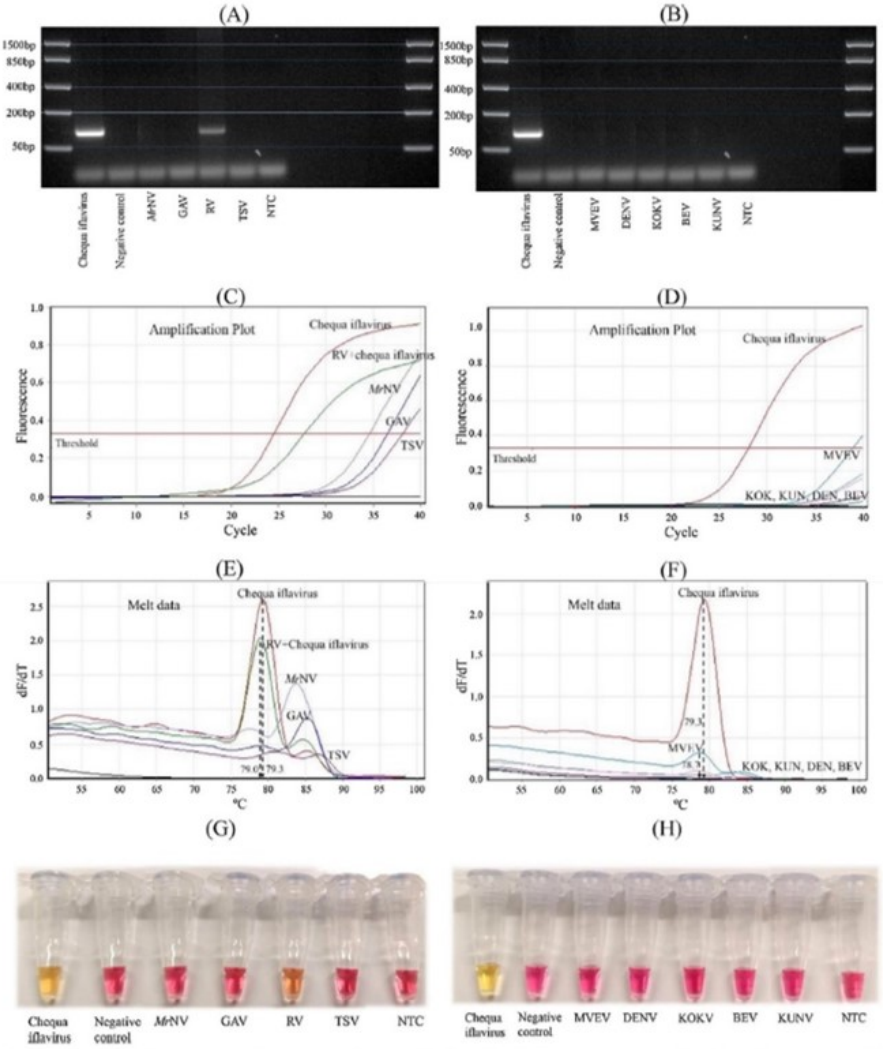

Fig. 3. Analytical specificity of the RT-PCR, RT-qPCR and RT-LAMP assays of the viruses listed in Table 2. (A–B) RT-PCR products on 1.8% gel electrophoresis, (C–D) Amplification plots obtained from RT-qPCR, (E–F) Melt data from RT-qPCR, (G–H) RT-LAMP products. NTC: no-template control.

Table 4
Limit of detection of the RT-PCR assays using 10-fold serial dilution of linearized plasmid DNA containing 618 bp chequa iflavirus insert. RT-PCR results were from amplicons after 35 cycles on 1.8% gel electrophoresis and the RT-LAMP was measured after 60 mins.

	Linearized plasmid with chequa iflavirus insert (copies/μl)							
	10^7	10^6	10^5	10^4	10^3	10^2	10^1	NTC
RT-qPCR	3/3	3/3	3/3	3/3	3/3	2/3	0/3	0/1
RT-PCR 35 cycles	3/3	3/3	3/3	3/3	2/3	2/3	0/3	0/1
LAMP 60 min	2/2	1/3	0/3	1/3	1/3	1/3	2/3	0/1

Table 5
Diagnostic performance (Abramson and Abramson, 2001) of the RT-PCR and RT-qPCR assays with clinical crayfish haemolymph.

		RT-qPCR	
		Positive	Negative
RT-PCR	Positive	19 (TP)	0 (FP)
	Negative	1 (FN)	1 (TN)
	Sensitivity	95.0%	
	Specificity	100%	
	Accuracy	97.4%	

TP, true positive; FP, false positive; FN, false negative; TN, true negative.
Sensitivity = [TP/(TP + FN)]*100, specificity = [TP/(TN + FP)]*100.
Accuracy = [(TP + TN)/(TP + TN + FN + FP)]*100.

iflavirus (nominal negatives); crayfish #10 (Supplement Table S1); the negative control crayfish used in NextGen sequencing (Sakuna et al., 2017) and others from farms and research institutions (2/68 negative with serial, quintuplicate sampling) indicating that production of viral free broodstock may be possible. A realistic on-farm program of testing would consist of non-destructive bleeding, identification of crayfish and testing by RT-qPCR. All iflavirus-positive crayfish would be returned for on-growing whilst all negative crayfish would be further serially tested in the laboratory with RT-qPCR. Only those crayfish negative at each subsequent testing would be used for broodstock or selective breeding.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2017.10.013.

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APPENDIX C

C3: Assessment of a cricket, *Acheta domesticus*, bioassay for Chequa Iflavirus and bunya-like virus from redclaw crayfish *Cherax quadricarinatus*.

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Assessment of a cricket, *Acheta domesticus*, bioassay for Chequa Iflavirus and bunya-like virus from redclaw crayfish *Cherax quadricarinatus*

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ABSTRACT

Chequa iflavirus and a bunya-like virus infect redclaw crayfish (*Cherax quadricarinatus*) and they may cause mortality reaching 20–40% after about three weeks after a stress event. To complete River's postulates for viruses, virus-free animals are needed. Due to a lack of chequa iflavirus and bunya-like virus-free crayfish (testing shows > 85% infection rate) coupled with the facts that iflavirus and bunyaviruses are found in insects and that crickets had been successful alternate hosts for crustacean viruses before, *Acheta domesticus* was trialled as a bioassay animal. There was no significant difference ($P > 0.05$) in mortality rates between uninfected control crickets and infected crickets. Reverse transcriptase polymerase chain reaction for both viruses failed to find any trace of the RNA viruses in fed or inoculated crickets after 30 days. The search for an alternative bioassay host will have to be widened.

1. Introduction

Novel Chequa Iflavirus and bunya-like virus were discovered in redclaw crayfish (*Cherax quadricarinatus)* from farms in northern Queensland, Australia (Sakuna et al., 2017a, 2017b). 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, to prevent viral disease outbreaks, an understanding of how viruses replicate and cause asymptomatic or symptomatic conditions in a species-specific model is required (Nomaguchi and Adachi, 2010). However, due to a lack of chequa iflavirus and bunya-like virus-free crayfish (preliminary testing shows > 85% infection rate) and with no cell lines, an alternative bioassay is needed.

Since almost all iflavirus are naturally found in terrestrial arthropods and most bunyaviruses have insect vectors, it was reasoned that insects might be a good place to look for an alternative host. 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 in several studies. House crickets (*Acheta domesticus*), that can be naturally be infected with their own densovirus were successfully shown to be a potential model to study the crustacean virus, Penaeus merguiensis hepandensovirus (PmeHDV) (La Fauce and Owens, 2008), and were subsequently used for testing a gene silencing via RNAi against PmeHDV (La Fauce and Owens, 2013). Furthermore, crickets have been tested successfully in their ability to take up plasmids expressing green fluorescent protein in bacterial cells (La Fauce and Owens, 2012). Also, crickets have been used as experimental intermediate hosts for larval stages of physalopteran nematodes (Cawthorn and Anderson, 1976). In addition, insects have been used as biomedical model animals for *Candida* yeast infections to save the sacrifice of mammals in experiments (Cotter et al., 2000). Clearly, crickets have been successful models in biomedicine and perhaps, the house cricket might be suitable to use as an alternative bioassay for chequa iflavirus and/or bunya-like virus from redclaw crayfish. This paper describes the investigation of the usefulness of *A. domesticus* as a model for the new RNA viruses from redclaw crayfish.

2. Materials and methods

2.1. Viral inoculum

Muscle tissue collected from crayfish that gave chequa iflavirus-positive sequences in RNA next-generation sequencing (Sakuna et al., 2017a, 2017b) was cut into 5 mm^2 pieces, then homogenised at a 1:4 ratio with insect media Sf-900^{h} III SFM (Life Technologies, US). The homogenate was centrifuged at 3300*g* for 30 min at 4 °C to remove coarse cellular debris. The supernatant was further clarified by centrifugation at 15,200*g* for 30 min at 4 °C and then was filtered through

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Table 1
RT-PCR and RT-qPCR primers used to detect chequa iflavirus and bunya-like virus.

	Primer name	Sequences (5'-3')	Position	Annel temp.	Product
1	Chequa iflavirus forward	CTCCTTCTGGGTGCGCTTTA	9305–9324	59 °C	104 bp
	Chequa iflavirus reverse	ATACTCTGGCGCATGCTCTC	9408–9389		
2	Bunya-like virus forward 1	AACACAGGGTTCAAGGCGAA	761–780	58 °C	276 bp
	Bunya-like virus reverse 1	GATTGGGGTCAGTGTCTGCA	1036–1017		
3	Bunya-like virus forward 2	GATCCGGCAGAATACGAGGG	2889–2908	59 °C	207 bp
	Bunya-like virus reverse 2	ACAACTGTCTGGCTACTGGC	3095–3076		

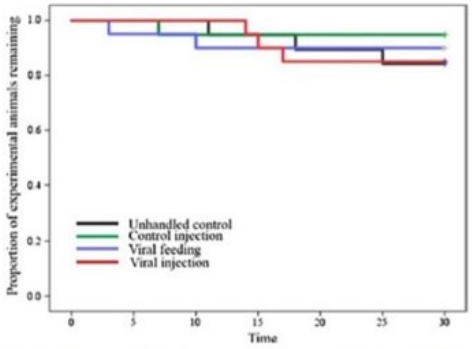

Fig. 1. Survival curves of *Acheta domesticus* exposed to an extract from crayfish infected with Chequa Iflavirus and bunya-like virus during a 30-day experiment trial.

a 0.45 μm syringe filter into a sterile container. One drop of the filtered supernatant was plated onto blood agar and tryptic soy agar and incubated at 25 °C for 48 h to confirm the absence of bacterial contamination.

2.2. Crickets

An infection experiment was conducted on the house cricket (*Acheta domesticus*) purchased from Pisces Enterprises (Queensland, Australia). Crickets were housed in plastic aquaria and supplied with dried chicken food, carrots *ad libitum* and water changed every day.

2.3. Experimental infection

Crickets were randomly distributed between four experimental treatments: (1) unhandled controls, (2) controls injected with 20 μl Sf-900th III SFM, (3) injected with 20 μl viral inoculum suspended in Sf-900th III SFM, and (4) fed with dried chicken food and carrots containing infected crayfish tissue. Experimental treatments contained two replicates of ten crickets each. All injections were performed using Ultra-Fine™ II Short Needle Insulin Syringe (0.25 mm (31 G) × 8 mm) discarded after each inoculation to minimise cross-infection. Crickets were injected by inserting the needle into the membrane below the pronotum. Control crickets were injected with the same volume of insect medium (Sf-900th III SFM). The experimental period began on the day of injection (day 0) and concluded on day 30. Experimental aquaria were monitored daily. Dead crickets were removed, recorded and

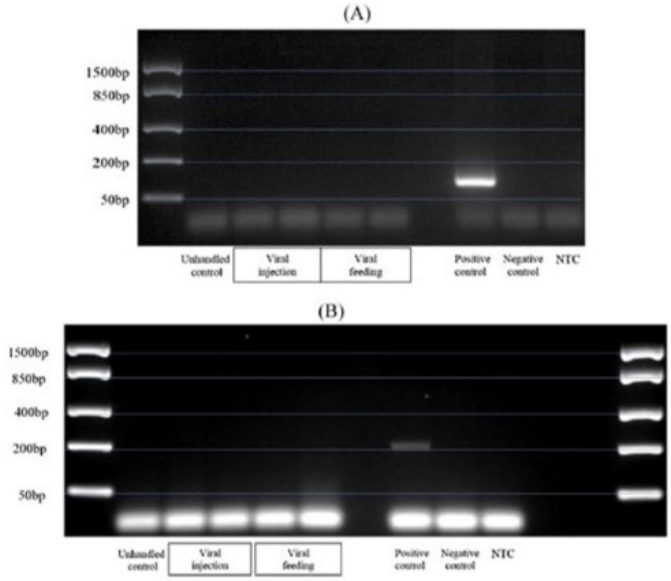

Fig. 2. RT-PCR products on 1.8% gel electrophoresis. (A) using Chequa iflavirus primers (104 bp amplicon) and (B) using Bunya-like virus primers 2 (207 bp amplicon). NTC = no template control.

prepared for reverse transcription polymerase chain reaction (RT-PCR). Approximately 50 mg of tissue was removed from crickets (flight muscle, gut and reproductive tissue) after longitudinal ventral dissection. Tissues were stored frozen until processed. At the end of the experimental period, all remaining crickets were sacrificed and the tissue removed for RT-PCR.

2.4. RNA extraction and cDNA template preparation

Total RNA was extracted from cricket tissue (flight muscle, gut and reproductive tissue) using total RNA Purification Kit (NorgenBiotek*, CA) according to the manufacturer's instructions.

A positive and a negative control were prepared from muscle of chequa iflavirus positive and negative crayfish (identified in RNA next-generation sequencing) respectively. cDNA was produced using Tetro cDNA Synthesis Kit (Bioline, UK) following the manufacturer's protocol and used as a template for reverse transcription polymerase chain reaction (RT-PCR).

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

DNA amplifications were undertaken in a C1000 Touch thermal cycler (BIO-RAD, US) using MyFi™ Mix (Bioline, UK) with chequa iflavirus forward and reverse primers developed by Sakuna et al. (2017a, 2017b) and with 2 sets of bunya-like virus forward and reverse primers (Table 1). Two sets of PCR primers for the bunya-like virus detection were designed from the Large (L) segment (6870 bp, RNA dependent RNA polymerase) by Primer 3, Geneious software (version 9.1.8). The PCR profile was 95 °C for 1 min followed by 30 cycles of 95 °C 15 s, 59 °C 15 s and 72 °C 15 s, with the exception of primer set 2 (Bunya-like virus 1) where 58 °C 15 s was used as an annealing condition. Samples were polymerised for an additional 5 min at 72 °C following the last cycle. The obtained PCR products were electrophoresed on 1.8% agarose gel with GelRed™ (Biotium, CA) to visualize the products on a UV transilluminator.

2.6. Statistical analyses

All statistical analyses were performed using IBM Statistical Package for the Social Sciences (SPSS) Version 23. The effect of treatment (route of infection), and replicate number was determined by a univariate analysis of variance (ANOVA). Post-hoc comparisons between significant means were performed using least significant differences (LSD). Survival was analysed by Kaplan-Meier method. Log Rank, Breslow and Tarone-Ware were used as statistic comparisons.

3. Results

3.1. Survival analysis

No abnormal sign were seen in any treatments with only a few of crickets dying during a 30-day experimental trial. Three crickets died in the unhandled control and the viral injection groups; two in the viral feeding and one in the control injected cricket groups (Fig. 1). There was no statistically significant difference ($P > 0.05$) between treatments (ANOVA) or in survival time (survival analysis).

3.2. Reverse transcription polymerase chain reaction (RT-PCR)

No amplicons positive for chequa iflavirus or bunya-like virus were seen in any treatments using both sets of primers treatments (examples of gels using chequa iflavirus (Fig. 2A) and bunya-like virus set 2 primers shown in Fig. 2B).

4. Discussion

This study investigates the use of the house cricket (*Acheta domesticus*) as an alternative bioassay for chequa iflavirus and bunya-like virus in redclaw crayfish. When challenged with chequa iflavirus and bunya-like virus, the viruses were not detected in tissue from house crickets by reverse transcription polymerase chain reaction (RT-PCR). According to our previous studies (Sakuna et al., 2017a, 2017b), there were no differences in sensitivity, specificity and accuracy in application to clinical samples between the RT-PCR and reverse transcription quantitative polymerase chain reaction (RT-qPCR), which was used as a standard reference. Therefore, these negative results being caused by low sensitivity of the RT-PCR appears very unlikely. Although, the sensitivity, specificity and accuracy of the RT-PCR for bunya-like virus have not been comprehensively studied, two set of primers were used to confirm the results. Unfortunately, these results demonstrate the house cricket has no capacity of being used as an alternative bioassay for chequa iflavirus or bunya-like virus from redclaw crayfish. The search for an alternative model will have to be broadened.

When *A. domesticus* was inoculated with the crustacean virus, PmeHDV, the copy number rose to about 10^5 at day 5, thereafter it slowly decayed to about 10^3 copies at day 20 (La Fauce and Owens 2008). In contrast using RT-PCR for Chequa iflavirus and bunya-like virus, we found no trace of the inoculum of either RNA virus after 30 days, suggesting total clearing of the viruses by the immune system of *A. domesticus*. This is also comparable to the rapidly declining copy number of PmeHDV in mealworms, *Tenebrio molitor* over 30 days (La Fauce and Owens, 2008). These results are supported by the fact when Bohle *Ranavirus* is injected into *Cherax quadricarinatus*, the ranavirus is undetectable in cell culture after 7 days from the haemolymph of those crayfish (Field, 1993). Combined, these results suggest pattern recognition of the virus to allow the immune system of these invertebrates to destroy and leave no viable virus or enough traces of RNA to be detected by RT-PCR. Clearly, not all invertebrates are passive carriers of viruses due to active removal of viruses.

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APPENDIX C

C4: A novel virus (order *Bunyavirals*) from stressed redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Australia.

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A novel virus (order *Bunyavirales*) from stressed redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Australia

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ABSTRACT

Athtabvirus, a bunya-like virus and chequa iflavirus infect redclaw crayfish (*Cherax quadricarinatus*) and they may cause mortality reaching 20–40% after about three weeks following transportation stress. Lesions were seen in the muscles of broodstock and juveniles and nerve cords of craylings. Using NextGen sequencing, the whole transcriptomes of a farmed case crayfish and a tank-reared, unaffected crayfish were assembled producing over 500,000 contigs. The average depth of reads was 18 replicates with a range from 15 to 44. The near complete sequences of the large and middle genome segments of a bunya-like virus were detected along with chequa iflavirus. The internal bunya-like motifs; RNA-dependent RNA polymerase on the L segment, and glycoprotein n (Gn) on the M segment were easily identified. In the opposite, positive-sense direction on the M segment, another presumed glycoprotein (glycoprotein c) with a low-density lipoprotein receptor (cysteine-rich) motif was identified by position specific iterated (psi)-BLASTp. The athtabvirus was related to Wenzhou Shrimp Virus 2 (E = 0.0, 43% amino acid identity), an unassigned, −ve sense ssRNA virus, and to peribunyaviruses ($E = 10^{-50-20}$). In descending order of the number of RNA copies/0.2 mg of tissue, the organs most heavily infected were muscle (9.4×10^6), nerve cord (5.24×10^6), heart (4.07×10^6), gills (3.96×10^6), hepatopancreas (1.58×10^6) and antennal gland (6.6×10^5). Given the tissue tropism (muscle and nerves) of athtabvirus and the original lesions, this virus is implicated in being involved in the mortalities in crayfish after transportation.

1. Introduction

In 2014, a new syndrome of mortalities associated with stress was recognised in redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Queensland, Australia (Sakuna et al., 2017a). Crayfish that were stressed by predominantly transportation and translocation, started to die with mortality reaching 20–30% in approximately three weeks and then mortalities waned. Crayfish from one farm had heavier mortalities reaching 40% within three weeks and 65% within 11 weeks. Histological examination of broodstock and juveniles failed to detect heavy viral or bacterial pathogens normally associated with crayfish deaths (Hayakijkosol et al., 2017; Sakuna et al., 2017a). However, the striated muscles were brittle and friable on dissection. With histological examination, the muscle fibres were fractured with haemocytic infiltration reminiscent of a viral infection or perhaps vitamin E/ selenium deficiencies. Craylings had lesions with karyorhectic and pyknotic nuclei in their nerve cords. The description of the new chequa iflavirus (Order Picornavirales, Family *Iflaviridae*) from these crayfish has already been published (Sakuna et al., 2017a). This paper reports the discovery of a second virus from these crayfish with unique large and medium segments of a presumed bi-segmented, bunya-like, unassigned, negative-sense ssRNA virus (Order Bunyavirales).

2. Materials and methods

2.1. Discovery of the −ve ssRNA bunya-like virus

Details of the crayfish, histopathology, the next-generation sequencing sample generation and *de novo* assembly were published in Sakuna et al. (2017a). Briefly, the transcriptomes of a case and a negative control crayfish were prepared using Illumina TruSeq stranded total RNA library preparation with Ribozero treatment followed by Illumina Hiseq™ 2 × 125 bp paired-end reads (N2GL, Otago Genomics Facility, University of Otago, New Zealand). Case and control crayfish sequences were pooled and assembled using Trinitymaseq (version r20140413p1) at the High Performance Computing Facility at James Cook University. Specific adapters were trimmed using Trinity trimmomatic. All contigs were imported into Geneious for analysis. All

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contigs greater than 2000 bp and their translated proteins were further compared with both single-stranded and double-stranded RNA and DNA viruses published in NCBI GenBank using bioinformatic tools (BLAST) by Geneious. The bunya-like sequences were confirmed to be in the case crayfish and not the control crayfish by back-mapping and by newly designed primers used in reverse transcription polymerase chain reaction (RT-PCR) (see below).

2.2. RNA extraction and cDNA template preparation for polymerase chain reaction

Total RNA was extracted from 10 mg crayfish tissue (muscle and/or haemolymph) using total RNA Purification Kit (NorgenBiotek®, CA) according to the manufacturer's instructions. A positive and a negative control samples of crayfish were prepared from muscle of −ve sense ssRNA virus positive and negative crayfish (identified in RNA next-generation sequencing and confirmed by RT-PCR and back mapping of sequences from Next Gen sequencing to the virus) respectively. cDNA was produced using Tetro cDNA Synthesis Kit (Bioline, UK) following the manufacturer's protocol and used as a template for RT-PCR.

2.3. Polymerase chain reaction (PCR)

DNA amplifications were undertaken in a C1000 Touch thermal cycler (BIO-RAD, US) using MyFi™ Mix (Bioline, UK) with 2 sets of bunya-like virus forward and reverse primers developed herein (Table 1). Two sets of PCR primers were designed from the Large (L) segment (6868 bp, RNA-dependent RNA polymerase) by Primer 3, Geneious software (version 9.1.8). The PCR profile for amplification with bunya-like virus primer set 1 was 95 °C for 1 min followed by 30 cycles of 95 °C 15 s, 58 °C 15 s and 72 °C 15 s. Samples were polymerised for an additional 5 min at 72 °C following the last cycle. For bunya-like virus primer set 2, conditions were the same except annealing temperature was set at 59 °C. The obtained PCR products were electrophoresed on 1.8% agarose gel with GelRed™ (Biotium, CA) to visualize the products on a UV transilluminator.

2.4. Quantitative assay for measuring the load of athtabvirus by reverse transcription quantitative polymerase chain reaction (RT-qPCR)

A DNA plasmid containing a 207 bp athtabvirus insert was linearized by EcoRI (Promega, US) digestions. An aliquot of the digested plasmid was run in a 1.8% agarose gel to confirm the digestion before purifying the remaining digestion reactions by Isolate II PCR and Gel kit (Bioline, UK). DNA was quantified using a spectrophotometer NanoPhotometer™ Pearl (IMPLEN, GE) and standards were prepared by 10-fold serial dilutions in nuclease-free water to prepare stocks containing 1×10^7–10^1 copies/µl. The same primers (set 2; Table 1) were used as in the conventional RT-PCR. The amplification was in 20 µl of reaction volume containing two µl of template, 10 µl of 2 x SensiFast SYBR No-ROX Mix (Bioline, UK), and 0.4 µM of each bunya-like forward and reverse primers. The thermal profile was 95 °C for 1 min followed by 40 cycles of 95 °C 5 s, 59 °C 10 s and 72 °C 10 s. The data acquisition and analysis were carried out with Rotor-Gene Q Series Software 2.3.1 (QIAGEN, GE). Triplicate reactions were conducted to assess the analytical performance of the RT-PCR.

2.5. Phylogenetic trees

Relationship trees were built with Geneious version 9 Tree Builder using Jukes-Cantor genetic distance model with neighbour-joining, no outgroup, which gives emphasis to site-specific identities in nucleotides or amino acids. The Jukes–Cantor model has the advantage that it is robust and depends on the minimal number of model parameters (only the equal substitution rate is taken as a parameter). In Geneious, Jukes-Cantor trees are built directly from a set of unaligned sequences, with the alignment built as part of the tree building process so they do not allow for bootstrapping of the tree.

2.6. Viral tissue tropism

To understand the loading of athtabvirus in various tissues of crayfish and to identify the optimum tissue for finding the viral load on farm, five moribund female broodstock crayfish from the farm where the index case was found (Sakuna et al., 2017a) were tested. These crayfish were moribund after the stress of being moved from the growout pond to the hatchery for breeding. Individual tissues were aseptically dissected from five frozen carcases and the RNA extracted and RT-qPCR performed as outlined above. The tissues included the muscle, heart, gill, antennal gland, hepatopancreas and nerve cord.

To give an indication if the virus was horizontal or vertically transmitted, the following protocol was used. Haemolymph from a further three live females was extracted as were the fertilised eggs (eyed, with a yolk sac stage). The eggs were either washed or surface sterilised with 0.5% formalin (5000 ppm) for 1 min (Edgerton and Owens, 1997) and then RNA extraction and RT-qPCR were performed as described previously.

3. Results

3.1. Post-assembly identification of reads

Unless otherwise stated, all references are to the antigenome, the reverse and complement of the negative viral genomic RNA so that the first ORF is at the 5′ end of the mRNA. The average depth of reads was 18 replicates with a range from 15 to 44.

The designed primers produced exactly the correct products in RT-PCR. The Large (L) segment of a bunya-like virus was 6868 bp long encoding for 2235 amino acids in which 2 motifs were recognized; an arenavirus-like RNA endonuclease cap-snatching motif (aa no. 152–187, 4.53e-03) (Fig. 1a) and a RNA-dependent RNA polymerase (RdRp) (aa no. 701–1417, 3.18e-49; Supplementary Fig. S2a). The estimated molecular weight is 259.6 kDa. The NCBI GenBank accession number is MG654468. As well as the sequence of the viral genome being found in the crayfish, the presumptive subgenomic mRNA was found by Next Gen sequencing as well with it being 6805 bp; shortened by 64 bp at the 3′ end. As yet, we do not know if we have the complete sequence of the noncoding ends of the L and M segments (see below).

The Medium (M) segment is 3537 bp long (Fig. 2). An encoded glycoprotein (putative envelope) starts at nt 37 from the virus complementary 3′ end until nt 2445 stop codon; It is 802 aa (2409 bp including the stop codon) long with an estimated 90.6 kDa molecular weight. The bunyavirus Gn or in the old terminology, G1 glycoprotein

Table 1
RT-PCR (set 1) and RT-qPCR (set 2) primers used to detect −ve ssRNA bunya-like virus.

	Primer name	Sequences (5′–3′)	Position	Anneal temp.	Product
1	bunya-like virus forward 1	AACACAGGGTTCAAGGCGAA	756–775	58 °C	276 bp
	bunya-like virus reverse 1	GATTGGGGTCAGTGTCTGCA	1,012–1,031		
2	bunya-like virus forward 2	GATCCGGCAGAATACGAGGG	2,884–2,903	59 °C	207 bp
	bunya-like virus reverse 2	ACAACTGTCTGGCTACTGGC	3,071–3,090		

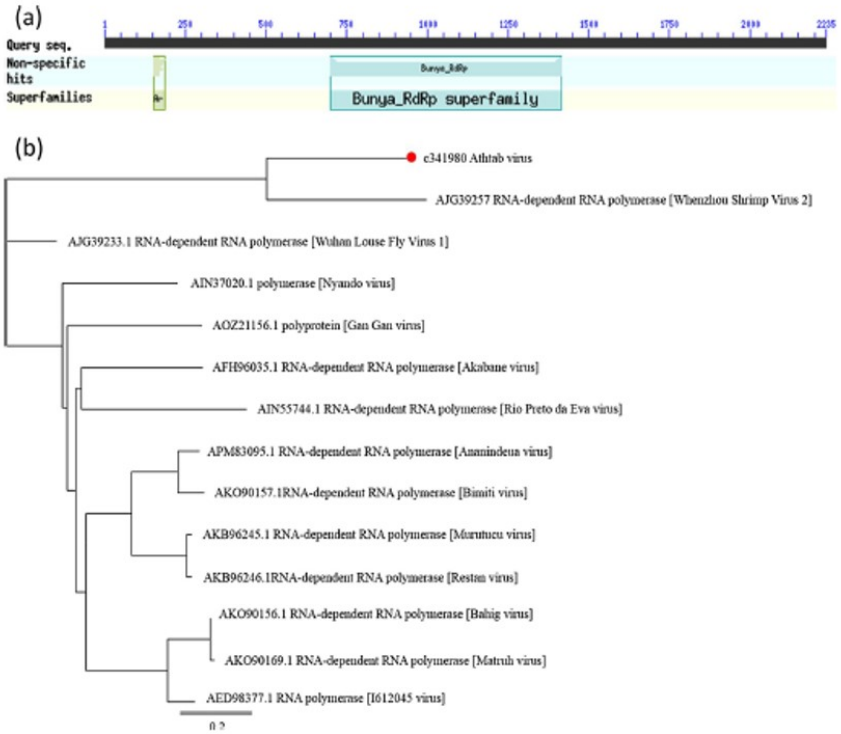

Fig. 1. The motifs and relationships of the Large segment of athtabvirus. Fig. 1a. The amino acid motifs of the Large segment of athtabvirus. 1b. The nearest neighbour relationship between athtabvirus and order Bunyavirales (where $E < 10^{-50}$). Trees were built with Geneious version 9 Tree Builder using Jukes-Cantor genetic distance model with neighbour-joining. C341980 athtabvirus is the name of the contig that contains the L segment.

motif (pfam03557) is identifiable (1.49e-16) at amino acids 371–668 (Supplementary Fig. S2b). The NCBI GenBank accession number is MG674172.

In a non-overlapping manner, on the antigenomic positive–sense strand another open reading frame is at 3507–2541 bp; 963 bp and 321 aa long which may produce a protein with an estimated molecular weight of 35.3 kDa (Supplementary Fig. S2c). This possible protein's function is unidentified but probably related to cholesterol metabolism as it has a low-density lipoprotein receptor (cysteine-rich) with a Ca^{2+} binding site at 297–321 aa. A restricted, position-specific iterated protein (psi)-BLAST limited to the order *Bunyavirales* hits (39% positives) with rice stripe tenuivirus (2976–2580 bp).

The search for a potential Small (*S*) segment has not been successful. Many different published automatic computer pipeline searches (e.g. Hidden Markov Models, Skewes-Cox et al. 2014; ProBlast2GO) as well as a few in-house developed searches have not found a candidate sequence. Manual searches have not been successful. At this stage, we suggest this virus maybe bi-segmented like the unassigned group of negative-sense RNA viruses including Whenzhou Shrimp Virus 2 (WZSV2) which do not have the S segment identified yet (Li et al. 2015).

We name the virus, athtabvirus after the ***Ath***erton ***Tab***lelands, the geographical area where the case crayfish came from.

3.2. Phylogenetic analysis of the genome segments

When the L segment protein (65–6770 bp; 2235 amino acids long) containing the RdRp was searched for homology using BLASTp, all the greatest hits except one (see below) were with genus *Orthobunyavirus* ($E < 10^{-50}$) (Fig. 1b). The best hit was with the unassigned, –ve ssRNA virus, WZSV2 ($E = 0.0$). Athtabvirus and WZSV2 formed their own cluster and were the last to join the main orthobunyavirus cluster.

Similarly, when the putative glycoprotein Gn within the M segment was searched for homology using BLASTp, most of the top hits were with genus *Orthobunyavirus* ($E < 10^{-20}$) or unassigned members of the family *Peribunyaviridae* (Khurdun, Akhtuba viruses) to which the orthobunyaviruses belong (Fig. 2b). Again the best hit was with the unassigned, –ve ssRNA virus, WZSV2 ($E = 0.0$). Athtabvirus and WZSV2 formed their own cluster as did the unassigned peribunyaviruses and both were late to join the true orthobunyavirus cluster.

3.3. Infected crayfish

The RT-PCR with both sets of primers worked efficiently and the sequences of the amplicons produced were identical to the athtabvirus sequence. With extremely limited sampling so far, crayfish from two commercial farms (9/9; 16/16), and one mixed population of James Cook University (JCU) crayfish (4/4), were RT-PCR positive for the athtabvirus, whilst *Cherax destructor* (0/3) were negative by RT-PCR. Crickets, *Acheta domesticus* were not susceptible to infection by

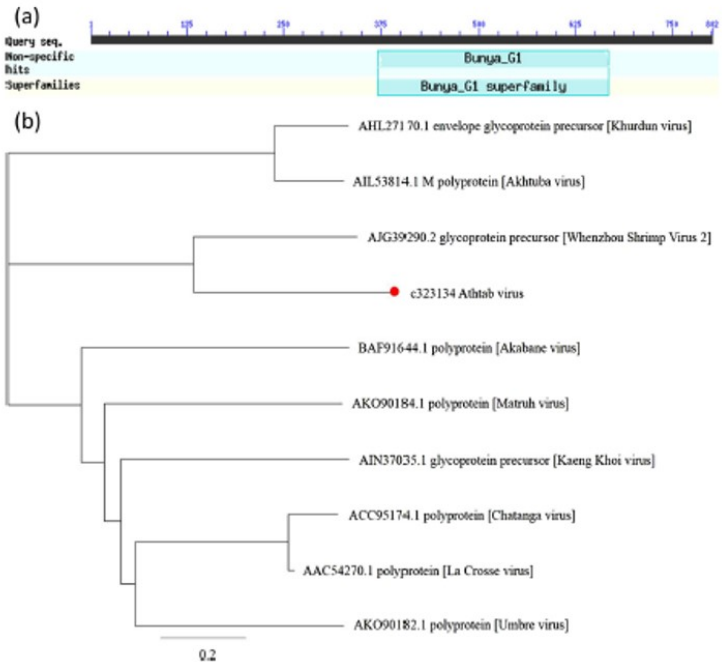

Fig. 2. Relationship of the Middle segment genome of athtabvirus translated into amino acids from the 3′ end. (a) Motif similarity to bunyavirus glycoprotein G1 (b) Dendrogram of nearest neighbour joining of unassigned negative-sense ssRNA virus and bunyaviruses where $E < 10^{-20}$. Trees were built with Geneious version 9 Tree Builder using Jukes-Cantor genetic distance model with neighbour-joining.

athtabvirus (Sakuna et al. 2017b).

3.4. Quantitative assay for measuring athtabvirus load by reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The assay by RT-qPCR was used for the detection of athtabvirus (Fig. 3A). The melt data indicated that the amplicons had a melting temperature at 83.2 °C (Fig. 3B). A strong linear correlation ($r^2 = 0.996$) was obtained between threshold cycles (Ct) and viral quantities over a 6-log range from 10^7 to 10^2 viral copy numbers per microliter (Fig. 3A and C). The amplification efficiency was 0.88. The coefficient of variation (CV) was 0.58%.

3.5. Potential viral tissue tropism

There was a large variation in RT-qPCR values across the six tissues (Fig. 4). In descending order of number of RNA copies/µl copies, the tissues were muscle (9.4×10^6), nerve cord (5.24×10^6), heart (4.07×10^6), gills (3.96×10^6), hepatopancreas (1.58×10^6) and antennal gland (6.6×10^5). This probably represents tissue tropism for the muscle and neuro-muscular junctions with further distribution by the haemolymph.

The haemolymph of female crayfish had a mean RNA copy numbers/µl of 1.7×10^6; after washing eggs, the mean copy number was 8.6×10^2 and after surface sterilisation of the eggs, the mean copy number was 8.1×10^2.

4. Discussion

The negative-sense, ssRNA athtabvirus is clearly closely related to WZSV2 with both the L and M segments having a relationship value of E = 0.0. Even the positive-sense encoded protein in the M segment of WZSV2 has exactly the same motifs (a low-density lipoprotein receptor (cysteine-rich) with Ca^{2+} binding site) in the same place (amino acids 291–321) as the athtabvirus. As with our study, Li et al. (2015) could not find the S segment for WZSV2. In the order *Bunyavirales*, the S segment or the nucleocapsid-coding segment is the most variable in size, sequence, location and has no fixed motifs, so it is very difficult to identify. Whilst it might be present, it cannot be recognised using current knowledge or bioinformatical software. Li et al. (2015) document that almost 30% of negative sense RNA viruses that they discovered failed to have an S segment identified.

However, these two viruses are quite different indicating the viruses are not close sibling viruses. Even though the identified L and M motifs resemble bunyaviral motifs (Figs. 1a and 2a), again the late joining in the dendrogram of these viruses to their closest bunyaviral neighbours (Figs. 1b and 2b) show that these viruses do not belong in the family *Peribunyaviridae* (the old family *Bunyaviridae*, genus *Orthobunyavirus* (Lefkowitz et al. 2017)). However, an exciting review of the new bunya-like viruses by Guterres et al. (2017) gives credence to the order *Bunyavirales* and places WZSV2 in the bunya-like virus supergroup closest to the current genus *Hantavirus*. As WZSV2 and athtabvirus are closely related, by inference athtabvirus will be in a cluster with Jianxia mosquito virus 2, Shuangao mosquito virus, Shuangao bedbug virus and WZSV2 (Guterres et al. 2017).

Of considerable interest is the inclusion in this supergroup of Wuhan insect virus 3 from the freshwater isopod crustacean, *Asellus* sp. As an isopod is a crustacean not an insect, therefore the virus' name should correctly be Wuhan isopod virus 3 (WIV3). Whilst there is no close homology between WIV3 and the WZSV2/athtabvirus, but they are in the same supergroup (Guterres et al. 2017), the finding of yet another crustacean member of the order *Bunyavirales* enforces Guterres et al. (2017) query; …"could bunya-like viruses first appeared in the sea?". Other crustacean marine bunyaviruses (Mourilyan virus = syn.

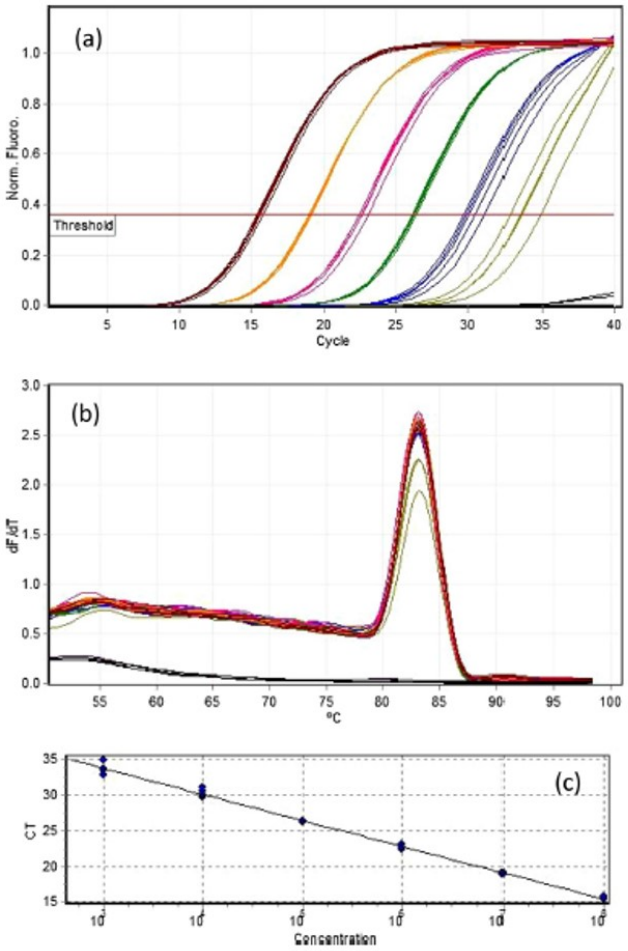

Fig. 3. Quantitative RT-PCR assay. (a) Amplification plot, (b) melt data (Mean 83.2, SD 0.58, CV 0.69%) and (c) standard curve from 10-fold serial dilutions of linearized plasmid DNA containing 207 bp athtabvirus insert.

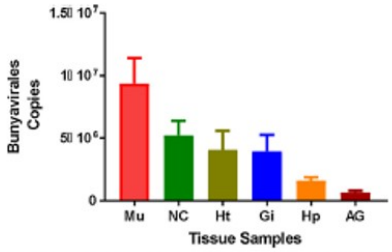

Fig. 4. The RNA copy numbers of athtabvirus in 0.2 mg of various tissues as measured by RT-qPCR.
Muscle = Mu, nerve cord = NC, heart = Ht, gills = Gi, hepatopancreas = HP, antennal gland = AG.

Whenzhou Shrimp Virus 1) from *Penaeus monodon* and *Penaeus japonicus* (Cowley et al. 2005, Li et al. 2015) and crab bunyavirus (Cancer pagurus systemic bunya-like virus) (Corbel et al. 2003.) add further imperative to this question.

It is possible that these two segments (athtabvirus L and M) are from two different bunya-like viruses but it would be curious and unlikely that Li et al. (2015) and ourselves both independently came to the same conclusions and neither group of researchers found likely other candidate sequences. i.e. we have both missed other L and M segments to go with the segments we have found. It seems that athtabvirus and WZSV2 both belong to the bi-segmented, unassigned, – ve sense ssRNA viruses, order *Bunyavirales* at present.

Of interest is the nature of the muscle lesions in the original crayfish case and the nerve lesions in the craylings (Sakuna et al. 2017a). The RNA copy number for athtabvirus was highest in muscle followed by nerve cord (Fig. 4). These lesions need to be investigated by techniques such as *in situ* PCR or gene probes. With the information available to us

at present, it is impossible to prove that athtabvirus had the dominant role in the original, stress-related mortalities in crayfish. Experimental infections to prove River's postulates for viruses still have to be undertaken as the first attempt using crickets (Sakuna et al. 2017b) was unsuccessful.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2018.03.012.

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APPENDIX C

C5: Therapeutic trials against pre-existing Chequa iflavirus in redclaw crayfish (*Cherax quadricarinatus*).

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Therapeutic trials against pre-existing Chequa iflavirus in redclaw crayfish (*Cherax quadricarinatus*)

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ABSTRACT

Chequa iflavirus is associated with mortalites reaching 20–40% after about three weeks following a stress event in farmed redclaw crayfish (*Cherax quadricarinatus*). Farmers need cost-effective interventions that can reduce the impact of this virus. This study was to identify strategies that would reduce the viral copy number as measured by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). With increasing viral copy number, there was significantly ($P < 0.002$) less weight gain at −16% reduction in growth. Chequa iflavirus ($> 10^4$ copies) was distributed widely in all crayfish tissues sampled suggesting dispersion throughout the body via the haemolymph. In two experiments each of 70 crayfish in 2 × 5 treatments, the source of the crayfish, the polyphenol flavonoid quercetin, inhabiting water temperature at 32 °C all had statistically significant effects ($P < 0.001$; $P < 0.018$; $P < 0.05$ respectively). Viral copy numbers dropped 10-fold to $10^{4.1}$ with quercetin and 82% to $10^{2.75}$ with 32 °C water temperature. Unexpectedly, delivery of specific RNA interference (RNAi) within a plasmid allowed viral copy number to initially increase due to presumed activation of the inflammasome via the dsDNA plasmid (unmethylated CpG) or endotoxin (lipopolysaccharide) inclusion and immunosuppression. Nevertheless, RNAi followed its predicted half-life and by the end of the experiment, a 5-fold reduction of viral copy numbers had occurred. A one minute, 5000 ppm formalin dip of eggs dropped viral copy numbers by 99.997% to 2.4×10^3. Five crayfish out of 140 that were repetitively bled and tested 5 or 6 times were test-negative with RT-qPCR that had a sensitivity of 100 copies. They were likely to be virus-free and could be used to start a specific pathogen free breeding program. This study demonstrated a number of ways forward for the crayfish farmers with the inflammasome suppressant, quercetin being the most favoured as it is abundant, cheap and readily incorporated into the diet and should ameliorate the original, transport-stress mortality which started this study.

1. Introduction

Infection with chequa iflavirus, a positive-sense, single-stranded RNA virus, might cause mortality reaching 20–40% after about three weeks after a stress event in redclaw crayfish (*Cherax quadricarinatus*) (Sakuna et al., 2017). Against viral infection, innate and adaptive immune responses play potential roles in vertebrates. A high degree of memory and specificity, which are characteristic of the adaptive immune response have been reported in some invertebrates (Rowley and Powell, 2007; Musthaq et al., 2014). However, convincing evidence of enhancing specific protection via putative protein vaccines is currently lacking in invertebrates. Conversely, experimental nucleic acid interventions via RNA interference (RNAi) against viruses have been widely reported (La Fauce and Owens, 2012). In addition, interventions such as raising the water temperature to 32 °C for crayfish lead to apparently less cherax bacilliform virus in crayfish (Claydon et al., 2004) and statistical significant drops in copy number of Penstylidensovirus in *Penaeus vannamei* held at 32.8 °C (Montgomery-Brock et al., 2007). Furthermore, chemotherapeutics like ivermectin have been shown to be functional in crayfish in statistically significantly reducing the effect of two parvoviruses, presumptive gill parvo-like virus and cherax ambidensovirus (Nguyen et al., 2014). Clearly, some interventions to reduce the impact of viruses in crayfish are possible but farmers need the most cost-effective methods even if this means the crayfish are not, ultimately, viral free. This study was undertaken to shotgun trial some possible viral reduction strategies for farmers to allow some on-farm follow-ups of potential treatments in the future.

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2. Materials and methods

2.1. Crayfish

Redclaw crayfish (14–20 g) were obtained from an isolated population held at James Cook University (JCU) since ~1995 (Experiment I all crayfish (n = 70); Experiment II, n = 55) and from a crayfish farm in northern Queensland (Experiment II, n = 20) to make up sufficient numbers. This smaller group of crayfish was from the same farm mentioned in Sakuna et al. (2017) that had 65% mortality within 11 weeks after transport.

Crayfish were housed individually in plastic cages 20 cm in diameter and 18 cm in height. Crayfish were assigned to treatments randomly (both experiments) but equally from the sources in Experiment II. Fifteen cages were placed in aquaria 110 cm in width, 215 cm in length and 50 cm in height. Crayfish were fed a commercial chicken diet once a day. Water exchanges were performed weekly to maintain appropriate water quality.

2.2. Short hairpin RNA (shRNA) design and construction

Short hairpin RNAs (shRNA) were designed online using siRNA Wizard v3.1 (InvivoGen, United States) against RNA-dependent RNA polymerase (RdRp) of chequa iflavirus, according to the parameters indicated on the siRNA Wizard website (www.invivogen.com/sirnawizard/design.php). The target siRNA sequence (5′GGGTGCGCTTTAACT GTATAT3′ with TCAAGAG of Loop sequence) was synthesised and cloned into psiRNA-h7SKneo G1 plasmid (InvivoGen, United States) by the manufacturer at BbsI/BbsI cloning sites. Non-specific inserted sequence (131 bp:AGCCTGTACTGTCTGCACAGACAGC CT ATGCTGTCT-GCCAGCTTAGTTCATATTTCGCRGCACTAAGGTGCTGCCCTCTCTAGR-CCTGCCCAGGTCAGTGGGACGCTGGTCCCACTCGCTCACTCACTCAG) was cloned into pGEM-T easy plasmid (Promega, United States) and used as a control plasmid with non-specific insertion. The pBK-CMV Phagemid was used as a control plasmid with no insertion. The recombinant plasmids or non-recombinant plasmids (Table 1) were transformed into *Escherichia coli* JM109 cells according to the manufacturer's instructions. Two colonies were selected for screening of recombinant plasmids. Colonies were seeded into 1 l Luria Bertani (LB) medium supplemented with 100 μg/ml IPTG, 100 μg/ml X-Gal and 50 μg/ml kanamycin or 100 μg/ml ampicillin in a shaking incubator at 150 rpm overnight at 37 °C. Recombinant plasmids were extracted from bacteria using Isolate II Plasmid Mini Kit (Bioline, UK).

2.3. Experimental design

2.3.1. Experiment I (Exp I)

The logic of Exp I (Table 2) was to see if shRNA specific against the RdRp of chequavirus delivered in a plasmid, which has the least manipulations for manufacture, would produce a meaningful reduction in viral titre. RNAi inside *Escherichia coli* bacterial cells had worked efficiently for penaeid hepandensovirus I; strain PmeHDV in the banana prawn, *Penaeus merguiensis* previously (Owens et al., 2015). The 8 ppt salinity treatment was to induce mild stress in crayfish (c.f. Claydon et al., 2004) to mimic the stress-associated mortality being seen on farms (positive control).

Seventy crayfish with pre-existing chequa iflavirus infection, as determined by RT-qPCR were randomly distributed between five experimental treatments, each containing fourteen crayfish (Table 2a). Crayfish received an injection on day 1 via sterile Terumo (1 ml) syringes and a 26-gauge needle, and were discarded after each inoculation to minimize cross-infections. The experimental period began on the day of the injection and concluded on day 30. To determine viral load, haemolymph was collected once weekly on days 0, 8, 15, 22 and 29 for RNA extraction.

Table 1
The design of the three experimental plasmid used in this study.

Plasmid	Insert sequence	Antibiotic selection
psiRNA-h7SKneo G1	Target siRNA	Kanamycin
pGEM-T easy	Non-specific insertion	Ampicillin
pBK-CMV phagemid	No insertion	Kanamycin

Table 2
Design for Experiment I (a) and Experiment II (b).

Treatment	Description
(a)	
Chequavirus shRNA	Injected with 2 μg/g BW chequavirus shRNA in psiRNA-h7SKneo G1
Non-specific shRNA plasmid control	Injected with 2 μg/g non-specific shRNA pGEM-T easy
Inoculation control	Injected with molecular grade water
Weighed control I	Blotted dry, weighed
(b)	
8 ppt salinity water (stress)	Living at 8 ppt salinity
Double dose chequa shRNA	Injected with 4 μg/g BW chequa shRNA in psiRNA-h7SKneo G1
Alternate plasmid control	Injected with 4 μg/g pBK-CMV Phagemid
Quercetin (polyphenol flavonoid)	Fed with quercetin 30 mg/kg body weight/day
Weighed control II	Blotted dry, weighed
Warm water culture	Living at 32 °C

2.3.2. Design for Experiment II (Exp II)

The logic for the design of Exp II was to double the dose of the RNAi (shRNAa-cv + p) given in Exp I to clarify its' apparent intermediate position seen in Exp I (see below). Also, the plasmid control was changed to a different plasmid, pBK-CMV phagemid to further test the role of dsDNA (unmethylated CpG) in the inflammasome (see below). In addition, the polyphenol flavonoid, quercetin was trialled to further examine the role of the inflammasome in crayfish viral infections and to test whether this cheap intervention might help farmers. The quercetin was purchased form Sigma-Aldrich (St Louis, MO, USA) and the dose was identical to Haleagrahara et al. (2017). To prepare quercetin containing feed, 30 mg of quercetin was dissolved in 30 ml of fish oil first; then the mixture was added to 1 kg of crayfish pellets, well mixed and dried.

Seventy crayfish with pre-existing chequa iflavirus infection were randomly distributed between five experimental treatments, each containing fourteen crayfish (Table 2b). The experiment was performed as the same as in Exp I. The crayfish not from JCU were transported to JCU and put directly into the trial to simulate the stress seen after movement of crayfish at the farm.

2.3.3. Viral tissue tropism

To understand the load of chequavirus in various tissue of crayfish and to identify the optimum tissue for sampling for viral load on farm, five moribund female broodstock crayfish from the farm where the index case was found (Sakuna et al., 2017) were sampled. These crayfish had become moribund from the stress of being moved from the growout pond to the hatchery for breeding. Individual tissues were aseptically dissected from five frozen carcases and the RNA extracted, reverse transcribed and qPCR performed as outlined below. The tissues included the muscle, heart, gill, antennal gland, hepatopancreas and nerve cord.

To understand if the virus was horizontal or vertically transmitted, the following protocol was used. Haemolymph from a further three live females was extracted as were the fertilised eggs (eyed-yolk stage). The eggs were either washed or surface sterilised with 0.5% formalin (5000 ppm) for 1 min (Edgerton and Owens, 1997) and then RNA

extraction, reverse transcription and qPCR was performed as described below.

2.4. RNA extraction and cDNA template preparation

Total RNA was extracted from 10 mg crayfish tissue (muscle and/or haemolymph) using total RNA Purification Kit (NorgenBiotek®, CA) according to the manufacturer's instructions. After dilutions and manipulations, viral copy numbers were expressed as either number/0.2 mg of tissue or/μl for volumes. Individual haemolymph was collected by sterile Terumo (1 ml) syringes with a 26-gauge needle and mixed with 1-part sodium citrate (3.8%) per 9 parts haemolymph. These haemolymph samples were subjected to total RNA extraction using total RNA Purification Kit (Norgen Biotek®, Canada) and further cDNA synthesis using Tetro cDNA Synthesis Kit (Bioline, UK) following the manufacturer's protocol.

2.5. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Viral titre was determined from crayfish from all treatments by reverse transcription quantitative polymerase chain reaction (RT-qPCR) performed according to the protocol of Sakuna et al. (2018).

2.6. Statistics

The copy number of the virus was tested for Normality using Q–Q plots and failed. Therefore, the copy number was $\log_{10}$ transformed and a one-way analysis of variance (ANOVA) was conducted with least significant difference (LSD) post-hoc tests applied with a $P < 0.05$ considered significant. Mortality data was analysed using a one-way ANOVA and LSD post-hoc test applied. All statistics were conducted using Statistical Program for the Social Sciences version 23.

3. Results

3.1. Survival analysis

A few of crayfish died during the two 30-day experimental trials. Four crayfish died in the double dose chequavirus shRNA; two in the weighed control part II and one in single dose chequavirus shRNA and plasmid control. There was no statistically significant difference ($P > 0.05$) between the number of deaths in treatments (ANOVA) or in survival time (survival analysis).

3.2. Treatment effects as measured by RT-qPCR

3.2.1. Experiment I

There were significant ($P < 0.002$) changes to the means of viral copy numbers with both treatment ($F = 4.35$; $df = 343,4$; $P < 0.002$) and week of the experiment ($F = 7.04$; $df = 343,4$; $P < 0.0001$) being significant. The statistical model accounted for ~14% ($R^2 = 0.137$) of the variability in chequavirus copy number. The viral copy numbers rose significantly in all groups, strongly in week 1 and strongly in week 2 in only the weighed and injected controls. The treatments effectively clustered into two subgroups, the additional stress groups of plasmid control, 8 ppt salinity and shRNA anti-chequavirus plus plasmid (shRNAa-cv+p) (subgroup A Fig. 1) and the less stressed, control subgroup, weighed only and injected plus weighed (subgroup C Fig. 1). The shRNAa-cv+p treatment group effectively straddles both subgroups suggesting the benefit of the shRNA slightly ameliorates the negative effect of the dsDNA plasmid.

There was a significant (Spearman's rho; $P < 0.002$) lowered weight increase with increasing viral copy number at around 16% reduction.

3.2.2. Experiment II

The alternate sources of the crayfish had massively significantly different chequavirus loads ($F = 32.9$; $df = 304,1$; $P < 0.001$) (JCU = $10^{3.5}$, farm = $10^{5.4}$ copies) which dominated the analysis, so the data from the two sources were analysed separately (Fig. 2a). In the JCU population of crayfish, all treatments lowered the viral copy number relative to the weighed control with only the warm-water culture (32 °C) being statistically significantly ($P < 0.05$) lower (subgroup B only, Fig. 2b). Moderate viral loads (averaging $10^{3.5}$ copies) were statistically significantly lowered ($10^{2.75}$, 82% drop) by the warm water culture (32 °C). The statistical model was weak, only accounting for ~5% ($R^2 = 0.054$) of the variability. In the crayfish from the stressed, farmed population, only the quercetin had a statistically significant ($P < 0.018$) lowering of viral copies from $10^{5.1}$ to $10^{4.1}$, a 10-fold (90%) reduction (subgroup D only, Fig. 2c) compared to all others except the weighed control. The statistical model accounted for ~23% ($R^2 = 0.226$) of the variability. There was no significant effect across weeks.

3.3. Viral tissue tropism

There was very little variation in RT-qPCR values (~5×10^4 copies) across the six tissues (Fig. 3). In descending order of viral copies, the tissues were hepatopancreas, nerve cord, antennal gland, heart, a slight drop to muscle and least in gills. This probably represents the systemic nature of the virus distributed in the haemolymph.

The female crayfish had a mean haemolymph chequavirus copy number of 7.1×10^5; after washing eggs, the mean copy number was 4.0×10^5 and after surface sterilisation the copy number was 2.4×10^3.

4. Discussion

4.1. Experiment I

Due to the significant rise in viral copy number early in the experiment, we surmised that the weighing procedure which involved gently blotting dry the crayfish and a period out of water whilst being weighed was enough of a stress to immunosuppress the crayfish and raise viral copy number. Stress is scientifically measured as immunosuppression (Dohms and Metz, 1991). Plasmid preparations could possibly have bacterial endotoxin from the lysis of bacterial cells for recovery of the plasmids, but this was not anticipated in the design and therefore not tested. In previous plasmid experiments (La Fauce and Owens, 2013; Owens et al., 2015), this was not seen to be a problem, but it is possible. Nevertheless, both double stranded DNA (plasmids, unmethylated CpG stimulation) and endotoxin (lipopolysaccharide) bind to Toll receptors (TR9 and TR4 respectively) in crayfish and signal through the myeloid differentiation factor 88 (MyD88) pathway to the NFκB suite of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-18); stimulating the detrimental inflammasome, allowing viral copy numbers to rise. This was our hypothesis for testing in Exp II.

4.2. Experiment II

In the farmed, stressed crayfish, quercetin significantly reduced the chequavirus copy number 10-fold accounting for ~23% of the variability. Polyphenol flavonoids (e.g. cocao, grape skin, onion skin, quercetin, rutin) have been shown extensively to dampen the inflammasome (Roopchand et al., 2015; Magrone et al., 2017) hence their inclusion as a treatment in Exp II to test our hypothesis on the inflammasome. Quercetin itself was shown to interact with the vitamin D receptors (VDR) (Lee et al., 2016) that directly turn on a number of genes. In a rheumatoid arthritis model in mice, quercetin significantly reduced TNF-α, IL-1β, IL-17 and monocyte chemoattractant protein-1 (MCP-1), all members of the inflammasome (Haleagrahara et al., 2017). We

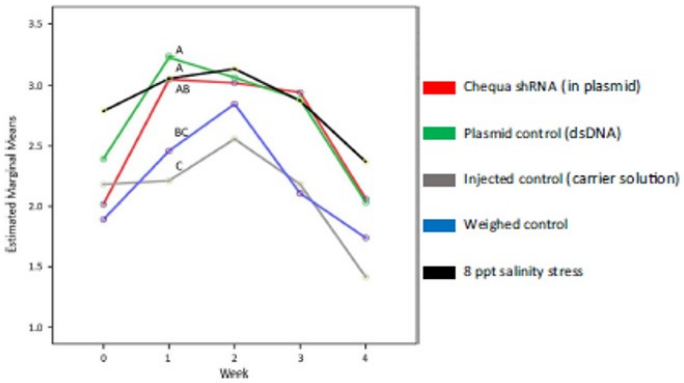


Fig. 1. The $\log_{10}$ copies of Chequa Iflavirus with treatments over time in Experiment I. Groups with the same letters are not statistically significantly different.

speculate that the mode of action of quercetin in crayfish is to bind to VDR and down-regulate the inflammasome thus reducing stress-triggered immunosuppression and the subsequent viral copy number.

In the JCU population of crayfish which was the same source to those in Exp I, the plasmid control mimicked the first experiment with a dramatic rise in viral copy number over the first couple of weeks after which it subsided to starting levels. In the JCU crayfish that had moderate viral loads (averaging $10^{3.5}$ copies), only the warm water culture (32 °C) statistically significantly lowered the viral copy number ($10^{2.75}$, 82% drop) in line with published literature (Claydon et al., 2004; Montgomery-Brock et al., 2007). Toll receptor 3 which does not use MyD88 signalling is the only temperature-independent Toll pathway. Raising water temperature (32 °C in this case) turns on all other Toll receptor pathways which would result in strong interferon regulatory factor (IRF 3 and 7) up-regulation leading to trans-acting interferon γ, a potent antiviral cytokine (Syahidah and Owens, 2011).

In the JCU population of crayfish, the number of viral copies in the double dose sa-Cv + p group mirrored the drop in the warm water group for the first two weeks after the single inoculation at day 1 before rising slightly, exactly as expected due to the two-week half-life of the shRNA (Rajeshkumar et al., 2009). Nevertheless, the final copy number at 750 copies was only ~20% (5-fold drop) of the starting load (3724 copies) suggesting the RNAi treatment had an effect even if experimental variability prevented it from being statistically significant. This result is in line with a now vast literature demonstrating the effectiveness of RNAi against viruses in crustacea (La Fauce and Owens, 2012) with most reductions being between 5 and 20 fold reductions. RNAi delivery has been declared by regulatory organisations to be a non-genetically modifying procedure, so the current bottleneck is cost-efficient manufacture and delivery most likely by diet. Plasmid constructs offer sufficient stability to get through the hostile environment of the hepatopancreatic milieu to absorption (La Fauce and Owens, 2013; Owens et al., 2015). Unfortunately, the double stranded DNA nature of plasmids or perhaps unmeasured endotoxin content appeared to trigger the inflammasome, there still was enough overall benefit to investigate further.

4.3. Implications for farmers

Transportation of live crayfish both to market and to hatcheries leads to outright mortality of somewhere between 20 and 65%, usually dependent on the time in transit. Furthermore, as viral copy number increased, growth was statistically reduced by approximately 16%. Therefore, the presence of these viruses, Chequa iflavirus and a bunyavirales (Sakuna et al., 2017) necessarily have to be dealt with to allow profitability and growth to the crayfish industry.

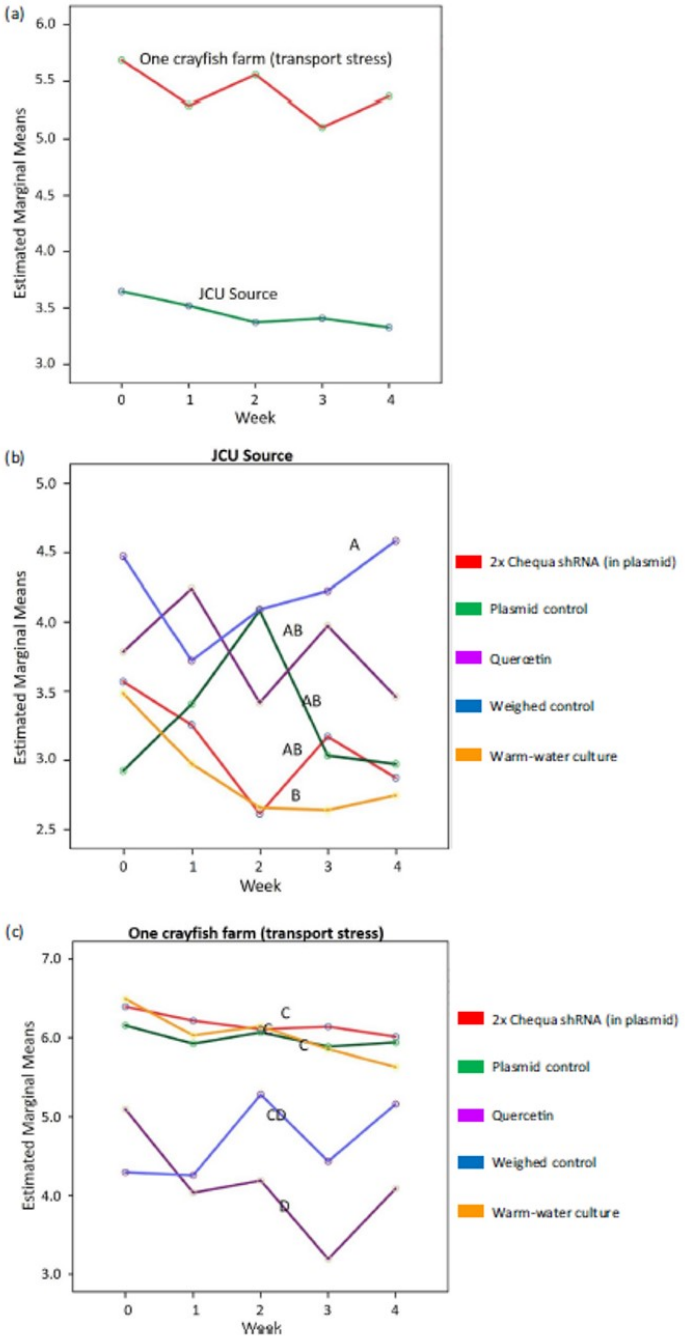
Quercetin dropped viral copy numbers by ~90%. For delivery at the pond level, flavonoids could be incorporated into the food during pelleting. Quercetin is one of the most widespread flavonoids found in plants. Flavonoid-containing grape skin is a by-product of crushing grapes for wine production; it is available at industrial quantities and should be cheaply available. Onion skin waste is a major industrial source of quercetin. It would be relatively easy and cheap to incorporate quercetin or rutin from citrus waste into pelleted diets at the dose rate of 30 mg/kg body weight/day found effective herein. There would be limited leaching as flavonoids are fat soluble, not water soluble.

Temperature at 32 °C reduced viral copy numbers by ~82–85% from about 2 weeks onwards. Broodstock could be warmed to this temperature in the hatchery via thermocouple-controlled solar hot water systems relatively efficiently. Unfortunately, initially, this measure by itself would not stop the distressing loss of crayfish that are moved into a hatchery for breeding. This continual eroding of numbers of broodstock makes planning quite difficult as farmers never know exactly how many broodstock will die and need replacing in an ever decreasing spiral of deaths.

Whilst RNAi appeared to drop viral copy number to about 80% at four weeks, this treatment was not statistical significant. It is technologically the most complex control and therefore most expensive to implement. It could be delivered at the hatchery which would limit the amount of RNAi to be manufactured, injected or fed relative to treating the ponds. The hatcheries already dip eggs into formalin to control *Saprolegnia* fungi. Practically, this has not removed the virus from the farms and when used at 5000 ppm, our qPCR results confirm a massive drop (99.997%) in copy number compared to the female's haemolymph copy number, but not eradication.

Five crayfish that were bled and tested 5 or 6 times always had viral copy number below detectable levels (< 100 copies) (Sakuna et al., 2018). At the prevalence rate seen in crayfish, the probability of the crayfish being infected but remaining test-negative is 5.8e−8. In spite of their common co-habitation history and these crayfish being in daily water-borne contact in the experiments, it seems reasonable to assume these crayfish are truly uninfected and could form the nucleus of a specific pathogen free selective breeding program. However, the tiny number of crayfish that qualify (5/140 tested) would mean it will take years of breeding to have sufficient for industry-wide stocking and it is likely inbreeding suppression of other traits will occur.

The positive sense, ssRNA viruses which includes the iflavirus, the rhinoviruses (De Palma et al., 2008) and hepatitis C virus (Yang et al., 2008) have a huge cornucopia of pharmaceutics (e.g. Pleconaril,



(caption on next page)

Fig. 2. a)The log_{10} copies of Chequa Iflavirus from two source populations in Experiment II.b) The log_{10} copies of Chequa Iflavirus with treatments over time in Experiment II in JCU crayfish. Groups with the same letters are not statistically significantly different. c) The log_{10} copies of Chequa Iflavirus with treatments over time in Experiment II with farmed crayfish. Groups with the same letters are not statistically significantly different.

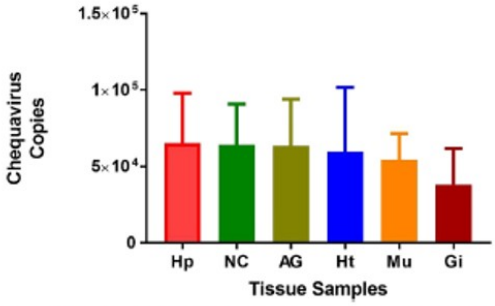


Fig. 3. The Chequa iflavirus load in 0.2 mg of various tissues as measured by RT-qPCR.
Hepatopancreas = HP, nerve cord = NC, antennal gland = AG, heart = Ht, muscle = Mu, gills = Gi.

Ribavirin, Rupintrivir) developed for treatment in humans. It would be very interesting to see if these drugs could be used to remove chequa iflavirus from crayfish stocks either alone or in combination with any of the above methods.

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