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Reverse transcription polymerase chain reaction (RT-PCR) detection for Australian Cherax reovirus from redclaw crayfish (*Cherax quadricarinatus*)

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ABSTRACT

Reoviruses have been isolated from many aquatic animals including fish and crustaceans. Viral inclusion bodies of reovirus have been found in the cytoplasm of the hepatopancreatic cells of redclaw crayfish (*Cherax quadricarinatus*). In the past, reverse transcription polymerase chain reaction (RT-PCR) designed from across other hosts was attempted to detect reovirus in redclaw crayfish but no specific set of primers successfully identified infected crayfish. In this study, two new sets of primers (Reo35F - Reo585R and Reo 35F - Seq.R1) producing a 551bp product and a 1370bp product respectively were designed using *C. quadricarinatus* reovirus partial sequence (NCBI GenBank accession no. KM405245). After the removal of primer sequences, the smaller PCR product was a 99.21% match to KM405245 while the larger product was a 99.32% match. Only three amino acid differences were observed between the Australian and Chinese sequences. The Australian sequence is the ancestral sequence so changes are reported in that order: i.e. Australian>Chinese: 19Arg>Lys; 363Leu>Met; 423Gly>Asp. The longer Australian Cherax reovirus sequence inclusive of primer sequence has been submitted to NCBI GenBank number MN308286. The second set of primers was used in the world's first RT-PCR diagnostic method to detect the Australian reovirus isolate from redclaw crayfish. The method has the detection limit of 1000 reovirus genome equivalents, and showed no cross-reactions with other prawn pathogens indicating the its high sensitivity and specificity for Cherax reovirus diagnosis.

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Keywords: Cherax reovirus, Australian isolate, Redclaw crayfish, *Cherax quadricarinatus*

1. Introduction

Reoviruses are classified under the family *Reoviridae* which contains fifteen different genera in many aquatic species including crustacea (Mohd et al., 2008; Kibenge and Godoy, 2016). Some genera such as *Cardoreovirus* have only been reported in crustaceans (Shen et al., 2015) while the *genus Aquareovirus* has been found in bony fish and crustaceans (Mohd et al., 2008). Aquareoviruses are double stranded RNA viruses composed of 11 genomic segments. The reovirus from redclaw crayfish (*Cherax quadricarinatus*) is presently unclassified.

In Australia, reoviruses in crustacea were first discovered in 1980 in 20% of wild caught, red-tailed endeavour prawns (*Metapenaeus ensis*) from Torres Strait (Owens and Hall-Mendelin 1990). The histopathology is clearly that of an RNA reovirus in the ovary and electron microscopy supported viral proliferative changes. Peer reviewers at that time would not allow the diagnosis of a reovirus infection due to lack of a precedent of reoviruses in crustacea and the term RNA proliferative syndrome was deemed descriptive (Owens, 1997). With the passage of time and accumulation of knowledge, clearly this is a pathognomic lesion for reovirus in *M. ensis*.

Evidence of the presence Cherax reovirus was first confirmed in 1997 but suspected as early as 1993 (Edgerton et al., 2000). In the early to mid-1990s, an ill-advised genetic improvement program for *C. quadricarinatus* was attempted which lead to dissemination of Cherax reovirus and *Coxiella cheraxi* to participating farms. Since that time over the years, Cherax reovirus has recrudesced to be associated with mortalities in 1997, 2005, 2006, 2008, 2012, 2014 and 2017 and to be found widespread in wild crayfish and farms from Richmond, Townsville and the Atherton Tablelands. To better understand the role of Cherax reovirus, the pathogenicity of Cherax reovirus was further explored (Hayakijkosol et al., 2017). *C. quadricarinatus* reovirus infected hepatopancreatic cells and inflammatory haemocytes were present around the infected hepatopancreatic tubules (Hayakijkosol and Owens, 2011). The viral inclusion bodies were found in the cytoplasm of the hepatopanceatic cells. *Scylla serrata* (giant mud crab) reovirus showed a similar pathogenesis, causing tissue necrosis in the hepatopancreas (Weng et al., 2007).

Reverse transcription polymerase chain reaction (RT-PCR) has been used to provide specific and sensitive diagnosis of reovirus in aquatic animals (Guo et al., 2008; Zhang and Bonami, 2012). A previous study attempted to characterize *C. quadricarinatus* reovirus and determined it had approximately 55 nm diameter icosahedral virions. Primers which were designed from partially sequenced RNA genome of the *Eriocheir sinensis* (Chinese mitten crab) reovirus were tested (Hayakijkosol and Owens, 2011). That study was unable to amplify the genetic material using those primers indicating insufficient homologies in the nucleic acid sequences of *E. sinensis* reovirus and *C. quadricarinatus* reovirus.

Partial and complete genomic sequences of phylogenetically similar reoviruses across several different species of crustaceans have recently been sequenced (Guo et al., 2008; Chen et al., 2011; Chen et al., 2012; Deng et al., 2012; Flowers et al., 2016) and may provide further information on reovirus in crustacea which can be used to design more specific primers for *C. quadricarinatus* reovirus. Of special interest, is the partial coding sequence of the RNA-dependent RNA polymerase gene (RdRP) (KM405245.1) of *Cherax quadricarinatus* reovirus by Zheng in 2015 (unpublished). This study aimed to develop an RT-PCR assay for *C. quadricarinatus* reovirus which can be used to rapidly detect reovirus in redclaw crayfish with high specificity and sensitivity.

2. Materials and methods

2.1. Source of reovirus

Infected juvenile redclaw crayfish (*C. quadricarinatus*, infected parent stock from Richmond, Queensland) were obtained from the breeding facility at the College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Queensland, Australia. Five crayfish approximately 3 to 5 cm in length were selected. The crayfish were anaesthetized in ice cold water until unresponsive. Haemolymph was collected into 10% (v/v) sodium citrate for RT-PCR and the crayfish placed back into ice cold water. The crayfish were divided into two by splitting the

cephalothorax longitudinally for histopathology and RT-PCR detection. One half of the cephalothorax was placed in Davidson's fixation for histopathology while the other half of the cephalothorax was stored frozen at −80 °C for RT-PCR detection. Another population of redclaw crayfish were also sampled for testing with PCR2 (see below); seven redclaw crayfish were sacrificed as above for hepatopancreatic samples and another 10 used for haemolymph extraction as above.

2.2. Histology

After 48 hours in the Davidson's fixation, the cephalothorax tissue including gills and hepatopancreas was transferred into histological cassettes. Cassettes with the tissues was transferred to 70% ethanol and processed for histology before being embedded in paraffin wax. Histopathological sections were cut at 5 µm and stained with Mayer's haematoxylin and eosin (H&E) (Hayakijkosol and Owens, 2011). Light microscopy (Olympus BX43 microscope) was used to screen for the pathognomic histopathological lesions including viral inclusion bodies of reovirus. Histopathological pictures were taken using a digital camera (Olympus DP26).

2.3. Primer design

Primers (Table 1; primer set 1) were designed using Geneious 9.1.8 against sequence KM405245.1 using default settings, with product size of 500-1000bp. The total expected length of the product was 551bp. A second primer set (Table 1; primer set 2) using the same forward primer was developed as above with a maximum whole gene size to increase the sequence available. The total expected length of the product was 1370 bp. The three primers were examined using Blastn. The closest matches apart from the sequence they were designed from were; Reo35F (the amphibian *Geotrypetes seraphini* E=7.3), Reo585R (the rice *Oryza brachyantha* E=1.9) and Seq.R1 (the fungus *Aaosphaeria arxii* E=4.6)

Table 1. Primers designed from sequence KM405245.1.

Primer set names	Primer names	Sequences
Primer set 1	Reo35F	GTGGCAGGAAACAATTGCGT
	Reo585R	CTCAGCTGCCAAACCTACCA
Primer set 2	Reo35F	GTGGCAGGAAACAATTGCGT
	Seq.R1	CTGCTCTTGATCGAGCACATTCTTCA

2.4. RNA extraction

Frozen hepatopancreai was subjected to nucleic acid extraction using Total RNA purification kit (Norgen, Canada) according to the manufacturer's instructions (protocol 1B). Briefly, 10mg of hepatopancreas was ground with liquid nitrogen in a microfuge with a microfuge pestle, prior to use of the kit. One hundred µl of haemolymph was also extracted using this kit without a liquid nitrogen step (protocol 1C).

2.5. RT-PCR detection

RT-PCR detection using primer set 1 utilised the following procedure. cDNA was produced using Tetro cDNA Synthesis Kit (Bioline, Australia) following the manufacturer's protocol for cDNA. Briefly,

12µl of RNA was mixed with 1µl Reo 585R primer, 1µl of 10mM dNTP, 4µl of 5X RT, 1µl of RNAse inhibitor and 1µl of RT to make a 20µl solution. This was heated at 45 °C for 30 min, 85 °C for 5 min and cooled to 4 °C prior to the PCR step. For PCR, a SensiFast SYBR No-Rox kit (Bioline, Australia), with 2µl of the cDNA as a template and using primers Reo35F and Reo585R (0.5µM) (Table 1) in a total volume of 20µl. Molecular biology grade water was used as a no template control. The PCR profile consisting of initial denaturation at 95 °C for 10 min, 40 cycles at 95 °C for 5 s denaturation, 56 °C annealing for 10 s and polymerization at 72 °C for 1 min. Samples were polymerized for an additional 5 min at 72 °C following the last cycle. The obtained PCR products were electrophoresed on a 2% agarose gel using Hyperladder 100bp plus (Bioline, Australia) as a marker. RT-PCR detection using primer set 2 followed a modified procedure to that above. Briefly cDNA was produced using Tetro cDNA Synthesis Kit (Bioline, Australia) following the manufacturer's protocol for cDNA, using random hexamers prior to DNA amplification. Briefly, 10µl of RNA was mixed with 1µl 40uM random hexamers, 1µl of 10mM dNTP, 4µl of 5X RT, 1µl of RNAse inhibitor, 1µl of RT and 2 µl water to make a 20µl solution. This was heated at 45 °C for 30 min, 85 °C for 5 min and cooled to 4 °C prior to the PCR step. The PCR step consisted of 2µl of cDNA 1X SensiFast SYBR No-Rox premix, forward and reverse primers (primer set 2) at 0.4µM (Table 1) in a total volume of 25µl. Molecular biology grade water was used as a no template control. The PCR profile consisted of initial denaturation at 95 °C for 10 min, followed by 35 cycles of 95 °C for 5 s, 56 °C for 10 s, and 72 °C for 10 s, with a final extension at 72 °C for 10 min. The resulting PCR amplicons were visualised by 2% agarose gel electrophoresis.

2.6. Cloning and sequencing

Bands were cut out from the gel and extracted using Wizard SV gel and PCR clean up kit (Promega, Australia) and transformed into *Escherichia coli* JM 109 High Efficiency cells using pGEM®-T Easy Vector System (Promega, Australia) 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 overnight. White colonies were inoculated to universal vials with 10 ml LB broth containing ampicillin (100 µg/ml) and incubated at 37 °C overnight. LB broths were purified using Wizard plus SV miniprep DNA purification System (Promega, Australia) according the manufacturer's instructions. Plasmids with DNA inserts of the PCR product were sent to Macrogen Inc (Seoul, Korea) for Sanger 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 the database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST).

2.7. Sensitivity of RT-PCR by total RNA template and plasmid standard

Ten-fold serial dilutions of total RNA extracted from reovirus-infected crayfish (100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg) were amplified by our RT-PCR using primer set 2 under standard primer set 2 conditions, including a cDNA step using random hexamers as described above. Similarly, quantitated plasmid containing the PCR product of primer set 2 was serially diluted from 10^7 copies per reaction to 10^2 copies per reaction and was amplified by the PCR step of the RT-PCR using primer set 2 under standard primer set 2 conditions.

164

165 *2.8. Specificity of RT-PCR*

166 A range of viruses was selected based on availability to the researchers and their common presence
167 in either crayfish or prawns. One hundred ng of total RNA was extracted from crustacea (either
168 crayfish or prawns) infected with reovirus, athtab bunyavirus, chequa iflavirus, *Macrobrachium*
169 *rosenbergii* nodavirus (*Mr*NV), gill-associated virus (GAV) or Taura syndrome virus (TSV). One
170 hundred ng of total DNA was prepared from prawns infected with white spot syndrome virus
171 (WSSV), and *Penaeus stylirostris* densovirus (*Pst*DV). The RNA extracted were amplified as per primer
172 set 2 RT-PCR conditions while the DNA used the same process without the RT step.

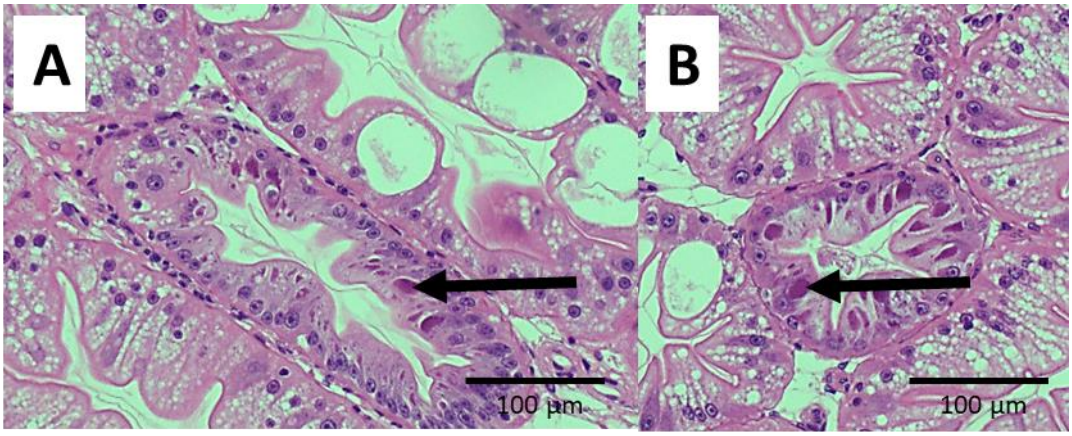
173

174 **3. Results**

175 *3.1. Histology*

176 Histology and RT-PCR using primer set 1 were conducted on the same individual redclaw crayfish for
177 accurate comparisons. Histopathological lesions from the reovirus infection were found in crayfish
178 2, 4 and 5. Histopathological sections showed infected hepatopancreatic tubules with reovirus
179 inclusion bodies in the cytoplasm of the hepatopancreatic cells (Fig. 1.A and B). Also, haemocytic
180 inflammatory infiltration was observed around the infected hepatopancreatic tubules of some of the
181 redclaw crayfish (Fig. 2.A and B). No histopathological lesion was found in the gills or other organs in
182 reovirus-infected crayfish.

183



184

185 **Fig. 1.** Two examples (A and B) of histopathology of reovirus-infected hepatopancreatic tubules in
186 Australian redclaw crayfish (*Cherax quadricarinatus*) and stained with Mayer's haematoxylin and
187 eosin (H&E). Reovirus inclusion bodies (arrows) are within the cytoplasm of the hepatopancreatic
188 cells.

189

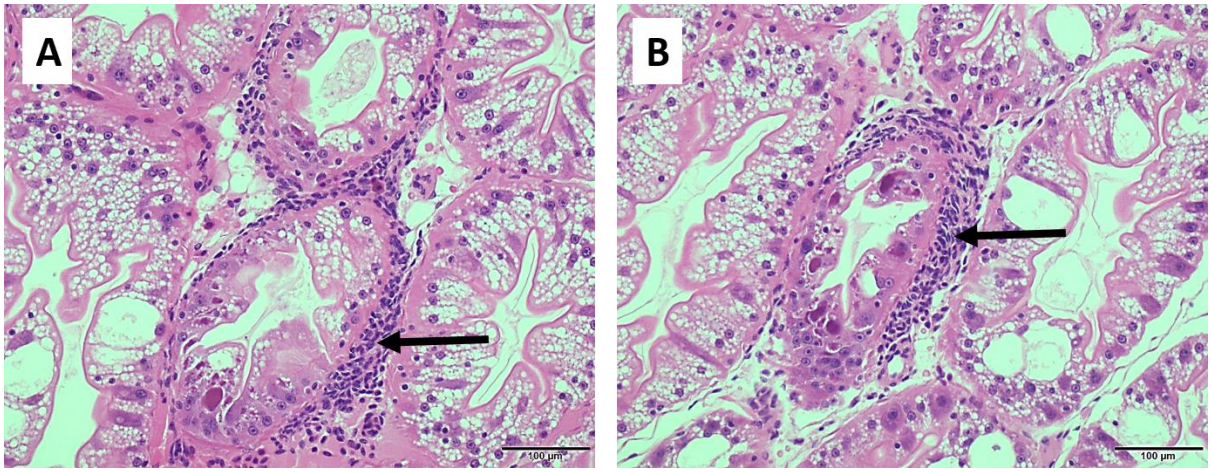


Fig. 2. Two examples (A and B) of redclaw crayfish (*Cherax quadricarinatus*) with reovirus-infected hepatopancreatic tissues stained with Mayer's haematoxylin and eosin (H&E). Viral inclusion bodies in the cytoplasm of the hepatopancreatic cells surrounded by inflammatory haemocytic infiltration (arrows) probably caused by the reovirus producing limited necrosis attracting bacteria and subsequently haemocytes.

3.2. RT-PCR and sequencing

For primer set 1, gel electrophoresis resulted in clear bands of the expected size (551bp) (Fig. 3.A) in three of the five hepatopancreatic samples (2, 4 and 5) as well as a possible faint band in sample 3. A non-specific band was found in all samples from the hepatopancreas at about 350bp. Another very faint band was found in all haemolymph samples at about 950bp and in some samples at about 250bp (Fig. 3.B). No bands were produced for no-template control samples. The sequencing results of the PCR products (eight replicates) confirmed the 551bp product was similar to the KM405245.1 original sequence with 99.21% match (e=0.0) after removal of primer sequences. The first amino acid (R arginine) in the Australian reovirus changed to a K, lysine at position 19 in the amino acid sequence of the Chinese isolate. The non-specific band at about 950bp in the haemolymph (Fig. 3.B) was confirmed to be host genome (28S rRNA gene). Other non-specific bands were not sequenced. Primer dimers were evident in negative and low positive sample. These were not examined further. Primer set 1 was not further optimised as primer set 2 was far superior with less spurious bands and potentially more informative.

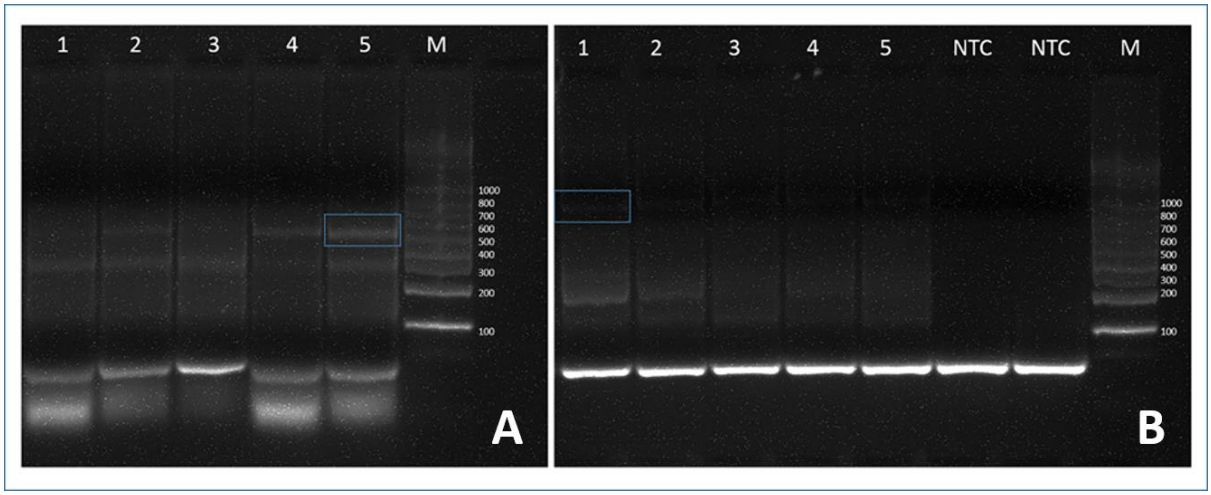
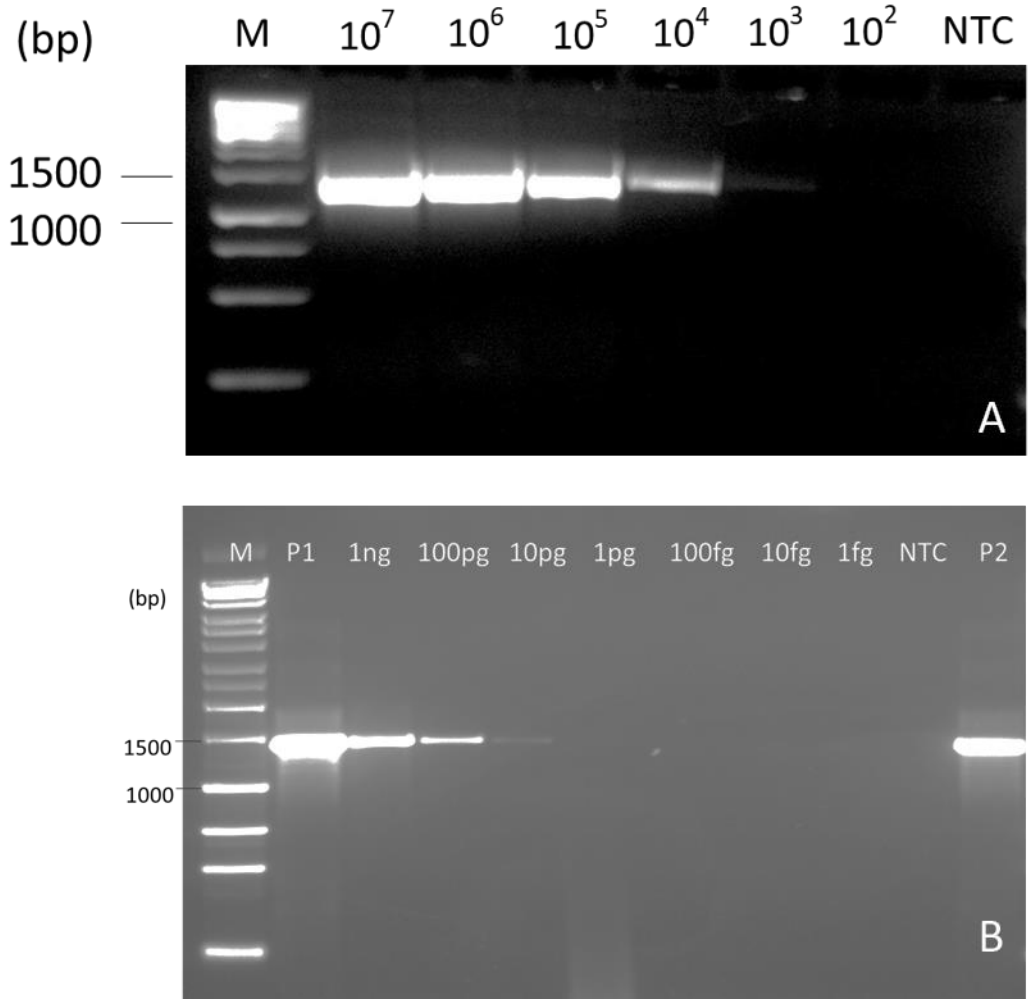


Fig. 3. PCR1 amplicons in gel electrophoresis of A) hepatopancreas from crayfish 1-5 and B) haemolymph from crayfish 1 to 5 with No Template Control (NTC) samples. Expected band size is 551bp. Band of expected size highlighted in blue rectangle on Gel A. Faint band at about 950bp from haemolymph (Gel B) is also highlighted.

Primer set 2 was initially developed to increase the amount of sequence information available for the Australian reovirus isolates. For primer set 2, gel electrophoresis resulted in clear bands of the expected size (1370 bp: Fig. 4B). No non-specific bands were observed. The sequencing results of the PCR product confirmed the 1370 bp product was similar to the KM405245.1 sequence with 99.32% match after removal of primer sequences and an e value of 0. There were only three amino acid changes between the Australian and Chinese isolates. This longer sequence identified the same first amino acid change mentioned above along with an additional two amino acid changes at Chinese isolate position 363 (from leucine in the Australian isolate to methionine) and position 423 (glycine to aspartic acid). The 1370 bp Australian Cherax reovirus sequence inclusive of primer sequences has been submitted to NCBI GenBank as number MN308286.

As the second primer set produced one specific band, this was selected as the preferred RT-PCR to analyse for diagnostic purposes. Analysis of sensitivity levels, using serially diluted plasmid containing an insert of the PCR product, identified a detection limit of 10^3 copies per reaction (Fig. 4A) while RT-PCR of dilutions of total crayfish RNA known to contain reovirus resulted in a sensitivity down to 10pg RNA (Fig. 4B). RNA from the same five crayfish examined by histology and used for PCR1 was also tested with PCR2. Similar to PCR1, 3/5 of the hepatopancreatic samples were positive while no haemolymph samples were positive. RNA from another crayfish population not know to carry reovirus was also tested with 0/7 hepatopancreas samples and 0/10 haemolymph samples positive.

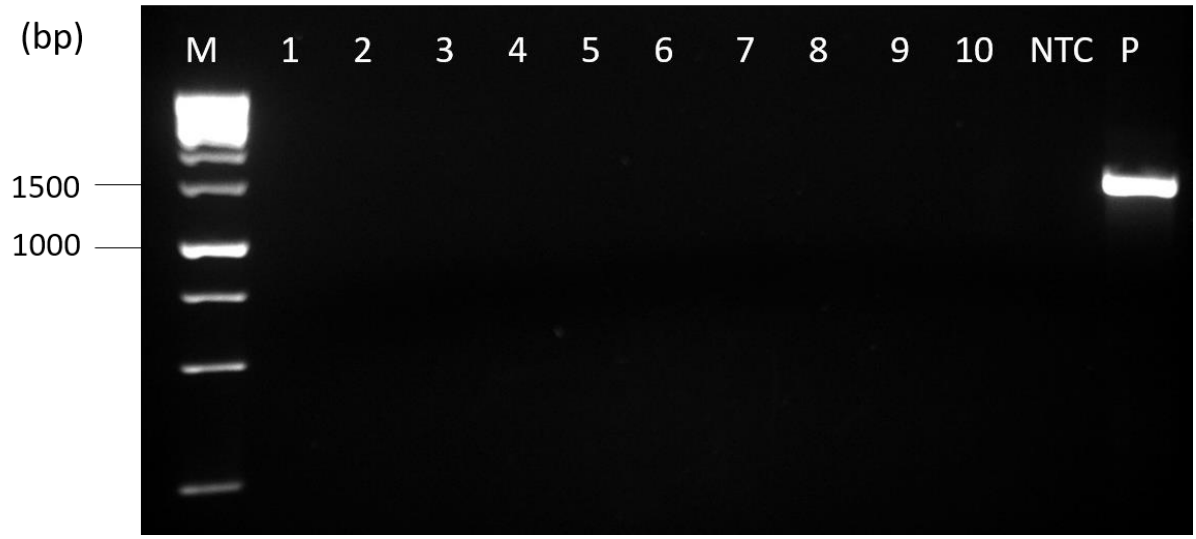


238

239 **Fig. 4.** Sensitivity of test results of RT-PCR primer set 2 using A) 10-fold serially diluted plasmid
240 standard DNA. B) 10-fold serially diluted total RNA (1ng to 1fg) extracted from reovirus-infected
241 crayfish as template (M: marker, P1: 10^6 copies plasmid standard DNA. NTC: no template control,
242 P2: 10^5 copies plasmid standard DNA. The expected product is 1370 bp.

243

244 Specificity of primer set 2 was determined against a range of viruses known to infected crayfish or
245 prawns. There was no cross reaction with any of these viruses (Fig. 5).



246

247 **Fig. 5**. Specificity test results of RT-PCR using primer set 2. M: DNA marker (Bioline HyperLadder 1kb
248 Plus, 1. WSSV, 2. IHHNV, 3. TSV, 4. GAV, 5. *Mr*NV, 6. Athtab Bunyavirus, 7. Chequa Iflavirus, 8.
249 combined infection with Bunyavirus and Iflavirus, 9. Uninfected *C. quadricarinatus* total RNA, 10.
250 *P. monodon* RNA, NTC: Nuclease-free water (negative control) and P: 10^7copies plasmid DNA. The
251 expected product is 1370 bp.

252

253 **4. Discussion**

254 This is the first time primers have been successfully designed to detect the Australian Cherax
255 reovirus previously identified by histopathology, although this is not the first time primer design has
256 been attempted (Hayakijkosol & Owens, 2011). Success was attributed to a single segment of the
257 RNA-dependent RNA polymerase from the reovirus sequence of *C. quadricarinatus* from China being
258 made available on NCBI in 2015. Also, the intentional use of an annealing temperature at the lower
259 end of the optimal temperature and longer annealing and extensions times to encourage successful
260 amplification in the event of base variation helped. Previous failed attempts were dependent on
261 reoviruses from other crustacean hosts like *Eriocheir sinensis* (Chinese mitten crab) from a different
262 crustacean genus.
263
264 Both histology and RT-PCR (primer set 1) identified the presence of reovirus in crayfish 2, 4 and 5
265 indicating the RT-PCR is as least as sensitive as histology. This does not exclude the presence of
266 reovirus in crayfish 1 and 3 as low levels of infection may not be identified, particularly given the PCR
267 using primer set 1 has not been optimised, but the independent processing of each protocol
268 provides more evidence that both methods can be used equally well to identify reovirus in redclaw
269 crayfish. Unfortunately, the lack of PCR-positive haemolymph samples means we do not yet have a
270 non-destructive screening technique for this reovirus. Sequencing of the expected band of the
271 reovirus-positive Australian crayfish showed a very high similarity to the reovirus sequence from
272 Australian *C. quadricarinatus* grown in China which was used to design the primers. Redclaw crayfish
273 were exported to China around 1992 by which time reovirus was already found in them (see
274 introduction above), indicating the Chinese reovirus sequenced had very likely originated from
275 Australia. This is very similar to the exportation of the pathogens *Coxiella cheraxi* to Ecuador
276 (Jimenez and Romero, 1997), Cherax bacilliform virus the USA (Groff et al., 1993) and Cherax
277 Iflavirus to Israel (Sakuna et al., 2017) in non-pathogen-free crayfish around the same time.

Of interest, around the same time in the early to mid-1990s, was the practice of feeding cheap, frozen crustaceans to live broodstock crustaceans to improve their fecundity. This practice has been implicated in allowing white spot syndrome virus to move from frozen, crushed up crabs into penaeid broodstock. It will be interesting, in the fullness of time, to see if the reovirus from cheap *M. ensis* (see introduction above) is closely related to Cherax reovirus thus also implicating the same pathway.

The non-specific bands produced in the first RT-PCR indicated further optimisation of the PCR and primers was needed to improve specificity of this Cherax reovirus diagnostic. An expansion of the sequence, to confirm geographically specific primer design, was carried out and it was noted that the new PCR conditions that were used eliminated non-specific bands. This was likely influenced by optimal annealing temperatures and times. RT-PCR2 has been confirmed to be both specific to reovirus and sensitive to 10^3 copies per reaction, while producing similar results to RT-PCR1 with the 5 redclaw crayfish from a reovirus-positive population. Extra samples from a crayfish population that was not known to carry reovirus were also examined using RT-PCR2 and no reovirus was found in either hepatopancreas or haemolymph. While the PCR reported here used real time reagents, the product size is too large for use in RT-qPCR and further primer design within the local known sequence would be required for conversion to a RT-qPCR.

Haemolymph was tested to see if a non-destructive sampling technique would be possible to be used on valuable broodstock, using both PCR1 and PCR2. While these confirmed reovirus-positive samples were negative by RT-PCR when using haemolymph, there is a possibility of haemolymph being positive but below detection limits. Nevertheless, this is the world's first RT-PCR for Cherax reovirus which is a major step forward and opens the pathway to improved diagnostics and understanding the true disease implications of Cherax reovirus.

Declaration of competing interest

All the authors have declared that no conflict of interest exists in this research study.

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