Establishing a gold standard method for the detection of Cherax reovirus using reverse transcriptase, quantitative, polymerase chain reaction

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Abstract

Cherax reovirus infects redclaw crayfish (*Cherax quadricarinatus*) and it may be involved in mortalities between 5-20% and stunting of up to 40% of survivors. The sequence of the RNA-dependent RNA polymerase was used to develop a reverse transcription, quantitative, PCR (RT-qPCR) which was specific against seven other crustacean viruses (*Athtab bunyavirus, Chequa iflavirus,* Macrobrachium rosenbergii nodavirus*, Gill-associated virus*, *Taura syndrome virus*, *White spot syndrome virus*, and *Penaeus stylirostris Penstylhamaparvovirus*) although GAV produced a reaction that was easily separated by melt curve analysis. A strong linear correlation ($r^2 = 0.9965$) was obtained between viral quantities ranging from 10^7 to 10 viral copies/reaction with an amplification efficiency of 0.92. This RT-qPCR is 2-times faster and 100 times more sensitive than a standard RT-PCR using agarose gel electrophoresis with the potential to detect the virus down to 7.64 copies/reaction in clinical samples. In clinical crayfish samples, it was able to detect Cherax reovirus in crayfish when the traditional RT-PCR was negative. Its' measurement of uncertainty was less than 2% (0.02–1.9), similar to PCRs for other crustacean viruses. This RT-qPCR is proposed as the gold standard and should be used for the screening of populations of *C. quadricarinatus* for broodstock before being used in hatcheries or on farms.

Keywords: Real-time PCR, Cherax reovirus, Redclaw crayfish, Australian *Cherax quadricarinatus*

1. Introduction

Redclaw crayfish (*Cherax quadricarinatus*) is native to northern Australia and Papua New Guinea, and has been increasingly and economically farmed, particularly in Queensland where suitable land and water could support aquaculture production of several thousands of tonnes (Jones, 1998; La Fauce and Owens 2007). However, an expansion of such farming comes with emerging novel viruses including iflavirus (Sakuna et. al., 2018a), bunyavirus (Sakuna et. al., 2018b) , and Cherax reovirus (Hayakijkosol and Owens, 2011) . Cherax reovirus sits in the *family Reoviridae* but has yet to be identified to genus level due to the lack of knowledge of the number of dsRNA segments and the dearth of sequence data. The areas of the genome available for analysis are limited to the RNA-dependent RNA polymerase (RdRp) gene. In Australia, Cherax reovirus has been detected in on-farm mortality of its' *Cherax* host, even though the viruses' role in the mortalities is perhaps circumstantial (Hayakijkosol and Owens, 2011, Hayakijkosol et. al., 2021). Furthermore, Cherax reovirus has been exported to China and perhaps elsewhere around the world in live *C. quadricarinatus* as identified by Sanger sequencing of crayfish in China. In experimental studies in *C. quadricarinatus*, oral exposure to Cherax reovirus led to 5% mortality with 23% stunting of survivors (Hayakijkosol and Owens, 2011). Injected reovirus caused 20% mortalities and 41% stunting of exposed crayfish. More definitive studies have been hampered by the lack of better diagnostic tests than destructive histopathology (Hayakijkosol and Owens, 2011).

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 designed from partially sequenced RNA genome of the *Eriocheir sinensis* (Chinese mitten crab) reovirus were tested but failed to amplify RNA of *C. quadricarinatus* reovirus indicating insufficient homologies in the nucleic acid sequences of the virus and *E. sinensis* reovirus (Hayakijkosol and Owens, 2011). Recently, a nearly completed Cherax reovirus's RNA-dependent RNA polymerase gene (RdRP) has been sequenced (GenBank accession no. KM405245) and from this, the first RT-PCR to detect Cherax reovirus has been established (Hayakijkosol et. al., 2021). This allows the scientific progression to quantitative RT-PCR to be developed which can aid the unravelling of the effect of Cherax

reovirus on its host. This paper describes the first RT-quantitative (q) PCR developed for Cherax reovirus and compares it to the other diagnostics known for this virus.

2. Materials and methods

2.1. Source of reovirus and total RNA template preparation

All samples in this study came from crayfish killed during previous studies (Hayakijkosol et. al., 2021). Reovirus-infected redclaw crayfish (*C. quadricarinatus*) were originally obtained from the breeding facility at the College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Australia and were progeny of crayfish from Richmond, north-western Queensland, Australia. The crayfish were tested for the presence of Cherax reovirus using our recently reported RT-PCR (Hayakijkosol et. al., 2021) as well as being used in this study. Hepatopancrei of four individual infected crayfish were subjected to RNA extraction using Total RNA purification kit (Norgen, Canada) according to the manufacturer's instructions. The concentration of RNA from each crayfish was measured by spectrophotometer, and then adjusted to 100 ng μl^{-1} by nuclease-free water followed by diluting serially in a 10-fold manner to prepare stocks containing 10 ng μl^{-1} - 1 fg μl^{-1}. Each RNA stock was kept at -20 °C until used. The conventional RT-PCR and RT-qPCR described herein were carried out using 1 µl of the RNA solution as template unless otherwise stated.

2.2. RT-PCR amplification, cloning and sequencing of Australian isolate of reovirus' RdRp gene

To extend the current sequence of the RdRp gene of the Australian reovirus, complementary (c) DNA of four infected crayfish-derived RNA extracts (100 ng each) was first produced using Tetro cDNA Synthesis Kit (Bioline, Australia) following the manufacturer's protocol. Each cDNA solution was subject to PCR using RdRp.F1/R1 primers designed against the RdRp sequence of the Chinese isolate of Cherax reovirus (Genbank No. KM405245.1) (Table 1). The 25 µl PCR reactions contained 1× SensiFast SYBR No-Rox premix (Bioline, Australia), 0.4 µM each forward and reverse primer, nuclease-free water and the specified amount of cDNA. 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 visualized by 2% agarose gel electrophoresis (AGE). After ligating the PCR product into plasmid pGEM®-T Easy Vector (Promega, Madison, USA) according to the manufacturer's protocol, the recombinant plasmids were transformed into 100 µl of JM109 *Escherichia coli* competent cells followed by blue–white colony selection using the primers and PCR method mentioned above. Positive colonies were cultured for 16 h in 2 ml of LB broth medium containing 100 µg/ml of ampicillin. The DNA plasmids were purified using Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, USA). Their concentrations were measured by spectrometric analysis, and then adjusted to 10^8 copies μl^{-1}. Sequencing of both strands of the plasmids (5-6 clones/PCR fragment) was performed by Macrogen Inc. (South Korea) using M13F-pUC and M13R-pUC universal primers. All obtained sequences were assembled into one continuous sequence using Sequencer 4.9, and compared with other viral sequences published in the database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST).

2.3. Primers and standard condition of RT-qPCR assay

Three pairs of primers to detect reovirus via RT-qPCR were designed according to the RdRp's consensus sequence of Chinese Cherax reovirus (Genbank No. KM405245.1) and our obtained sequence (Section 2.2) using Geneious 9.1.8. Two pairs were rejected due to non-specific amplification (data not shown) and the remaining RT-qPCR primer set (17Aus.F/257R) (Table 1) was optimised for concentration of primers, a melting temperature (Tm) and use of separate or combined annealing and extension steps under RT-qPCR conditions. The optimum conditions are reported here. The standard RT-qPCR consisted of 2 steps: (1) cDNA production using Tetro cDNA Synthesis Kit (Bioline, Australia) and (2) qPCR amplification. Briefly, a 9 µl cDNA premix containing 2 µl of 5× buffer, 0.5 µl each of random hexamer (50 ng μl^{-1}), dNTP mix (10 ng μl^{-1}), RNase inhibitor (200 units μl^{-1}), Tetro reverse transcriptase (200 units μl^{-1}), and 5 µl of nuclease-free water were prepared. After adding 1 µl of the RNA template, the reaction was incubated at 45 °C for 30 min. The resulting cDNA (1 µl) was further amplified by qPCR (Step 2) performed on Rotor-Gene Thermal cycler (QIAGEN, Germany) in a 25 µl volume containing 1× SensiFast SYBR No-Rox premix (Bioline, Australia), 0.4 µM each of forward (17Aus.F) and reverse primers (257R) and nuclease-free water. The optimized thermal profile was 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s and 68 °C for 10 s. The data acquisition and analysis were carried out

with Rotor-Gene Q Series Software 2.3.1 (QIAGEN, Germany). A standard RT-PCR protocol previously reported [2] was also used to compare with the RT-qPCR assay developed here. Primers are listed in Table 1.

Table 1. Primers used in RdRp gene sequencing, RT-PCR and RT-qPCR assay.

Primer name	Sequence (5'-3')	Nucleotide position on RdRp gene (Genbank No. KM405245.1)	Product size
For RdRp gene sequencing			
RdRp.F1	GGTATGGATATGTTGGGAAGACC	1-23	1456 bp
RdRp.R1	TCGTCTGAAAGTCCTCGAATGA	1456-1435	
For RT-PCR comparison [2]			
Reo35F	GTGGCAGGAAACAATTGCGT	35-54	1370 bp
SeqR1	CTGCTCTTGATC GAGCACATTCTTCA	1404-1379	
For RT-qPCR			
17Aus.F	GCGTAGACGGGACAGAGCCA	51-70	241 bp
257.R	CGCATCGCCTAGTACTCGAGC	291-271	

2.4. Standard curve and detection limit of RT-qPCR by plasmid DNA

The recombinant plasmid DNA containing the 1370 bp Cherax reovirus insert previously developed [2] was selected as the standard for this experiment as the RT-qPCR primers were designed within the range of the insert. Three replicates of 10-fold serial dilutions (10^7 – 1 copy μl^{-1}) of the plasmid were made and subjected to the RT-qPCR assay with the cDNA synthesis step omitted (see Section 2.2). The data acquisition and analysis were carried out with Rotor-Gene Q Series Software 2.3.1 (QIAGEN, Germany) in order to determine the standard curve and the detection limit (DL) of the protocol.

2.5. Molecular sensitivity of RT-qPCR determined by total RNA template

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-qPCR. The same set of templates were tested by the conventional RT-PCR/AGE concurrently (Hayakijkosol et. al., 2021) so that the

results by both methods could be compared. The RT-PCR is selected for comparison because it was the only reported molecular protocol that detects Cherax reovirus so far.

2.6. Molecular specificity of RT-qPCR

The molecular specificity of our RT-qPCR protocol was examined using 100 ng of total RNA template extracted from crustacea (either crayfish or prawn) infected with Cherax reovirus (species not approved yet; Order Reovirales, Family *Reoviridae*, probably a new genus), *Athtab bunyavirus* (Order *Bunyavirales*, unclassified family and genus), *Chequa iflavirus* (Order *Picornavirales*, Family *Iflaviridae*, Genus *Iflavirus*), Macrobrachium rosenbergii nodavirus (species not approved yet; MrNV, Order *Nodamuvirales*, Family *Nodaviridae*, Genus *Gammanodavirus*), *Gill-associated virus* (GAV, Order *Nidovirales* Family *Roniviridae* Genus *Okavirus*), and *Taura syndrome virus* (TSV, Order *Picornavirales*, Family *Dicistroviridae*, Genus *Aparavirus*), and 100 ng of total DNA template prepared from prawns infected with *White spot syndrome virus* (WSSV, Order *Incertaesedis*, Family *Nimaviridae*, Genus *Whispovirus*), and *Penaeus stylirostris Penstylhamaparvovirus* (PstHPV, Order *Piccovirales*, Family *Parvoviridae,* Genus *Penstylhamaparvovirus*). The viruses tested were shortlisted based on the common presence in either crayfish or prawns, and the availability to our research group. Reactions containing plasmid template (10^4 copies) and 100 ng RNA of from crayfish previously identified as free of reovirus were also included as positive and negative control, respectively.

2.7. Reliability of RT-qPCR by statistical sensitivity and specificity analysis

Screening crayfish and prawns with unknown reovirus status by RT-qPCR was conducted using 43 samples obtained from various sources in Queensland, Australia and one sample from Western Australia (Table 3). The results were confirmed by conventional RT-PCR (Hayakijkosol et. al., 2021). The results were used to compare the diagnostic performance of the RT-PCR protocol with the RT-qPCR protocol following the equations given in Table 4.

3. Results

3.1. RdRp gene sequencing

The sequencing results of the PCR products (4 replicates) confirmed the 1456 bp product was highly similar to the RdRp sequence of Chinese isolate of Cherax reovirus (Genbank No. KM405245.1) with 99.45% match ($e^{-0.0}$). The new sequence designated as Australian Cherax reovirus has been submitted to NCBI GenBank number MN308286.2. It was 86 bp longer than our previous reovirus RdRp sequence (1370 bp) (Genbank No. MN308286.1): 34 bp, and 52 bp expanded from 5' and 3' end, respectively. The three amino acid differences between first identified between KM405245.1 and MN308286.1 remain the only variation when comparing KM405245.1 to the extended sequence MN308286.2. Primers for RT-qPCR assay used herein were designed based on this sequence. The RT-qPCR product of a positive clinical sample was also sequenced and confirmed to be the expected reovirus product.

3.2. Standard curve and detection limit of RT-qPCR by DNA plasmid

Three replicates of 10-fold serial dilutions of plasmid DNA containing the 1370 bp Cherax reovirus insert were amplified by our RT-qPCR (Fig. 1A) where there was a positive signal in all template concentrations except at 1 copy. The melt curve peak of the target amplicons was at 83.5 °C (Fig. 1B). Although the peak shifted slightly towards 84 °C at 10 copies, it was still considered as reovirus-positive as it generated from the correct amplicon size (241 bp) (Fig. 1B, inset). Thus, 10 copies of plasmid is the reliable lower detection limit (DL) of our assay. The equation used to construct the standard curve was $Y = -3.542X + 34.302$ (Fig. 1C). A strong linear correlation ($r^2 = 0.9965$) was obtained between threshold cycles (Ct) and viral quantities ranging from 10^7 to 10 viral copies/reaction (Table 2). The amplification efficiency was 0.92. The coefficient of variation (CV), and the measurement of uncertainty within each concentration was less than 1.0% except those of 10^6 viral copies showing approximately 1.9% (Table 2).

Table 2. The cycle threshold (Ct) values of replicate assays for Cherax reovirus in relations to the uncertainty value and the coefficient of variation (CV). nr = no result, below detection level.

Plasmid copy / reaction	Ct values			Mean Ct (M)	Standard deviation (SD)	[a]Measurement of Uncertainty (%)	[b]CV (%)
	1st	2nd	3rd				

10^7	10.08	10.09	10.03	10.07	0.03	0.29	0.32
10^6	12.80	12.47	12.95	12.74	0.25	1.88	1.93
10^5	15.82	16.02	16.03	15.96	0.12	0.66	0.74
10^4	20.27	20.46	20.49	20.41	0.12	0.54	0.58
10^3	23.39	23.51	23.63	23.51	0.01	0.51	0.51
10^2	27.22	27.73	27.44	27.46	0.16	0.93	0.59
10	30.81	30.82	nr	30.815	0.007	0.02	0.02
1	nr	nr	nr	-			

[b]CV (%): (SD/M)*100

[a]Measurement of Uncertainty (%): {[(HV-LV)/2]/M}×100 where HV and LV are the highest value and lowest value measured, respectively.

204

205 *3.3. Comparative molecular sensitivity of RT-qPCR and conventional RT-PCR by total RNA*
206 *template*

207

208 RT-qPCR/melt curve analysis using 10-fold serial RNA dilutions (100 pg, 10 pg, 1 pg, 100 fg,
209 10 fg and 1 fg) identified a detection limit of 100 fg (Fig. 2A). The inset figure 2A indicates the
210 viral copy number in each sample dilution, and their Ct values correspondent to the original
211 amplification plot data from which the melt curve was generated. The RT-qPCR detection limit of
212 100 fg corresponds to 7.64 copies/reaction. Using the same set of templates, carried out
213 concurrently, conventional RT-PCR/AGE (Hayakijkosol et. al., 2021) was 100 times less sensitive
214 (Fig. 2B) than the RT-qPCR.

215

216 *3.4. Molecular specificity of RT-qPCR*

217 When RT-qPCR assay was tested with either 100 ng of DNA or cDNA from the RNA of
218 various pathogenic viruses, the results were assessed by both melt curve and AGE. As seen in Fig.
219 3A, the melt curve data showed the specific-positive peak (83.5 °C) with Cherax reovirus only.
220 PstHPV gave a small, flattened signal at around 83 °C (pink line) without a sign of DNA
221 amplification on AGE (Fig. 3B). Re-amplification of the PstHPV-qPCR product failed to show
222 the Cherax reovirus-positive signal on both melt curve and AGE (data not shown) indicating that
223 the unclear melt curve was not a PstHPV derivative but reaction noise. GAV produced a non-
224 specific peak at 87 °C (Fig. 3A) corresponding to its amplicons of approximately 450 bp (Fig. 3B).

Sequencing of this product identified it as GAV (AF227196.2) with 96.80 % identity (Supplementary method 1 and supplementary Table 1). However, it did not alter reliability of the test result as it was sufficiently different (>3 °C) to the Cherax reovirus peak to easily differentiate it from Cherax reovirus.

3.5. RT-qPCR vs RT-PCR reliability test

To determine diagnostic reliability, a range of samples from different geographic locations was tested using both the previous RT-PCR and the new RT-qPCR. Table 3 summarizes the test results by both assays relative to background of the samples tested (i.e. origin and species of crustacea). As the RT-qPCR detected 100x less virus (section 3.3), this was selected as the gold standard for the purposes of calculation and the RT-PCR reported by Hayakijkosol et al. (2021) compared against it for determination of sensitivity, specificity and overall accuracy. Amongst 43 crustacean samples (Table 3) with unknown reovirus status, 4 were identified as positive and 39 identified as negative for Cherax reovirus by the RT-PCR. Sensitivity, specificity and overall accuracy of the RT-PCR is 57%, 100% and 93% respectively (Table 4). The sample No's 15, 16 and 19 were negative by the RT-PCR method (Fig. 4B) despite being positive by the RT-qPCR as confirmed by crossing the threshold and by the correct melt curve (Fig. 4A). The RT-qPCR has a reliable detection limit of 10 copies (Fig. 1) and is 100 times more sensitive than the RT-PCR (Fig. 2) with a detection limit of 1000 copies for the RT-PCR. Copy number (Fig. 4A, inset) of samples 15, 16 and 19 as determined by RT-qPCR range between 2.3, (below the reliable detection limit of the RT-qPCR) and 98 which lies below the expected RT-PCR detection limit. It is likely that templates at less than 10 copies/reaction require an increased number of replicates to be reliably detected by the RT-qPCR, so the expected limit of this assay should remain at 10 copies, although lower detection will occasionally occur, even with no replication of samples. Therefore, it is reasonable that these were missed by the RT-PCR and they should be considered true positives by the RT-qPCR.

254 Table 3. Origin, species, number of crustaceans and Cherax reovirus status of the crustaceans as
255 determined by the conventional RT-PCR (Hayakijkosol et. al., 2021), and the RT-qPCR protocol.
256 The data provided here was used to evaluate the diagnostic performance of the RT-PCR assay
257 (Table 4) in comparison to the RT-qPCR protocol.

Origin	Species	Sample No	Cherax reovirus status	
			RT-PCR	RT-qPCR
[a]JCU Breeding facility (11 samples)	*Cherax quadricarinatus*	1 to 4	+	+
		5 to 11	-	-
Crayfish farm 1 (5 samples)	*Cherax quadricarinatus*	12 to 14	-	-
		15 to 16	-	+
Crayfish farm 2 (7 samples)	*Cherax quadricarinatus*	17 to 18	-	-
		19	-	+
		20 to 23	-	-
Crayfish farm 3 (1 sample)	*Cherax tenuimanus*	24	-	-
Crayfish farm 4 (9 samples)	*Cherax quadricarinatus*	25 to 33	-	-
Creek near Gin Gin (2 samples)	*Cherax* c.f. *depressus*	34 to 35	-	-
Recirculating Facility (5 samples)	*Cherax quadricarinatus*	36 to 40	-	-
Frozen samples from our research group (3 samples)	*Penaeus monodon*	41 to 43	-	-
Total positive			4	7
Total negative			39	36
Total samples tested			43	43

[a]JCU: James Cook University, Townsville campus

Table 4. Diagnostic performance of the RT-PCR protocol (Hayakijkosol et. al., 2021) against the recommended gold standard RT-qPCR (this paper). This assumes the RT-qPCR is 100% sensitive and specific.

RT-PCR	Cherax reovirus status by RT-qPCR (gold standard)	
	Positive	Negative
Positive	4 (TP) [a]	0 (FP)
Negative	3 (FN)	36 (TN)
Totals	7	36

	57.1% sensitivity 93% (accuracy of RT-PCR test result) (see discussion)	100% specificity

Sensitivity = [TP/(TP + FN)]*100, specificity = [TN/(TN + FP)]*100.

Accuracy = [(TP + TN)/(TP + TN + FN + FP)]*100.

[a] TP, true positive; FP, false positive; FN, false negative; TN, true negative.

Of interest, was the fact that the *C. tenuimanus* sample (Table 3) was also RT-qPCR tested for Athtab bunyavirus and *Chequa iflavirus* (Sakuna et. al., 2018a, Sakuna et. al, 2018b). Three replicate samples were found to be positive for Athtab bunyavirus at 4 x 10^3, 5 x 10^4 and 2 x 10^6 copies but all were negative for *Chequa iflavirus.*

4. Discussion

This study reports the world's first RT-qPCR assay to detect Cherax reovirus. The study started from partially sequencing the RdRp gene of the virus isolated from crayfish cultured in northern Queensland, Australia. The sequence was submitted to GenBank database under the accession No. MN308286.2. BLAST searches showed the high similarity between this sequence and the RdRp sequence of Chinese isolate of Cherax reovirus (Genbank No. KM405245.1) with a few nucleotide substitutions. The conserved regions were used for designing the primers used for the RT-qPCR assay. When 10-fold dilutions of plasmid templates were tested (Fig. 1A) with this assay, the melt curve generated a Cherax reovirus-positive peak at 83.5 °C (Fig. 1B) with a slight shift towards 84 °C in 10-copies template (the detection limit). The phenomenon is common in the presence of a very low amount of target RNA (Sakuna et. al., 2018a). It may have arisen from a slight variation in concentration of salt in the template solution as a result of inconsistent template preparation, or single nucleotide polymorphisms (SNPs) occurring during qPCR amplification as a result of *Taq* polymerase error (McInerney et. al., 2014).

Comparative detection sensitivity tests showed that the assay detected Cherax reovirus RNA as little as 100 fg or 7.64 viral particles in a clinical sample (Fig. 2A), namely 100 times more sensitive than our reported conventional RT-PCR (Hayakijkosol et. al., 2021) (Fig. 2B). The protocol also was highly specific against other viruses commonly found in either crayfish or prawns (Fig. 3A). However, PstHPV gave an indistinct, flattened melt curve signal despite no sign

of amplification shown on AGE (Fig. 3B). Re-amplification of the qPCR product showed no Cherax reovirus-specific signal (data not shown), indicating that the unclear melt curve was not PstHPV derivative but reaction noise possibly from impurities of template prepared from poor quality prawns pooled from a previous study (Jaroenram and Owens, 2014a; Jaroenram and Owens, 2014b; Jaroenram et al, 2015). As such, this type of flattened peak can be rejected as being a positive sample. Although a peak was produced with the GAV positive sample, it was not a major problem in the assay as its melt peak (87 °C; >3 °C from the true melt peak) (Fig. 3A) and amplicon size (450 bp) (Fig. 3B) was not the same as that of Cherax reovirus. The signal noises by GAV and PstHPV did not alter the specificity of the RT-qPCR as they can by ruled out by melt curve analysis. Hypothetically, a mixed infection with GAV may require confirmation of the product on a gel. No mixed infections were available to test with this scenario and GAV has not been reported as being naturally present in *C. quadricarinatus* (see Supplementary discussion 1) indicating this may remain a hypothetical issue. However, if a broad peak encompassing both specific peak positions was identified in a clinical sample, two bands on a gel (450bp vs 241bp) would clearly identify a mixed infection. Thus, our assay is specific enough for Cherax reovirus detection. If mixed infections were found in future, further studies could focus on maximizing our RT-qPCR's detection efficiency for both viruses.

Having demonstrated that the RT-qPCR was specific and sensitive, we next validated it on clinical samples collected from various sources (Table 2) comparing the results to those of the previously reported RT-PCR (Hayakijkosol et. al., 2021). While both assays were equally specific, the RT-qPCR protocol reported had an improved sensitivity (~10 copies vs ~1000 copies). Use of the RT-qPCR identified several samples that were false negatives in the RT-PCR assay due to its' detection limit. Allocating the RT-qPCR as the gold standard, the statistical sensitivity of the RT-PCR was determined to be only 57.1% relative to the RT-qPCR. This leads to a reduction of test result reliability by 7% (93% accuracy) (Table 4). Thus, the RT-qPCR is more suitable for detecting low levels of infection of Cherax reovirus, which may be present prior to an outbreak.

In summary, we have developed a rapid (~90 min), sensitive (7-10 viral copies equivalent) and extremely reliable RT-qPCR assay to detect Cherax reovirus. The assay is 100 times more sensitive than conventional RT-PCR and 2 times faster without the need for extended workflows associated with gel electrophoresis required in the conventional RT-PCR. Moreover, confirmation by melt curve analysis improved the overall specificity of test results. We believe that our RT-qPCR can

be the gold standard for Cherax reovirus detection. However, the protocol is still laboratory-based, with the need of sophisticated thermal cycler and a separated cDNA synthesis step. To make it more practical, and fully utilizable in field application, exploring the use of other simpler detection platforms for a pre-screening process while keeping the RT-qPCR as a confirmatory method should be considered. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is an alternative as it detects target RNA under single temperatures (60 – 65 °C) (Wong et. al, 2018) using a simple and portable heating block without separating the cDNA synthesis step from cDNA amplification (i.e. both steps occur simultaneously in one reaction). To date, RT-LAMP has been proven practical and efficient in detecting diverse groups of pathogens covering major aquatic viruses i.e. yellow head virus (YHV) (Jaroenram et. al., 2012) and TSV (Teng et. al., 2007, Kiapathomchai, 2008). Thus, RT-LAMP may be a useful test to develop.

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Authorship contribution statement

Wansadaj Jaroenram: Investigation, Methodology, Data curation, Result analysis & interpretation, Writing - original draft, review & editing. **Orachun Hayakijkosol:** Methodology, Result analysis, Writing - review & editing. **Leigh Owens & Jennifer Elliman:** Conceptualization, Supervision, Resources, Samples, Formal analysis, Project administration, Writing, review & editing, Funding acquisition. **Jennifer Elliman & Orachun Hayakijkosol:** additional confirmatory laboratory work.

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Declaration of competing interest

None of the authors have any conflicts of interest.

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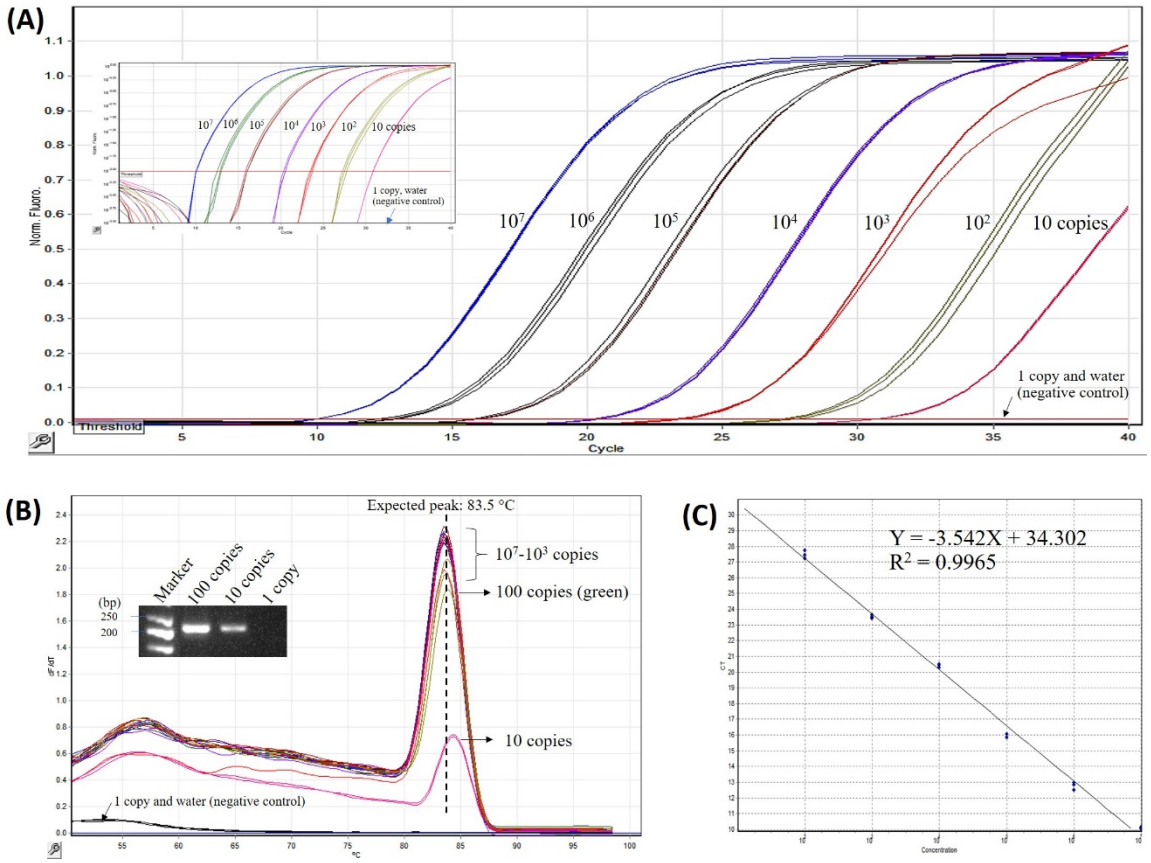

Fig. 1. Determination of RT-qPCR standard curve and detection limit using 10-fold serial dilutions (10^7 – 1 copy/reaction) of the plasmid DNA containing 1370 bp Cherax reovirus insert as templates. (A) Result by linear amplification plot with its original log-scale plot (inset). (B) Melt data of the samples as in (A) with the expected melt peak at 83.5 °C. The melt peak of 10 copies plasmid shifted forward slightly but was considered as reovirus positive as it generated from the correct PCR amplicons (241 bp) (inset). Therefore, the detection limit of the assay was 10 copies. (C) Standard curve generated from the data (A) and Table 2.

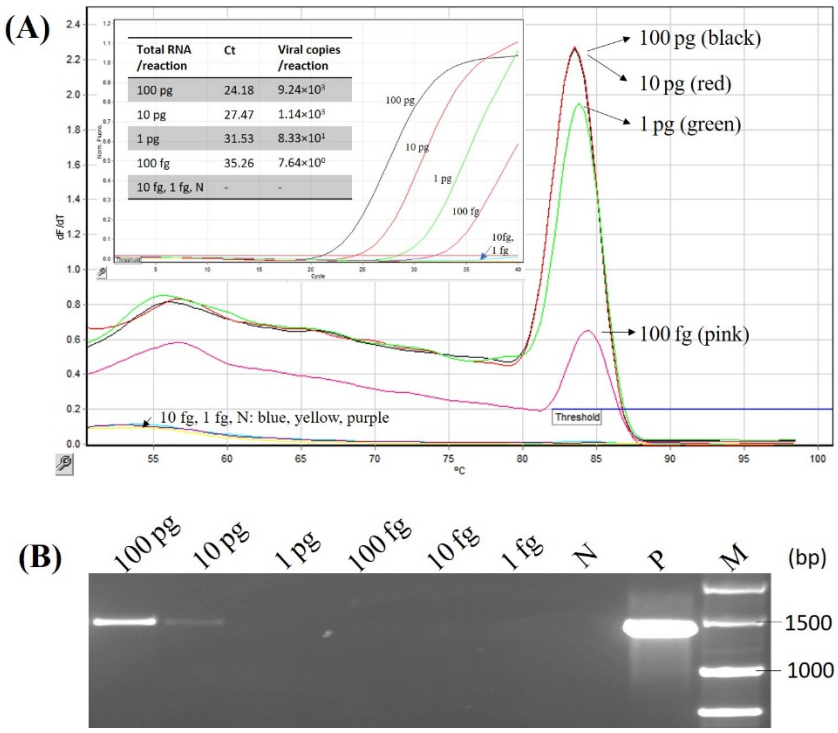

Fig. 2. Comparative molecular sensitivity test results of RT-qPCR and conventional RT-PCR using 10-fold serially diluted total RNA extracted from reovirus-infected crayfish as template. (A) Results by RT-qPCR/melt curve analysis corresponding to its original linear amplification plot (inset). (B) Result by RT-PCR/AGE (Hayakijkosol et. al., 2021) for the same templates as in (A). N, P, M: Nuclease-free water (negative control), 10^5 copies plasmid DNA (positive control, and DNA marker, respectively.

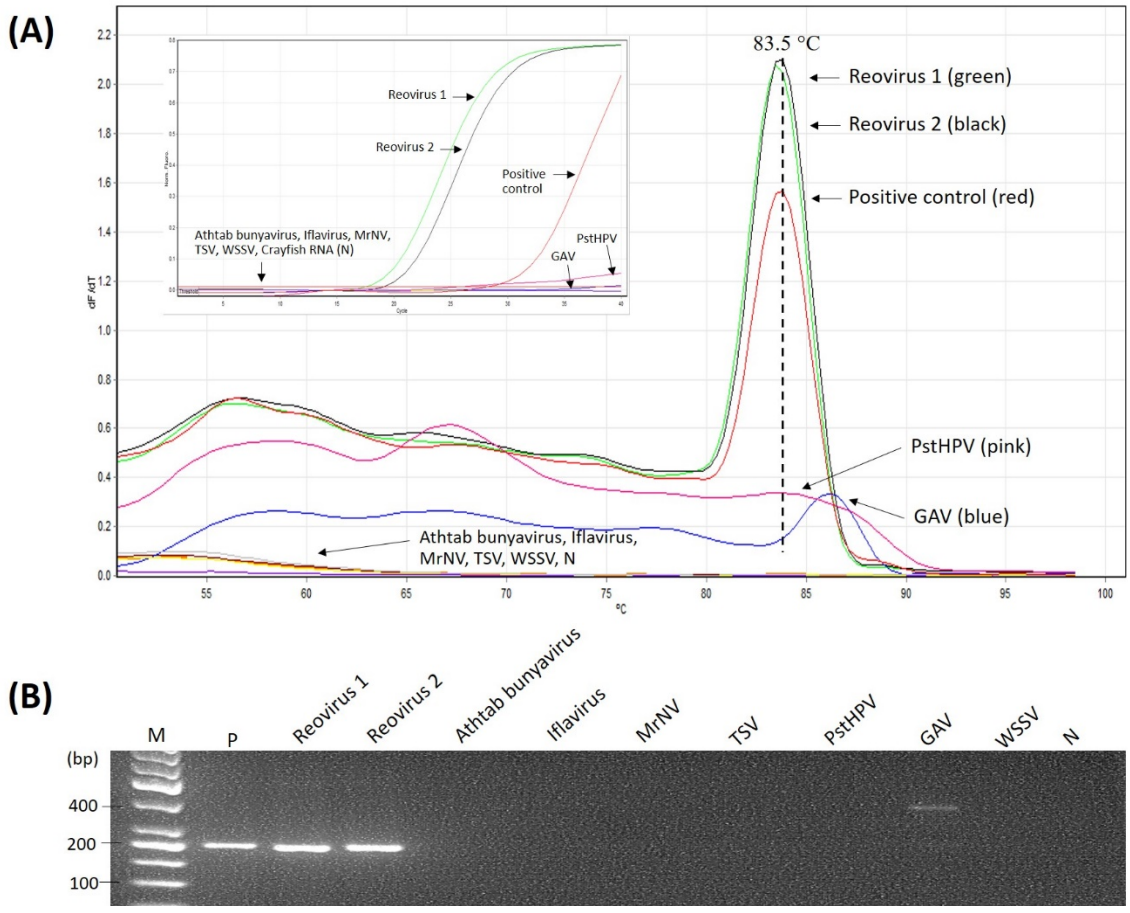

Fig. 3. Specificity of RT-qPCR for Cherax reovirus detection. (A) melt data corresponding to its amplification plot (inset) of RT-qPCR products obtained from amplifying 100 ng of either DNA or RNA extracted from various viruses indicated in the figure. The melt peak for Cherax reovirus was at 83.5 °C. (B) AGE results for the same RT-qPCR amplicons as in (A). Lanes M, P and N: Molecular marker, 10^4 copies of plasmid (positive control), and 100 ng RNA of clean crayfish (negative control), respectively.

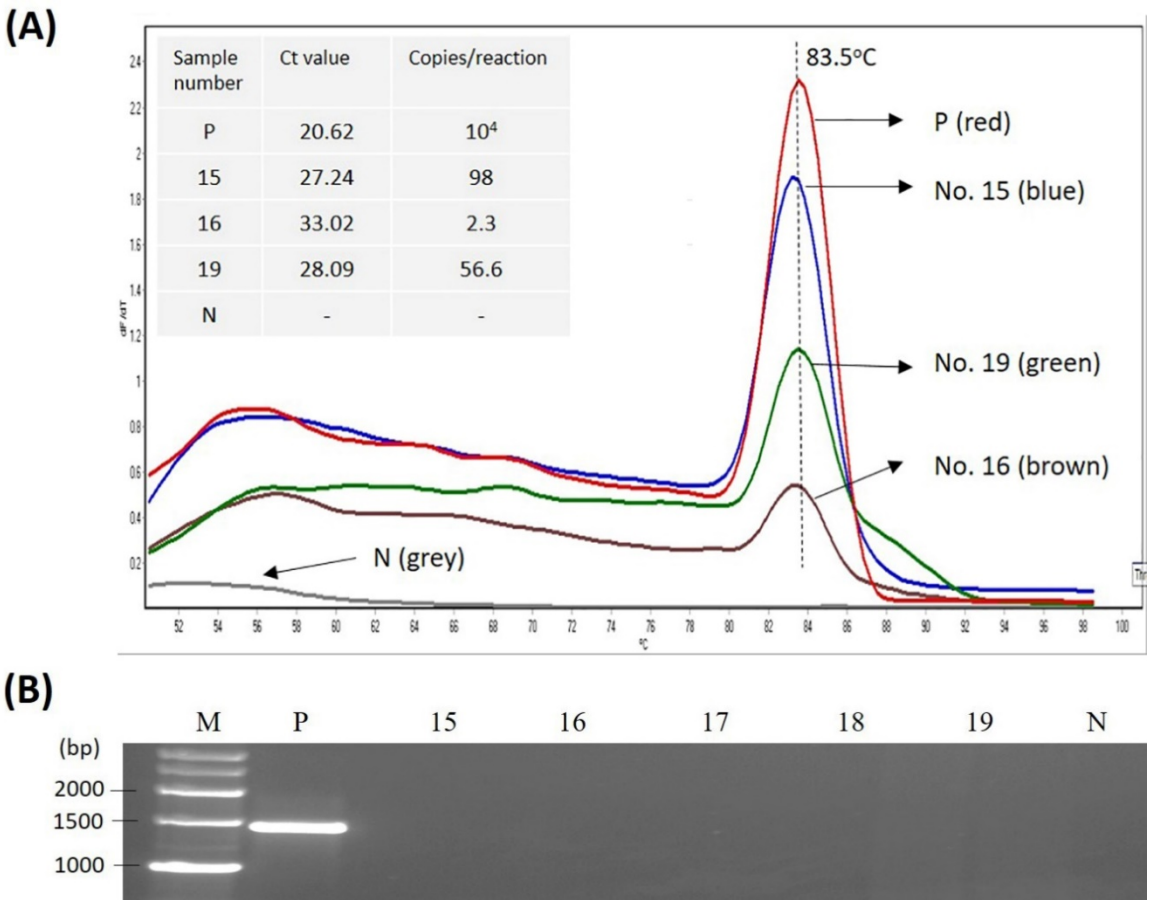

Fig. 4. Verification of disparate results (No. 15, 16, 19) between RT-qPCR and conventional RT-PCR for comparative reliability test of Table 3. (A) RT-qPCR melt data, its Ct values and calculated copy number of reovirus (inset) for the sample number 15, 16, 19 (Table 3). The melt peak for Cherax reovirus was at 83.5 °C. (B) AGE results of conventional RT-PCR for samples 15 to 19, which includes the three samples found to be positive in (A). Lanes M, P and N: Molecular marker, 10^4 copies of plasmid (positive control), and 100 ng RNA of clean crayfish (negative control), respectively.