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

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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	CTCCTTCTGGGTGCGCTTTA	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	ACGCGTATAACAGAATTGGTGT	9806–9827	
FIP (LAMP F1c + LAMP F2)	CCTACCATACGCTAACTCGCAAGGGTATGGAAGTCATCCGAT		
BIP (LAMP B1c + LAMP B2)	TTCGTATTGTTCAAACCATTCGCATGTAGTTCCTCCCACGTAG		
LAMP F2	GGTATGGAAGTCATCCGAT	9690–9708	
LAMP F1c	CCTACCATACGCTAACTCGCAAG	9765–9743	
LAMP B2	TGTAGTTCCTCCCACGTAG	9860–9842	
LAMP B1c	TTCGTATTGTTCAAACCATTCGCA	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 5s, 59 °C 10 s and 72 °C 10s. 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.