Aquaculture 319 (2011) 25–29

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Experimental infection of redclaw crayfish (*Cherax quadricarinatus*) with *Macrobrachium rosenbergii* nodavirus, the aetiological agent of white tail disease

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ARTICLE INFO

Article history:
Received 18 May 2011
Accepted 17 June 2011
Available online 24 June 2011

Keywords:
Cherax quadricarinatus
The Australian redclaw crayfish
Macrobrachium rosenbergii nodavirus
An experimental model
White tail disease (WTD)

ABSTRACT

Macrobrachium rosenbergii nodavirus (MrNV) or white tail disease has been reported as a new disease of crustacea in western Queensland, Australia. In Australia, *Macrobrachium* can be hard to source due to their need for a saltwater environment for breeding. No alternative animal experimental model for MrNV has been identified, so redclaw crayfish (*Cherax quadricarinatus*) were tested as a potential experimental animal in order to study MrNV infection. The highest mortality (35%) was in the groups injected with MrNV and the lowest mortality (0%) was in the control groups. Necrotic muscle and muscle degeneration with haemocytic infiltration were observed in infected crayfish. For the first time, a quantitative real-time polymerase chain reaction (qPCR) on clinical material was developed and it confirmed MrNV infection in infected animals. The mean viral titres (2.73×10^2 copies) and cycle times (Ct = 31.33) lead us to hypothesize that MrNV only poorly replicates in juvenile *C. quadricarinatus*. However, *C. quadricarinatus* may be a less than perfect but useable experimental animal model for MrNV infection in the future because of clinical signs, gross lesions, histopathological changes and qPCR titres present in experimentally infected *C. quadricarinatus*. This study determined that redclaw crayfish (*C. quadricarinatus*) had low susceptibility and were limited carriers of white tail disease.

1. Introduction

The giant freshwater prawn *Macrobrachium rosenbergii* is an economically important crustacean cultured on a large scale worldwide (Arcier et al., 1999; Qian et al., 2003; New, 2005). However, the *Macrobrachium* industry has suffered considerable economic losses from diseases such as mid-cycle disease and white tail disease (WTD), *M. rosenbergii* nodavirus (MrNV) with extra small virus (XSV). WTD has been reported in many countries such as Taiwan, China, India, Thailand and Australia (Sahul Hameed et al., 2004; Owens et al., 2009).

MrNV is a small, non-enveloped RNA virus with a genome consisting of 2 linear ssRNA fragments (RNA1 and RNA2) (Sahul Hameed et al., 2004). The clinical sign of MrNV infection which develops in postlarvae of *M. rosenbergii* is whitish muscle, particularly in the abdominal segments. MrNV lesions can progress to weakening of swimming and feeding ability. Mortalities may reach 100% within 2 to 3 days in some hatcheries (Sudhakaran et al., 2008).

The Australian redclaw crayfish (*Cherax quadricarinatus*) is a tropical crayfish that is native to northern Australia and Papua New Guinea. Redclaw crayfish farming in Australia is a developing aquaculture industry and redclaw is slowly becoming economically viable (Jones, 1998; La Fauce et al., 2007). Anderson and Prior (1992) first reported a virus, a presumed baculovirus, in *C. quadricarinatus*. Since then, a number of viruses have been described from farm-reared redclaw crayfish including bacilliform viruses, parvoviruses and reoviruses (Anderson and Prior, 1992; Groff et al., 1993; Edgerton et al., 2000; Owens and McElnea, 2000; Bowater et al., 2002). Redclaw crayfish are susceptible to many viral diseases; therefore, *C. quadricarinatus* may be susceptible to MrNV. Possibly, *C. quadricarinatus* may be developed as an experiment animal model for WTD. However, no report on WTD in *C. quadricarinatus* has been published.

Interfering ribonucleic acid (iRNA) is a regulatory process that can be used to control and limit the expression of some genes of the viruses. In 2009, iRNA was studied in order to control viral infection in an *in vivo* cricket model (La Fauce and Owens, 2009). In Australia, *Macrobrachium* can be hard to source due to their need for a saltwater environment for breeding and no alternative experimental animal model for MrNV has been reported. This study aimed to determine the susceptibility of the redclaw crayfish (*C. quadricarinatus*) to the Australian MrNV isolate in order to use *C. quadricarinatus* with iRNA studies in the future.

2. Materials and methods

2.1. Experimental animals

Juvenile *C. quadricarinatus* five to eight centimetres in length were obtained from a commercial crayfish farm in northern Queensland

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doi:10.1016/j.aquaculture.2011.06.023

and the breeding facility at James Cook University. Crayfish were housed in aquaria 45 cm wide, 150 cm in length and 40 cm high and fed commercial crustacean diet once a day. Water exchanges were performed daily to maintain appropriate water quality.

2.2. Preparation of inoculum

As a positive control, infected muscle from *M. rosenbergii* was confirmed by histopathology and PCR to contain MrNV. The inoculum was prepared from infected muscle which was homogenized in phosphate buffered saline (PBS) and filtered though a 45 μm filter. Thirty microliters of inoculum were injected intramuscular into the ventral side of the first abdominal segment of crayfish, just to the side of the ventral nerve cord.

2.3. Infectious challenge

Sixty redclaw crayfish were randomly distributed between six experimental aquaria divided into three treatments; control, feeding and inoculated crayfish with two replicates in each treatment. After 24 h starvation, feeding crayfish were orally challenged with MrNV only on day 0 with approximately 5% of body weight of muscles from infected *M. rosenbergii*. Prior to inoculation, crayfish were anaesthetized by being placed in chilled water. Each animal received approximately 30 μl of inoculum (intramuscular), and following injection placed immediately into the experimental aquaria; no further injections were given. Double strength PBS was injected intramuscularly into the control crayfish. The experimental period began on the day of injection (0 day) and concluded on day 60.

Redclaw crayfish were monitored daily for clinical signs and dead crayfish were removed. Dead crayfish were immediately prepared for histology and quantitative real-time polymerase chain reaction (qPCR) by splitting the cephalothorax longitudinally. The first half of the cephalothorax was split into two parts. The first part was placed in Davidson's fixation for histopathology while the second part was put in 95% ethanol for qPCR. The remaining half of the cephalothorax was stored frozen at −20 °C. At the end of the experiment period, all remaining crayfish were sacrificed, their length and weight recorded and processed appropriately for screening by histological examination and qPCR.

2.4. Histopathology

After 48 h in Davidson's fixation, the cephalothorax, gills and muscles of redclaw crayfish were collected and 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 (Olympus E C microscope) for the presence of muscle lesions and viral inclusion bodies. Digital photographs were taken with a MicroPublisher 5.0 RTV.

2.5. RNA extraction and qPCR for MrNV

RNA was extracted from approximately 30 mg of crayfish muscles in 95% ethanol for cDNA amplification using SV total RNA isolation system (Promega, Australia) according to the manufacturer's instructions. cDNA was produced using ImProm-II Reverse Transcriptase System (Promega, Australia) following the manufacturer's protocol before DNA amplification (Immomix, Bioline Australia). The specific primers and probe were designed from completed sequence of MrNV RNA1 (GenBank accession number AY222839 (The French West Indies), AY231436 (China) and FJ751226 (Thailand)) to amplify 198 bp of PCR product. The forward and reverse primers for MrNV were 5′-GAC CCA AAA GTA GCG AAG GA-3′ at positions 2860 bp to 2879 bp and 3′-GGC CTC TCC CTT TAG TGT T-5′ at positions 3040 bp to 3058 bp respectively. The probe sequence was 5′-[6FAM] AAG CAA CCG CCT TCA ATG CC [TAM]-3′ at positions 2915 bp to 2934. The cycle process consisted of incubation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 60 s. Three reactions of 20 μl were performed independently on different days to determine the specificity and reproducibility of the assay. Data acquisition and analysis were performed using Rotor-Gene 6000 (Corbett Robotics) and Microsoft Excel.

2.6. Standard curve for qPCR

In order to make a standard curve for qPCR, a positive control plasmid was prepared. The specific primer for the plasmid was designed from completed sequence of MrNV RNA1 (GenBank accession number AY222839 (The French West Indies), AY231436 (China) and FJ751226 (Thailand)). The forward and reverse primers for MrNV were 5′-CTC TTG ATC GTG TCA GTG GA-3′ at positions 2425 bp to 2445 bp and 3′-CAG GCA TTG CTT ACC ACG TT-5′ at positions 3185 bp to 3205 bp respectively. The PCR products were purified using Wizard® SV Gel and PCR Clean-UP system (Promega, Australia) and directly transformed into *Escherichia coli* JM 109 High Efficiency cells using pGEM-T® Easy Vector System (Promega, Australia), according the manufacturer's instructions. The ligation reaction was spread plated onto duplicated Luria Bertani (LB) agar plates containing ampicillin, 5-bromo-4-chloro-3-indoylgalactoside (X-Gal) and isopropyl-D-thiogalactopyranoside (IPTG) and incubated at 37 °C overnight. Blue and white bacterial colonies were screened for recombinant plasmids. Three white colonies of recombinant plasmids, putatively containing DNA insert, were inoculated to universal vials with 15 ml LB broth and incubated at 37 °C overnight. LB broths were purified using Wizard® Plus SV Minipreps DNA Purification System (Promega, Australia), according the manufacturer's instructions. Plasmids with DNA inserts of MrNV were sent to Macrogen Inc (Seoul, Korea) for sequencing to confirm the quality of the sequences.

2.7. Optimisation of $MgCl_2$ concentration

Optimised the $MgCl_2$ concentration was by 0.5 mM increments of $MgCl_2$ to the mastermix (Immomix, Bioline Australia) against an aliquot of MrNV positive control plasmid. The reaction mixture comprised 10 μl of Immomix reaction, 10 μM of forward primer, 10 μM of reverse primer, 10 μM of probe, 2.5 μl of plasmid template at concentration 1×10^5 copies for each reaction. Four replicates of each concentration of $MgCl_2$ from 0 mM to 3.5 mM were optimized.

2.8. Optimisation of probe concentration

Four replicates of mixture containing probe concentrations of 50, 60, 70, 80, 90, 100, 200 and 400 nM were optimized in the Taqman

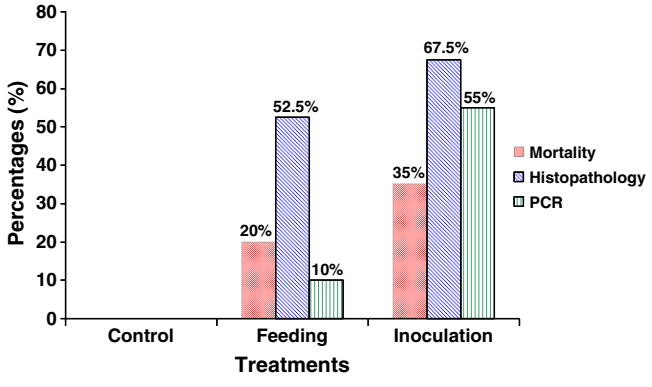

Fig. 1. Comparison of MrNV positives for mortality, histopathology (myolysis with inflammatory cells) and real-time PCR from *C. quadricarinatus* samples.