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B2 or not B2: RNA interference reduces *Macrobrachium rosenbergii* nodavirus replication in redclaw crayfish (*Cherax quadricarinatus*)

O. Hayakijkosol, L. Owens *

School of Veterinary and Biomedical Sciences, James Cook University, Solander Drive, Townsville, Queensland 4811, Australia

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ABSTRACT

Macrobrachium rosenbergii nodavirus (MrNV) which causes white tail disease (WTD) is one of the most serious viral infections in *M. rosenbergii.* In less than one week mortality has been reported in hatcheries. RNA interference (RNAi) is an innate immune response which can be triggered by the recognition of intracellular dsRNA and can inhibit viral RNA in a sequence specific manner in cells. RNAi has been used against viral infections such as yellow head virus and white spot syndrome virus in prawns. However, no study of RNAi against MrNV has been reported. In this study, RNAi against viral protein B2 and MrNV were inoculated into redclaw crayfish (*Cherax quadricarinatus*) which was developed as an experimental animal model. Mortalities were 10% with stealth RNAi plus MrNV and 60% with control stealth RNAi and MrNV exposure. Moreover, length and weight analyses of control stealth RNAi with MrNV were significantly smaller (8.7 cm) and lighter (17.69 g) ($F=8.106$, $df=5, 54$, $p<0.05$ and $F=2.943$, $df=5, 54$, $p<0.05$), respectively compared to other treatments (average 10.3 cm in length and 22.9 g in weight). In this study, clinical signs of MrNV and histopathological lesions such as myolysis with haemocytic infiltration in the muscle were found in infected redclaw crayfish. Using qPCR, two out of ten redclaw crayfish in the stealth RNAi with MrNV treatment and eight out of ten in control stealth RNAi with MrNV treatment were detected to have MrNV. Protein B2 is anti-RNAi mechanism of nodavirus. Down regulating the viral defence by targeting protein B2 gene with RNAi could be an effective tool to decrease mortality and limit MrNV infection. This approach could be very effective against the economical very important viral encephalopathy and retinopathy in fish caused by nodavirus.

1. Introduction

The cultured prawn industry including the giant freshwater prawn (*Macrobrachium rosenbergii*) has increased due to the worldwide demand for seafood. However, infectious viral disease is one of the most serious economical problems facing prawn industries (Sri Widada et al., 2004). White tail disease (WTD) is a significant viral infection for *M. rosenbergii* due to large scale mortalities in hatcheries, leading to subsequent production losses in many countries such as Taiwan, Thailand, France, India and People's Republic of China (Bonami and Sri Widada, 2011). *Macrobrachium rosenbergii* nodavirus (MrNV) was identified as the causative virus of WTD and is associated with a non-autonomous extra small virus (XSV). The main clinical sign in *Macrobrachium* gave the name to WTD: whitish coloration of the muscle in the tail. Mortality has been reported after the first clinical signs and up to 100% mortality has been seen, often in less than a week (Sahul Hameed et al., 2004)

Invertebrates lack the protein-based adaptive immunity that prevents viral infection but they are still capable of effective viral defence mechanisms such as RNA interference (RNAi) (Brennan and Anderson, 2004). RNA interference is the antiviral mechanism that allows cells to control the expression of undesirable messenger RNA (mRNA) using a double-stranded RNA (dsRNA) template (Tirasophon et al., 2005). The recognition of intracellular dsRNA triggers RNAi to degrade the sequence specific homologous viral RNA (Hannon, 2002). RNAi has been studied in many eukaryotic organisms such as protozoa (Bastin et al., 2001), fungi (Raoni and Arndt, 2003), algae (Wu-Scharf et al., 2000), nematode (Fire et al., 1998), insects (La Fauce and Owens, 2009), fish (Dang et al., 2008), mammals (Caplen et al., 2001) and plants (Fagard and Vaucheret, 2000). Also, RNAi has been used to protect against viral infection and viral replication of prawn viruses such as yellow head virus (YHV) (Tirasophon et al., 2005), white spot syndrome virus (WSSV) (Xu et al., 2007) and *Penaeus merguiensis* densovirus (La Fauce and Owens, 2009). However, no report has been published on RNAi against MrNV.

A recent study demonstrated that the Australian freshwater, or redclaw crayfish (*Cherax quadricarinatus*) can be used as an experimental animal model for MrNV (Hayakijkosol et al., 2011). MrNV protein B2 is produced to inhibit the degradation of sequence-specific viral RNA in cells. Therefore, RNAi was used in a sequence-specific manner against the viruses' protective mechanism to inhibit the production of protein B2, in effect an “arms race” between the virus and host cell. In this current study, we aim to investigate whether a triggered RNAi targeting

* Corresponding author.
E-mail address: leigh.owens@jcu.edu.au (L. Owens).

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protein B2 is protective, decreasing mortality and inhibiting MrNV replication in infected animals.

2. Materials and methods

2.1. Redclaw crayfish

Juvenile *C. quadricarinatus* or redclaw crayfish (five to eight centimetres in length) were obtained from a commercial crayfish farm in northern Queensland. *C. quadricarinatus* were housed in aquaria 50 cm in width, 50 cm in length and 40 cm in height. *C. quadricarinatus* were fed a commercial crustacean diet once a day. Water exchanges were performed daily to maintain appropriate water quality.

2.2. Preparation of inoculum

The inoculum was prepared from infected muscle of *M. rosenbergii* confirmed by histopathology and PCR (Hayakijkosol et al., 2011) to contain MrNV. This was homogenised in phosphate buffered saline (PBS) and filtered though a 45 μm filter. Thirty microliters of inoculum were injected intramuscularly into the ventral side of the first abdominal segment of crayfish, just to the side of the ventral nerve cord in accordance with the protocol described by Hayakijkosol et al. (2011).

2.3. Stealth RNAi design and stealth RNAi assay in redclaw crayfish

Stealth RNAi and stealth control RNAi duplexes were designed online using BLOCK-iT™ RNAi Designer (Invitrogen, Australia) against protein B2 of Australian MrNV (Table 1). Sixty redclaw crayfish (*C. quadricarinatus*) were randomly distributed between twelve experimental aquaria divided into six treatments: (1) untouched controls, (2) placebo controls, (3) control stealth RNAi + double strength PBS (2×PBS), (4) stealth RNAi + 2×PBS, (5) stealth RNAi + MrNV and (6) control stealth RNAi + MrNV (Table 2) with two replicates of five redclaw crayfish in each treatment. Redclaw crayfish in each treatment received two injections: the first on day 0 and the second 24 hours later while redclaw crayfish in the untouched control treatment were not inoculated. Redclaw crayfish were anaesthetized by being placed in chilled water (4 °C) before being inoculated with 2×PBS, control stealth RNAi, stealth RNAi and MrNV using sterile 1 ml syringes and 26-gauge needle. Redclaw crayfish in the appropriate treatments were challenged with 30 μl of 2×PBS and 2 μg of stealth control RNAi, 2 μg of stealth RNAi and 10^4 virions of MrNV. The infectious challenge experiment of MrMV began on the day of injection (0 day) and concluded on day 60.

Clinical signs of MrNV infection were monitored daily and dead redclaw crayfish were removed. Dead crayfish were immediately measured for weight and length and then prepared for histopathology and quantitative real-time polymerase chain reaction (qPCR) by splitting the whole body longitudinally. The first half of the cephalothorax and abdomen were 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 body was stored frozen at −20 °C. At the end of the experiment, all remaining crayfish were sacrificed, and their weight and length were recorded and processed appropriately as above for screening by histopathology and qPCR.

Table 1
Stealth RNAi and control stealth RNAi sequences were designed using BLOCK-iT™ RNAi Designer (Invitrogen, Australia) targeting protein B2 of the Australian MrNV isolate. Underlined, bold and letter "d" in the stealth RNAi and stealth control RNAi sequences indicate a different base pair, an additional base pair and a deleted base pair, respectively.

Stealth RNAi	Sequence of stealth RNAi (5′–3′)
Stealth RNAi (protein B2 target)	CACCGACAACCUACUUUCAAAGCCA
Control stealth RNAi (control)	CACAdACAAddUCCUUUCAAA**CG**GCC**C**A

Table 2
Redclaw crayfish (*Cherax quadricarinatus*) were divided into six treatments for knockdown of MrNV protein B2 using stealth RNAi.

Treatments	First inoculation (0 hour)	Second inoculation (24 hours)
1. Untouched controls	N/A	N/A
2. Placebo controls	2×PBS	2×PBS
3. Control stealth RNAi + 2×PBS	Control stealth RNAi	2×PBS
4. Stealth RNAi + 2×PBS	Stealth RNAi	2×PBS
5. Stealth RNAi + MrNV	Stealth RNAi	MrNV
6. Control stealth RNAi + MrNV	Control stealth RNAi	MrNV

2.4. Histopathology

The cephalothoraxes, gills and muscles of redclaw crayfish were collected after 48 hours in Davidson's fixation and transferred to 70% ethanol, then dehydrated through a series of alcohols to xylene and embedded in paraffin wax. Tissue sections were cut at 5 μm and stained with Mayer's haematoxylin and eosin (H&E). The histopathological sections were screened under light microscopy (Olympus E C microscope). Digital photographs of histopathological lesions were taken with a MicroPublisher 5.0 RTV camera.

2.5. RNA extraction and qPCR for MrNV

RNA was extracted from approximately 30 mg of redclaw crayfish muscles in 95% ethanol using SV total RNA isolation system (Promega, Australia) according to the manufacturer's instructions (Hayakijkosol et al., 2011). RNA was reversed transcribed to cDNA and DNA was amplified using SensiMix™ Probe One-Step Kit (Bioline, Australia) following the manufacturer's protocol. The forward and reverse primers for MrNV were 5′-GAC CCA AAA GTA GCG AAG GA-3′ and 3′-GGC CTC TCC CTT TAG TGT T-5′, respectively. The probe sequence was 5′-[6FAM] AAG CAA CCG CCT TCA ATG CC [TAM]-3′ (Hayakijkosol et al., 2011). The cycle process consisted of incubation at 42 °C for 10 min for the reverse transcriptase step and 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 analysis and acquisition were performed using Rotor-Gene 6000 (Corbett Robotics).

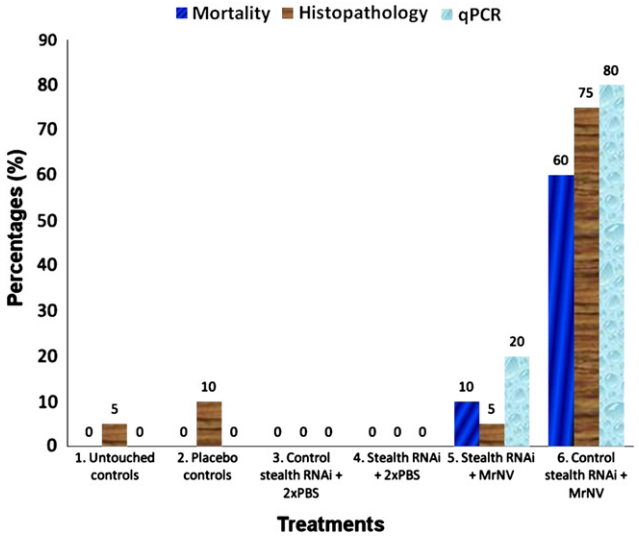

Fig. 1. Comparison of percentages of MrNV positive results; mortality, histopathology (myolysis with inflammatory cells) and quantitative real-time PCR (qPCR) from samples of redclaw crayfish (*Cherax quadricarinatus*).