Detecting of *Macrobrachium rosenbergii* Nodavirus (*MrNV*) of White Tail Disease (WTD) in Apparently Healthy Giant Freshwater Prawn, *Macrobrachium rosenbergii* in Korea

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Abstract: White tail disease (WTD) is caused by the *Macrobrachium rosenbergii* nodavirus (*MrNV*) and an extra-small virus (XSV). *MrNV* belongs to the *Nodaviridae* family. While the role of XSV in the pathogenicity of WTD remains unclear, *MrNV* is considered to be a significant factor in the disease. To study WTD infection in giant freshwater prawns (*Macrobrachium rosenbergii*), adult and post-larval (PL) prawns were collected from three giant freshwater prawn farms in Gyeongsangnam-do, Korea in 2021. Although the adult and PL prawns did not display any gross signs of WTD, *MrNV* was detected in both adult and PL in this study. However, XSV was not detected in both prawns. Phylogenetic analysis revealed that the capsid protein gene sequences of *MrNV* obtained in this study were robustly clustered with the *MrNV* group, and were clearly distinguished from *Alphanodavirus* and *Betanodavirus* groups of the family *Nodaviridae*. Although Zenker’s necrosis and myolysis were observed histopathologically in the abdominal striated muscle of adult and PL prawns, no gross signs associated with white tail were observed because of local lesions.

Keywords: *Macrobrachium rosenbergii*; white tail disease (WTD); *Macrobrachium rosenbergii* nodavirus (*MrNV*); extra small virus-like particle (XSV)

1. Introduction

The culture of the giant freshwater prawn *Macrobrachium rosenbergii* is a major aquaculture industry in Southeast Asia and some Caribbean countries [1]. *M. rosenbergii*, belonging to the *Palaemonidae* family, is generally considered to be more resistant to diseases than shrimp which belongs to the *Penaeidae* family [2].

Viral pathogens such as infectious hypodermal and hematopoietic necrosis virus (IHHNV), white spot syndrome virus (WSSV), *Macrobrachium hepatopancreatic parvo-like virus* (MHPV), *Macrobrachium muscle virus* (MMV), *Macrobrachium rosenbergii* nodavirus (*MrNV*), and extra-small virus-like particles (XSV) have been detected in *M. rosenbergii* [1]. The post-larvae (PL) of *M. rosenbergii* are often threatened by white tail disease (WTD) caused by the *Macrobrachium rosenbergii* nodavirus (*MrNV*) and extra small virus (XSV) [3].

The clinical signs of WTD-infected prawns are the appearance of white spots on the tail and white areas extending over the entire body [4]. The larvae, post-larvae (PL), and early juveniles are susceptible to white tail disease during the growth stages and demonstrate high mortality, while they are resistant in the adult stages [5,6].

WTD was first observed on Guadeloupe Island in the French West Indies and later in China, India, Taiwan, Thailand, Australia, Malaysia and Indonesia [5–12].
MrNV belongs to the Nodaviridae family and is distinguished from the genera Alphamodavirus and Betanodavirus [13,14]. Similar to other nodaviruses, MrNV is a small and non-enveloped icosahedron, with a diameter of 26–27 nm, and is composed of a nucleocapsid polypeptide of 43 kDa [7,15]. XSV, related to noda-like viruses, is an icosahedron with a diameter of 15 nm and is composed of a nucleocapsid, a polypeptide of 16 and 17 kDa [5,7]. The role of XSV in the pathogenicity of WTD is unclear, while MrNV is considered to be an important factor in this disease [3].

In this study, adult and PL stage M. rosenbergii were collected from three farms which were located geographical proximity between farms in Gyeongsangnam-do, Korea, in 2021. Molecular biological, and histopathological studies were performed as per the Manual of Diagnostic Tests for Aquatic Animals by the World Organization for Animal Health (WOAH) [16]. This study is the first to report the occurrence of WTD in M. rosenbergii in Korea.

2. Materials and Methods

2.1. Sample Collection and RNA Extraction

In Korea, there are sixteen M. rosenbergii farms in total and prawn production in all farms used a monoculture of giant freshwater prawn. In 2021, thirty adult prawns M. rosenbergii were collected from two M. rosenbergii farms and thirty PL prawns were collected from the other farm in Gyeongsangnam-do, Korea. The collected M. rosenbergii were then transported to the laboratory in oxygenated plastic bags. The muscles from the abdominal and tail regions extracted from the prawns were pooled (n = 5/pool) for RNA extraction. The pooled sample was then placed in a tube (Watson, Japan) containing 2 mm zirconia and 5 mm stainless beads, to which 200 µL of DEPC-treated water was added. The tissue was subsequently ground for 5 min using FastPrep®-24 (MP Biomedicals LLC, Solon, OH, USA). After RNA extraction using the RNeasy Mini Kit (Qiagen, Germany), the sample was stored at –80 °C and used for the next experiment.

2.2. Reverse-Transcription (RT)—PCR, Sequencing, and Phylogenetic Analysis

The cDNA synthesis and RT-PCR were performed using one-step AccuPower® RocketScript RT-PCR PreMix to detect MrNV and XSV in the post-larvae and adults (Bioneer, Korea) [4,6,16].

The reaction was performed in a 25 µL RT-PCR solution containing 20 pmol of each primer specific to MrNV or XSV and RNA template using the following cycles: reverse-transcription (RT) at 52 °C for 30 min; denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s, and elongation at 68 °C for 1 min, ending with an additional elongation step for 10 min at 68 °C (Table 1).

| Methods               | Primers         | Sequences                  | Product Size | References |
|-----------------------|-----------------|----------------------------|--------------|------------|
| MrNV (1st RT-PCR)     | Forward         | 5’-GCC-TTA-TAG-ATG-GCA-CAA-GG-3’ | 425 bp       | [4,6]      |
|                       | Reverse         | 5’-AGC-TGT-GAA-ACT-TCC-ACT-GG-3’ |              |            |
| XSV (1st RT-PCR)      | Forward         | 5’-GCC-GGA-TCC-GAT-GAA-TAA-GCG-CAT-TAA-3’ | 546 bp       |            |
|                       | Reverse         | 5’-CCG-GAA-TTT-CGT-TAC-TGT-GAG-TCC-CAA-3’ |              |            |
| MrNV (nested RT-nPCR) | Forward         | 5’-GAT-GAC-CCC-AAC-GTT-ATC-CT-3’ | 205 bp       | [17]       |
|                       | Reverse         | 5’-GTG-TAG-TCA-CTT-GCA-AGA-GG-3’ |              |            |
| XSV (nested RT-nPCR)  | Forward         | 5’-ACA-TTG-GCG-GTG-GGG-TCA-TA-3’ | 236 bp       |            |
|                       | Reverse         | 5’-GTG-CCT-GTT-GCT-GAA-ATA-CC-3’ |              |            |

Table 1. PCR primers for detection of MrNV and XSV in this study.

Nested RT-nPCR was performed using the AccuPower® HotStart PCR Premix (Bioneer, Korea) with the PCR product of one-step RT-PCR as a template [16,17]. The nested RT-nPCR method for both MrNV and XSV comprised an initial 95 °C for 10 min, followed
by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C with a final extension at 72 °C for 5 min (Table 1).

The amplified PCR products were confirmed using the QIAxcel Advanced automatic electrophoresis apparatus (Qiagen, Hilden, Germany), and then sequenced using a sequencing device in an Applied Biosystems sequencer (SG/3500). The capsid protein gene sequence of MrNV was obtained and compared against the GenBank database using BLASTN (http://www.ncbi.nlm.nih.gov/genbank (accessed on 19 September 2022)) [18]. The phylogenetic analysis of the obtained nucleotide sequences was performed using the MEGAX program [19]. A phylogenetic tree based on the neighbor-joining method [20] was constructed with one-thousand bootstrap replications.

The capsid protein gene sequences of MrNV obtained from the prawns were deposited in the GenBank database (accession numbers OP434475–OP434477).

2.3. Histopathology Analysis

Histopathological analysis was performed on the tissues of the abdominal and tail muscle from both the adult and PL prawns, even though no gross symptoms associated with muscle degeneration were observed. For light microscopic observation, the muscle tissues were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin wax, sectioned at 4 μm, and stained with hematoxylin and eosin (H & E staining) [21].

3. Results

3.1. Collected Samples

In 2021, giant freshwater prawns (M. rosenbergii) were monitored to detect WTD infections and others in Korea. The average lengths and weights of the 30 adult prawns collected from the M. rosenbergii farms were determined to be 9.2 cm and 7.9 g, respectively. The average weight of the 30 PL prawns was found to be 0.8 g. The adult and PL prawns collected from the prawn farms demonstrated no gross signs of WTD.

3.2. Sequence Analysis of MrNV

The analysis using the manual diagnostic tests for aquatic animals as per WOAH resulted in the detection of MrNV from 9 pooled samples in total from two adult farms and 3 pooled samples from a PL farm, but not that of XSV. Among the positive samples, one representative PCR product per farm was used for purification and sequencing.

In this study, the capsid protein gene similarities between the adult and PL prawns were determined to be 99.5–100%. The similarity in the capsid protein gene between MrNV from this study and MrNV using the BLAST analysis was determined to be <97.1%; specifically, Australia (96.6%), Thailand (96.1%), Taiwan (94.6%), and China (95.1–96.6%).

The phylogenetic analysis demonstrated that the capsid protein gene sequences of MrNV obtained in this study and the 17 related sequences belonging to the family Nodaviridae formed three groups—MrNV, alphanodavirus, and betanodavirus. The capsid protein gene sequences of MrNV were monophyletic with the MrNV group reported from Australia, China, Taiwan, Thailand, and India. This was distinguished from alphanodavirus and betanodavirus in the family Nodaviridae (Figure 1).

3.3. Microscopic Observation

Although histopathologically there were no severe lesions associated with mortality, local Zenker’s necrosis and myolysis were observed locally in approximately 100 μm diameters in the abdominal striated muscles of the adult and PL prawns without hemocytic infiltration (Figure 2). The expression rate ranging from 47% to 67% was revealed in histopathological analysis of giant freshwater prawns sampled from three farms (Table 2).
MrNV group

Korea_MR_MrNV_01 (OP434475)
Korea_MR_MrNV_02 (OP434476)
Korea_MR_MrNV_03 (OP434477)
Australian MrNV (JN619370)
China_MrNV (NC_005095)
China_MrNV (FJ751225)
Taiwan_MrNV (DQ521575)
Thailand_MrNN (EU150126)
India_MrNV (GU300102)
India_MrNV (JQ418298)
China_MrNV (AY231437)

Barfin flounder virus BF93Hok (EU826138)
Epinephelus tauvina nervous necrosis virus (AF318942)
Paracoto virus (AF171943)
Nodamura virus (X15961)
Boolarra virus (X15960)
Black beetle virus (X00956)
Flock House Virus (X15959)

Figure 1. The phylogenetic tree was constructed using the neighbor-joining method, using MEGA software (version X; http://www.megasoftware.net (accessed on 19 September 2022)). All reference sequences were acquired from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank (accessed on 19 September 2022)). Only bootstrap values above 60% are shown (1000 resampling processes) at branch points. Bar, 0.2 substitutions per site.

Table 2. Information of histopathological analysis of *M. rosenbergii* infected with MrNV.

| Samples                  | No. of Specimen | No. of Zenker’s Necrosis and Myolysis | Expression Rate of Histopathological Changes |
|--------------------------|-----------------|--------------------------------------|---------------------------------------------|
| Adult prawn (Farm 1)     | 17              | 8                                    | 47%                                         |
| Adult prawn (Farm 2)     | 10              | 5                                    | 50%                                         |
| Post-larval prawn (Farm 3)| 3               | 2                                    | 67%                                         |
Figure 2. Zenker’s necrosis and myolysis in the abdominal striated muscle of Macrobrachium rosenbergii infected with MrNV (M. rosenbergii nodavirus) with H & E staining. (A) Zenker’s necrosis (black arrows); (B,C) local myolysis (transparent arrows); (D) disseminated myolysis.

4. Discussion

In 2021, WTD was detected in M. rosenbergii from giant freshwater prawn farms in Korea. The infection was confirmed using molecular biology and histopathological experiments.

The MrNV was detected in both adult and PL samples in this study. Yoganandhan et al. (2006) also found MrNV only in some PL samples in Thailand. Previous studies of WTD on M. rosenbergii reported a close relationship between MrNV and XSV [5,22]. It has since been suggested that XSV is a satellite virus that lacks RNA-dependent RNA polymerase (RdRp) and relies on the RdRp of MrNV or other RNA viruses for self-replication [23,24]. Zhang et al. (2006) were unable to determine whether MrNV alone could cause prawn mortality considering it was not possible to isolate pure XSV or MrNV viruses from infected prawns [24]. Subsequently, Gangnonngiw et al. (2020) reported that MrNV alone could cause severe mortality in the PL stage, which was not significantly different from that caused by a combination of MrNV and XSV [3]. The mechanism of resistance to MrNV and XSV in adult prawns remains unknown [2,9,25].

The phylogenetic tree of MrNV groups using the capsid protein gene indicated that our MrNV is most closely related to the MrNV strains from Australia, China, Taiwan, Thailand, and India. Shekhar et al. (2011) and NaveenKumar et al. (2013) also found capsid protein gene of MrNV from India highly homology with MrNV detected from China, Taiwan and Thailand [14,26]. Two genera, alphanodavirus, which infect insects, and betanodavirus, which infect fish belong to the family Nodaviridae [13]. NaveenKumar et al. (2013) suggested that the two genotypes (MrNV and PnNV) were a part of the family Nodaviridae.
Nodaviridae through the phylogenetic analysis of the capsid protein genes [14]. Our study revealed that the MrNV genotype groups from this study could be distinguished from alphanodavirus and betanodavirus of the family Nodaviridae as per the phylogenetic analysis based on the comparison of the capsid protein gene.

Yoganandhan et al. (2006) reported that both MrNV and XSV were detected in the brooder prawns, but no gross lesions of WTD were observed [9]. The mechanism of no gross lesions might associate with low expression level of the viral protein genes in PL and adult prawns. When the population of PL was transferred to grow-out ponds which gave favorable conditions for viral replication, the expression level of the viral protein genes could be increased.

The gross and histopathological symptoms of *M. rosenbergii* caused by MrNV are known to be characterized by extensive muscle necrosis and myolysis leading to opacity of skeletal muscles [12,16,27] and basophilic cytoplasmic inclusion bodies of striated muscles, and oedema leading to abnormal open spaces among the affected muscle cells [7,8]. In this study, no gross lesions were observed due to muscle necrosis, but local muscle necrosis and myolysis were observed histopathologically. In this study, Zenker’s necrosis and myolysis which is not enough to lead gross lesions were observed locally without the inclusion bodies and oedema in striated muscles of abdominal region. This is presumed to be the result of infection that does not have enough time dose before sufficient viral infection leading to gross symptoms and mortality [28,29]. Zenker’s necrosis and myolysis were observed with expression rates from 47% to 67% of in giant freshwater prawns infected with MrNV in this study. There was no significant difference in these values between post-larval stage and adult stage with statistical analysis (chi-squared test). Although the expression rate of pathological changes is high, it was observed as mild infection with no mortality and gross sign because of limited local lesions of approximately 100 μm in diameter.

Cases of WTD outbreaks on shrimp farms in Asia and other countries, therefore, continue to be reported to WOAH. MrNV infectivity in various shrimps, as well as the association of XSV with pathogenicity and disease, should be further studied. Additionally, it is necessary to diagnose using several types of genetic marker such as RNA-dependent RNA polymerase (RdRP) gene to acquire more genetic information of WTD.

5. Conclusions

In this study, we investigated WTD infection in giant freshwater prawns, *M. rosenbergii*, collected from three giant freshwater prawn farms in Korea. Although MrNV was detected in the adult and PL prawns, XSV was not. Phylogenetic analysis revealed that the capsid protein gene sequences of MrNV obtained in this study were clustered with the MrNV group of the family Nodaviridae. In the histopathology analysis, Zenker’s necrosis and myolysis were observed in the abdominal striated muscles in giant freshwater prawn. We presumed that the routes of possible transmission of WTD were breeding water, surrounding organisms and through the imported prawns for culture. The continuous monitoring of hatcheries and post-larval farms is additionally required to prevent the spread of WTD.

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**Institutional Review Board Statement:** Giant freshwater prawns are invertebrates and do not require laboratory animal ethics according to the Laboratory Animal Act in Korea.

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