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RT-PCR amplification and sequence analysis of extra small virus associated with white tail disease of *Macrobrachium rosenbergii* (de Man) cultured in Taiwan

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Abstract

Post-larvae of *Macrobrachium rosenbergii* infected with white tail disease (WTD) have been reported in Taiwan. The causative agents have been identified as *M. rosenbergii* nodavirus (*Mr*NV) associated with extra small virus (XSV). The present study is the first report confirming the presence of XSV virus in *M. rosenbergii* displaying WTD symptoms in Taiwan by reverse transcription polymerase chain reaction (RT-PCR). A 772 bp amplified product was obtained by RT-PCR, cloned and sequenced. The nucleotide sequence analysis of the 772 bp DNA fragment revealed 98% and 97% identity with XSV isolated from China and India, respectively. Comparison of the deduced amino acid sequences of the XSV partial genome showed strong homology (99% and 97%) with isolates from China and India. Phylogenetic analysis revealed the XSV-Taiwan isolate was more closely related to the Chinese rather than the Indian isolates. The results demonstrated the presence of XSV virus co-infection in *M. rosenbergii* cultured in Taiwan suffering from WTD.

Keywords: Macrobrachium rosenbergii, RT-PCR, sequence analysis, white tail disease, XSV.

Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii* (de Man), is an economically important palaemonid in Taiwan. An epizootic disease designated as white tail disease (WTD) has been observed in hatcheries and nursery ponds since 1992. This disease has caused high mortalities and huge economic losses (Tung, Wang & Chen 1999). WTD has recently been reported in China (Qian, Shi, Zhang, Cao, Liu, Li, Xie, Cambournac & Bonami 2003), India (Sahul Hameed, Yoganandhan, Sri Widada & Bonami 2004a; Shekhar, Azad & Jithendran 2006), Thailand (Yoganandhan, Leartvibhan, Sriwongpuk & Limsuwan 2006) and the French West Indies (Arcier, Herman, Lightner, Redman, Mari & Bonami 1999). The affected prawns exhibit white opaque muscle in abdominal segments, commonly accompanied by progressive weakening of their feeding and swimming ability. The mortalities reached between 50% and 70% after transferring the post-larvae (PL) to the grow-out ponds (Tung *et al.* 1999).

The causative agents of WTD have been identified as *M. rosenbergii* nodavirus (*Mr*NV) associated with extra small virus (XSV) (Qian *et al.* 2003). *M. rosenbergii* nodavirus is a small icosahedral non-enveloped virus, 26–27 nm in diameter, observed in the cytoplasm of connective tissue cells in infected prawns. The viral genome is formed by two pieces of single-stranded, positive-sense RNA (RNA1 and RNA2), of about 2.9 and 1.3 kb in length, respectively. The viral capsid contains a single polypeptide of 43 kDa (Bonami, Shi, Qian & Sri Widada 2005). The XSV virus is icosahedral in shape, 15 nm in diameter. The genome consists of a linear single-stranded RNA of 796 nucleotides, encoding a single structural protein of 17 kDa

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(CP-17) (Sri Widada & Bonami 2004). Because the clinical signs exhibited are not specific to WTD, other methods are required to screen for the aetiological agent. A number of diagnostic methods have been developed for detecting *Mr*NV, including dot-blot hybridization, *in situ* hybridization, reverse transcription polymerase chain reaction (RT-PCR) and ELISA (Sri Widada, Durand, Cambournac, Qian, Shi, Dejonghe, Richard & Bonami 2003; Romestand & Bonami 2003). Dot blot hybridization and RT-PCR have also been developed for detection of XSV (Sri Widada, Richard, Shi, Qian & Bonami 2004). A single-tube, one-step multiplex RT-PCR has been developed for detecting *Mr*NV and XSV (Yoganandhan, Sri Widada, Bonami & Sahul Hameed 2005).

Although the relationships between *Mr*NV and XSV virus remains unknown, it is hypothesized that XSV constitutes a new species of satellite virus (Sri Widada & Bonami 2004). Similar clinical signs associated with *Mr*NV virus infection were reported in diseased *M. rosenbergii* from Taiwan (Tung *et al.* 1999) but the phenomenon of co-infection with the satellite virus was not demonstrated in WTD-infected prawns. The present study is the first report confirming the presence of XSV virus in WTD affected *M. rosenbergii* in Taiwan by RT-PCR. Furthermore, the RT-PCR amplified product of XSV virus was cloned and sequenced, and the phylogenetic relationships between different geographic isolates were investigated.

Materials and methods

Collection of infected post-larvae

Infected PL (24–32 days of age) of *M. rosenbergii* with WTD were collected from affected hatcheries located in Kaohsiung and Pington in southern Taiwan. More than 30 samples of diseased giant freshwater prawns, each consisting of 10–30 individuals were collected from the WTD–affected hatcheries. Healthy animals were obtained from a different hatchery with no record of WTD, and the absence of the disease was further confirmed by histological analysis. The PL were washed in sterile distilled water and stored at −20 °C before processing.

Total RNA extraction

Total RNA was extracted using TRIzol™ reagent (Invitrogen, Carsbad, CA, USA) following the manufacturer's protocol. Briefly, 30–50 mg of muscle from 3–5 PL was homogenized in 950 μL TRIzol™ reagent by power homogenizer. The homogenates were incubated for 5 min at room temperature, and then 0.2 mL chloroform was added. The samples were vigorously shaken for 2–3 min, and then centrifuged at 12 000 ***g*** for 15 min at 4 °C. The aqueous phase from the tube was transferred to a fresh tube. Total RNA was precipitated from the aqueous phase with isopropanol, washed with 75% ethanol and dissolved in 50 μL diethyl pyrocaronate-treated water and stored at −70 °C.

RT-PCR for XSV

Reverse transcription polymerase chain reaction was performed using a Reverse-iT™ one-step RT-PCR kit (ABgene, Surrey, UK), allowing RT and amplification to be carried out in a single reaction tube. The upstream primer XSV-1 5′-CCACGTCTA-GCTGCTGAC-3′ and the downstream primer XSV-772 5′-CGGAATAATGCCTAACCAAT-3′ specific to XSV were designed from sequence data of GenBank accession no.AY247793. The size of the amplified product was 772 bp. The reaction was performed in 50 μL RT-PCR buffer containing 10 pmol of each primer and RNA template, using the following steps: RT at 55 °C for 1 h; denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 40 s; annealing at 52 °C for 40 s and elongation at 72 °C for 1 min, ending with an additional elongation step of 10 min at 72 °C. The amplified product was analysed by electrophoresis on a 1.5% agarose gel.

Cloning and sequence analysis

The RT-PCR products were extracted from agarose gel, and purified using a PCR clean-up system (Promega, Madison, WI, USA). The DNA fragments were ligated into pGEM-T easy vector (Promega) and transformed into *Escherichia coli* DH-5α. The recombinant plasmid DNA was extracted, and automatic sequencing was performed commercially (Protech Technology Enterprise Co., Ltd, Taipei, Taiwan). Sequence analysis was done with other XSV isolates recorded at GenBank (GenBank accession no.DQ174318 for a Chinese strain and GenBank accession no.AY247793 for an Indian strain) using BLAST (Altschul, Madden, Schäffer, Zhang, Zhang, Miller & Lipman 1997) and Clustal W (Thompson,

Higgins & Gibson 1994). The phylogenetic analysis was performed using the neighbour-joining method (Saitou & Nei 1987).

Results

The diseased post-larvae with whitish muscle were collected from different hatcheries located in southern Taiwan. The clinical sign of infected PL was non-transparent muscle in abdominal segments. All 30 WTD affected cases were shown to be infected with XSV virus infection by RT-PCR analysis. However, the samples obtained from the unaffected hatchery were negative for the presence of XSV. As shown in Fig. 1, the 772 bp products were obtained using the primer pair (XSV-1/XSV-772) specific to the genome of XSV virus. The RT-PCR products were purified and cloned into pGEM-T plasmid, and then sequenced. The nucleotide sequence and deduced amino acid of XSV partial genome are shown in Fig. 2 (GenBank accession no.DQ521573). It contained a unique open reading frame with 522 nucleotides and could encode a structural protein with 174 amino acids. Comparisons by Clustal W analysis revealed that the nucleotide sequence of the XSV Taiwan isolate was very similar to those of XSV isolates from China and India (Fig. 3). BLAST analysis revealed 98% and 97% identity of nucleotide sequence of XSV with isolates from China and India, respectively. In addition, the deduced amino acid sequence of the XSV Taiwan isolate showed strong homology with Chinese and Indian isolates. Only one deduced amino acid substitution at position 139 (I → M) was observed which differed from the

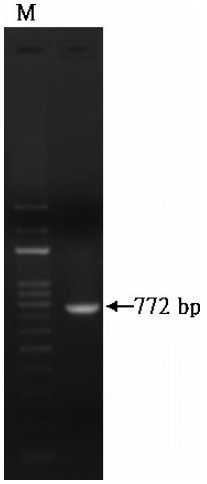

Figure 1 Agarose gel showing RT-PCR products of extra small virus extracted from post-larvae of white tail disease-infected prawn. M: 100 bp DNA ladder marker.

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1    ccacgtctagctgctgacgttaaatgcagccgggtggaaatgcgtattaatattttaac
60   aacatgaataagcgcattaataataatcggagaaccatgagatcacgtaggggacgtggt
        M  N  K  R  I  N  N  N  R  R  T  M  R  S  R  R  G  R  G    19
120  aggacaatgggatctaatctcattccttatgccaactcaccagtccctataccatataca
     R  T  M  G  S  N  L  I  P  Y  A  N  S  P  V  P  I  P  Y  T    39
180  ccacccgttaccccagttaccgtcattggtaatcctcggaaaactacttggattgacatt
     P  P  V  T  P  V  T  V  I  G  N  P  R  K  T  T  W  I  D  I    59
240  gatctttcaagtgaagagtccgggatttacactttgacggttgggtcataccgtaatagg
     D  L  S  S  E  E  S  G  I  Y  T  L  T  V  G  S  Y  R  N  R    79
300  attactaaacttggtccatctaaacctaactttattattgagaaggtcgcagcatatgct
     I  T  K  L  G  P  S  K  P  N  F  I  I  E  K  V  A  A  Y  A    99
360  gcaccaggagattataaggttgttctcaatgactttaaaactggtatacaagtcgttgat
     A  P  G  D  Y  K  V  V  L  N  D  F  K  T  G  I  Q  V  V  D   119
420  gaaggctcttatgctcatagagccgcagcaggtattctttatccacctgctgcacaaatg
     E  G  S  Y  A  H  R  A  A  A  G  I  L  Y  P  P  A  A  Q  M   139
480  ttttacggtatttcagcaacaggcacactcaacactattactaccactgctaaagatcca
     F  Y  G  I  S  A  T  G  T  L  N  T  I  T  T  T  A  K  D  P   159
540  gttccagtggttcgtgctttggtaacatattgggactccgaacagtaatgagcaggttct
     V  P  V  V  R  A  L  V  T  Y  W  D  S  E  Q  *               174
600  atgcttcgaactaaaacaagcaatgagttctagctccgaactaaaacgagccaaactctc
660  gcatctgagtataaatcatgccccatgatcctcgcatcgtatcgtactttgctggagaaa
720  acccttatggccaattgtggcaaggggagacgtattggttaggcattattccg
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Figure 2 Nucleotide sequence and deduced amino acid sequence of extra small virus (XSV) partial genome from Taiwan isolate (GenBank accession no. DQ521573). The numbering for the nucleotide sequences is given on the left and the numbering for the sequences of deduced amino acids is given on the right.

Figure 3 Multiple nucleotide sequence alignment of extra small virus (XSV) partial genome (XSV-Tw = XSV Taiwan isolate) with other geographical XSV isolates (China, GenBank accession no. DQ174318 and India, GenBank accession no. AY247793) using Clustal W. The degree of similarity is illustrated underneath the alignments with a series of consensus symbols. '*' Represents residues in that column identical in all sequences in the alignment.

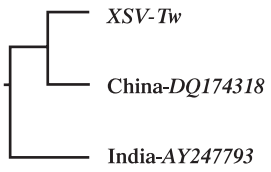

Figure 4 Phylogenetic neighbour-joining tree deduced from analysis nucleotide sequences of partial genome of extra small virus from different geographical isolates.

Chinese isolate. However, there were four changes at positions 49 (S → G), 72 (A → T), 129 (V → A) and 139 (I → M) which occurred in XSV-Taiwan in contrast to the Indian isolate. The nucleotide sequences were used to construct a phylogenetic tree to examine the relationships between the three isolates. The results revealed the XSV-Taiwan isolate was more closely related to the Chinese than the Indian isolate (Fig. 4).

Discussion

The occurrence of WTD in *M. rosenbergii* has previously been reported in Taiwan (Tung *et al.* 1999). That histopathological study revealed dense viral inclusion bodies in the affected muscle of diseased prawns. Furthermore, non-enveloped virions with a mean size of 23 nm were observed in the cytoplasm of infected cells in the infected prawns, and were tentatively named as *Macrobrachium* muscle virus (MMV). However, the genomic characters of MMV were not evaluated, and co-infection with the satellite virus was not demonstrated. The present study confirmed XSV infection associated with WTD of *M. rosenbergii* cultured in Taiwan by RT-PCR.

Reverse transcription-polymerase chain reaction is considered the most sensitive method for detecting *Mr*NV and XSV (Sri Widada *et al.* 2003). Using RT-PCR, it has been found that WTD samples from India are positive for XSV only (Sahul Hameed, Yoganandhan, Sri Widada & Bonami 2004b), but another report from Thailand recorded only *Mr*NV infection. It is hypothesized that failure to detect the dual infections was because single-step RT-PCR protocols were used (Yoganandhan *et al.* 2006). However, both *Mr*NV and XSV were present together in all positive samples in our study (data not shown). These apparently contradictory results may be because different RT-PCR reaction conditions for XSV were used. For example, the number of cycles, annealing temperatures and the primer pair for RT-PCR used in our study differed from the previous reports. The role of the two viruses in the development of WTD is unknown. Although it is hypothesized that XSV constitutes a new species of satellite virus, the pathogenicity of this virus and its relationship to WTD merits further study.

In the present study, a primer pair specific to XSV was designed based on sequence data of XSV isolated from India (GenBank accession no. AY247793). Using this primer pair (XSV-1/XSV-772), the 772 bp amplicon was successfully produced by RT-PCR. Sequence analysis of the partial genome demonstrated it contained 522 nucleotides that could encode a single structural protein with 174 amino acids. The results are similar to the previous reports from China and India. The deduced amino acid sequence of a 490-bp DNA fragment (GenBank accession no. DQ189991) of Thai XSV showed 100% identity to Indian isolates (Yoganandhan *et al.* 2006). Furthermore, only one and four deduced amino acid substitutions were observed when compared with the isolates from China and India, respectively. The conserved sequences observed from different geographical regions indicate that XSV isolates are closely related. A phylogenetic tree of the nucleotide sequences of XSV also demonstrated the close relationships between these isolates. The results suggest that geographical proximity of the areas from which isolates were obtained may influence their identity.

Based on a previous study, XSV possesses the characteristics of satellite viruses such as those described in the plant kingdom (Sri Widada & Bonami 2004). For example, the XSV genome only encodes structural protein CP-17, but does not possess the gene coding for viral RNA polymerase. It is hypothesized that XSV may depend on the RNA-dependent RNA polymerase of *Mr*NV for its replication. However, comparison of the deduced amino acid sequences of CP-17 of XSV with known structural proteins of satellite viruses showed that XSV was not affiliated to known virus families. Further study is needed to investigate the real role of structural protein CP-17 of XSV in WTD-infected *M. rosenbergii*.

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