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RT-PCR and sequence analysis of *Macrobrachium rosenbergii* nodavirus: Indian isolate

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Abstract

White tail disease is a recently reported disease of giant freshwater prawn, *Macrobrachium rosenbergii* associated with *M. rosenbergii* nodavirus (*Mr*NV). The present study is the first report on RT-PCR and sequence analysis of *Mr*NV of an Indian isolate confirming the nodavirus infection in *M. rosenbergii* from India. Total RNA was extracted and the cDNA was synthesized from the infected post larvae collected from a hatchery located in Chennai, India. An 850-bp amplified product could be obtained by RT-PCR. The nucleotide sequence analysis of this 850-bp segment showed 98% nucleotide and 99% amino acid sequence identity with the reported sequence of an *Mr*NV isolate from the West Indies.

Keywords: *Macrobrachium rosenbergii* nodavirus; RT-PCR; Sequence analysis

1. Introduction

Occurrence of white tail disease (WTD) in hatchery-reared *Macrobrachium rosenbergii* was first reported from the West Indies (Arcier et al., 1999). The proposed causative agent was identified as an icosahedral, non-enveloped virus of 26–27 nm called *M. rosenbergii* nodavirus (*Mr*NV). The genome of this virus contains two pieces of ssRNA, of 3.0 kb (RNA-1) and 1.2 kb (RNA-2) (Qian et al., 2003). Along with this virus, extra small virus particles (XSV) of 14–16 nm have been found in whitish muscle disease cases in prawns from China. XSV has been proposed to be a satellite virus dependent on the larger *Mr*NV particles (Qian et al., 2003). Actual role of XSV and its interactions with *Mr*NV in WTD is still unclear as some positively diagnosed *Mr*NV samples were found negative for XSV (Widada et al., 2004). In addition to *Mr*NV, two other viruses, parvo like virus (Anderson et al., 1990) and *Macrobrachium* muscle virus (Tung et al., 1999) have been reported to affect this species. The occurrence of WTD has also been reported from Taiwan (Tung et al., 1999) and China (Qian et al., 2003), but there are no published reports of it from any other part of the world.

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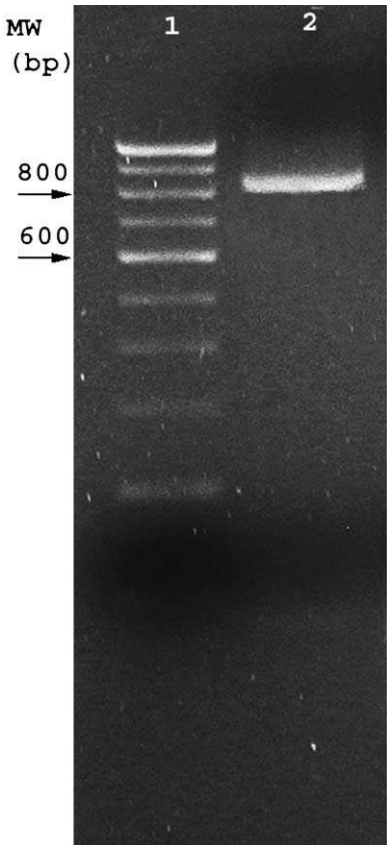

Fig. 1. RT-PCR product of *Mr*NV (Indian isolate). Lane 1: 100 bp marker. Lane 2: PCR product of 850 bp obtained by RT-PCR.

Mortalities due to WTD in *M. rosenbergii* have been recently noticed in India from hatcheries located at Nellore, Andrapradesh and Chennai, Tamil Nadu. The present work is the first report confirming the presence of *Mr*NV in *M. rosenbergii* in India by reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing of the PCR-amplified product.

2. Materials and methods

2.1. Samples

Post larvae (PL) of *M. rosenbergii* were obtained from a hatchery in Chennai, India. Mortality was recorded in the 8- to 10-day-old PL showing empty guts and whitish opaque tails.

2.2. RT-PCR

Total RNA was extracted from the infected PL using a NucleoSpin RNA II kit (Macherey-Nagel). The cDNA was synthesized and RT-PCR was carried out using a

```
   1 atggaagtccgccgattaattgaagcatttgtgaagaatgaaccg
     M  E  V  R  R  L  I  E  A  F  V  K  N  E  P     15
  46 accaataaatctggtcgcataatatcgtctttcgcggactcaaga
     T  N  K  S  G  R  I  I  S  S  F  A  D  S  R     30
  91 ttcttgttgaagttttccacatatacgcttgcctttcgagatgaa
     F  L  L  K  F  S  T  Y  T  L  A  F  R  D  E     45
 136 gtattacatgctgaacataatcgacattggttttgtcctggattg
     V  L  H  A  E  H  N  R  H  W  F  C  P  G  L     60
 181 acacctaatgagatagcagataaagtctgcgactatgttcgtggt
     T  P  N  E  I  A  D  K  V  C  D  Y  V  R  G     75
 226 gttgcgacacctgcagaaggtgattttagcaactttgacggaagg
     V  A  T  P  A  E  G  D  F  S  N  F  D  G  R     90
 271 gtatctgcttggtgtcaagagaacgtgatgaatgcggtttaccac
     V  S  A  W  C  Q  E  N  V  M  N  A  V  Y  H    105
 316 agatggtttaaccgtaagttttctaaggaattgcagaagtataca
     R  W  F  N  R  K  F  S  K  E  L  Q  K  Y  T    120
 361 tcaatgttggttagttgcccagctcgagctaagcgttttggtttc
     S  M  L  V  S  C  P  A  R  A  K  R  F  G  F    135
 406 cagtatgaaccgggagtgggggttaagagtggtagtccaaccacc
     Q  Y  E  P  G  V  G  V  K  S  G  S  P  T  T    150
 451 tgtgaccttaattcagttctaaataacttttactcaatacgcagca
     C  D  L  N  S  V  L  N  N  F  T  Q  Y  A  A    165
 496 gttaggctgactaaaccagacctctcaccacaagaagcctttgaa
     V  R  L  T  K  P  D  L  S  P  Q  E  A  F  E    180
 541 caaactggcttaagtttcggcgacgattcactatttgacaagcaa
     Q  T  G  L  S  F  G  D  D  S  L  F  D  K  Q    195
 586 tatcagctcagatggaattacgtcgtcgaacaacttggtatggaa
     Y  Q  L  R  W  N  Y  V  V  E  Q  L  G  M  E    210
 631 ctcaaggttgaacccttcgaccccaataacggtgtgacttttctt
     L  K  V  E  P  F  D  P  N  N  G  V  T  F  L    225
 676 gctcgtgtttttcctgatccttatagtacaaatactagttttcag
     A  R  V  F  P  D  P  Y  S  T  N  T  S  F  Q    240
 721 gatccactaagaacgtgg
     D  P  L  R  T  W
```

Fig. 2. Sequence of Indian *Mr*NV. The numbering for the nucleotide sequence is given on left side and the numbering for the corresponding amino acid residues are given on the right side.

RobusT II RT-PCR kit (Finnzymes). RT was carried out at 42 °C for 60 min. The PCR cycle consisted of initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 92 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min with a final cycle of 10 min extension at 72 °C. The upstream primer 5′CCACGTTCTTAGTGGATCCT 3′ and the downstream primer 5′CGTCCGCCTGGTAGTTCC 3′ (30 pmol each) specific to RNA-1 were used as reported by Widada et al. (2003). The PCR product was purified by gel extraction using a NucleoSpin Extract kit (Macherey-Nagel).

2.3. Sequencing and sequence analysis

Sequencing of the gel-purified PCR product was carried out using both upstream and downstream primers by Bangalore Genei, India. The sequence was aligned and edited using BioEdit Sequence Alignment Editor. Sequence analysis was done using BLAST

```
Epinephilus NNV          DEFTMMVKAFQKKEAYNAPNYPRNISTVPHT-QNVKLSSYTYAFKASVLQHVP---WYMP 56
Sevenband grouper NNV    DEFTMMVKAFQKKEAYNAPNYPRNISTVPHT-QNVKLSSYTYAFKASVLQHVP---WYMP 56
Redspotted grouper NNV   DEFTMMVKAFQKKEAYNAPNYPRNISTVPHT-QNVKLSSYTYAFKASVLQHVP---WYMP 56
Japanese flounder NNV    GEFTMIVKAFQKKEAYNAANYPRNISTVPHT-QNVKLSSFTYAFKASILQHVP---WYMP 56
Atlantic halibut NNV     DEFTMIVKAFQKKEAYNAPNYPRNISTVPHN-QNVKLSSYTYAFKASTLQHVP---WYMP 56
Striped jack NNV         DEFTMIVKAFQKKEAYNAPNYPRNISTVPHT-QNVKLSSYTYAFKEAVLQHVP---WYMP 56
MrNV (West Indies)       MEVRRLIEAFVKNEPTNKS--GRIISSFADSRFLLKFSTYTLAFRDEVLHAEHNRHWFCP 58
MrNV (China)             MEVRRLIEAFVKNEPTNKS--GRIISSFADSRFLLKFSTYTLAFRDEVLHAEHNQHWFCP 58
MrNV (India)             MEVRRLIEAFVKNEPTNKS--GRIISSFADSRFLLKFSTYTLAFRDEVLHAEHNRHWFCP 58
                          *.  :::** *:*. * .   * **:....   :*:*::* **:   *:      *: *

Epinephilus NNV          THTPAEIADAVQNLAASSTELVETDYSKFDGTFLRFMRECVEFAIYKRWVHLDHLPELTT 116
Sevenband grouper NNV    THTPAEIADAVQNLAASSTELVETDYSKFDGTFLRFMRECVEFAIYKRWVHLDHLPELTT 116
Redspotted grouper NNV   THTPAEIADAVQNLAASSTELVETDYSKFDGTFLRFMRECVEFAIYKRWVHLDHLPELTT 116
Japanese flounder NNV    THTPAEIADAVQNLAASSTELVETDYSKFDGTFLRFMRGSVEFAIYKRWVHLDHLPELTT 116
Atlantic halibut NNV     THTPAEIADAVQNLAASTTELVETDYSKFDGTFLRFMRECVEFAIYKRWVHLDHLAELSQ 116
Striped jack NNV         THTPAEIAEAVQSLAASSTELVETDYSKFDGTFLRFMRENVEFAIYKRWVHLDHLTELST 116
MrNV (West Indies)       GLTPNEIADKVCDYVRGVATPAEGDFSNFDGRVSAWCQENVMNAVYHRWFNRKFSKELQK 118
MrNV (China)             GLTPNEIADKVCDYVRGVATPAEGDFSNFDGRVSAWCQENVMNAVYHRWFNRKFSKELQK 118
MrNV (India)             GLTPNEIADKVCDYVRGVATPAEGDFSNFDGRVSAWCQENVMNAVYHRWFNRKFSKELQK 118
                           ** ***: * . . . :  .* *:*:*** .  : :  *  *:*:**.: ..  **

Epinephilus NNV          LLANEIQAPAVTR-LGIKYDPDCSRLSGSALTTDGNSIANAFVSYLAGRMAG--MDDDEA 173
Sevenband grouper NNV    LLANEIQAPAVTR-LGIKYDPDCSRLSGSALTTDGNSIANAFVSYLAGRMAG--MDDDEA 173
Redspotted grouper NNV   LLANEIQAPAVTR-LGIKYDPDCSRLSGSALTTDGNSIANAFVSYLAGRMAG--MDDDEA 173
Japanese flounder NNV    LLANEIQAPAVTR-LGIKYDPDCSRLSGSALTTDGNSIANAFVSYLAGRMAG--MDDDEA 173
Atlantic halibut NNV     LLANELQAPAVTR-LGLKYDPDCSRLSGSALTTDGNSIANAFVSYLANRLAG--MDDDEA 173
Striped jack NNV         LLGNELQAPAVTR-LGIKYDPDCSRLSGSALTTDGNSIANAFVSYLAGRQAG--MDDDEA 173
MrNV (West Indies)       YTSMLVSCPARAKRFGFQYEPGVGVKSGSPTTCDLNSVLNNFTQYAAVRLTKPDLSPQEA 178
MrNV (China)             YTSMLVSCPARAKRFGFQYEPGVGVKSGSPTTCDLNSVLNNFTQYAAVRLTKPDLSPQEA 178
MrNV (India)             YTSMLVSCPARAKRFGFQYEPGVGVKSGSPTTCDLNSVLNNFTQYAAVRLTKPDLSPQEA 178
                           .  :..** :: :*::*:*. .  ***. * * **: * *..* * * :   :. :**

Epinephilus NNV          WSWIGIVYGDDGLRSGNVSNELLTNTASSLGFDLKIVNRAPRGSPVTFLSRVYLDPWSSP 233
Sevenband grouper NNV    WSWIGIVYGDDGLRSGNVSNELLTNTASSLGFDLKIVNRAPRGSPVTFLSRVYLDPWSSP 233
Redspotted grouper NNV   WSWIGIVYGDDGLRSGNVSNELLTNTASSLGFDLKIVNRAPRGSPVTFLSRVYLDPWSSP 233
Japanese flounder NNV    WTWIGIVYGDDGLRSGNVSNGLLTET---------------------------------- 199
Atlantic halibut NNV     WSWIGIVYGDDGLRSGNVSDTLLTDTASSLGFDLKIVNRAPRGSPVTFLARVYLDPWSSP 233
Striped jack NNV         WTWIGIVYGDDGLRSGNVSDALLSKTASSLGFDLKIVNRAPRGSPVTFLSRVYLDPWSSP 233
MrNV (West Indies)       FEQTGLSFGDDSLFD-KQYQLRWNYVVEQLGMELKVEPFDPSNG-VTFLARVFPDPYSTN 236
MrNV (China)             FEQTGLSFGDDSLFD-KQYQLRWNYVVEQLGMELKVEPFDPSNG-VTFLARVFPDPYSTN 236
MrNV (India)             FEQTGLSFGDDSLFD-KQYQLRWNYVVEQLGMELKVEPFDPNNG-VTFLARVFPDPYSTN 236
                         :   *: :***.* . :  :   . .

Epinephilus NNV          ASVQSPLRTLLKLHTTCDTQSEIDDIGWAKTQAYLVTDSKTPFIGHWCRAYQRNCTARVV 293
Sevenband grouper NNV    ASVQSPLRTLLKLHTTCDTQSEIDDIGWAKTQAYLVTDSKTPFIGHWCRAYQRNCTARVV 293
Redspotted grouper NNV   ASVQSPLRTLLKLHTTCDTQSEIDDIGWAKTQAYLVTDSKTPFIGHWCRAYQRNCTARVV 293
Japanese flounder NNV    ------------------------------------------------------------
Atlantic halibut NNV     ASVQSPFRTLLKLHTTCDTQSEIEDIGWAKTQAYLVTDCKTPFIGHWCRAYQRNCTARVV 293
Striped jack NNV         ASVQSPLRTLLKLHTTCDTQSDIEDVGWAKTQAYLVTDCLTPFIGHWCRAYQRNCTARVV 293
MrNV (West Indies)       TSFQDPLRTWRKLNMTSRTCVPVESAALDRVSGYLVTDKYSPVTSEYCHMIERCYMNTAE 296
MrNV (China)             TSFQDPLRTW---------------------------------------------------- 246
MrNV (India)             TSFQDPLRTW---------------------------------------------------- 246
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Fig. 3. Multiple sequence alignment of *Mr*NV (Indian isolate) with *Mr*NV (West Indies and Chinese isolate) and other fishnoda viruses using ClustalW. GenBank accession nos. are *Epinephilus* NNV (AAO73138); Sevenband grouper NNV (AAQ90061); Redspotted grouper NNV(AAQ88211); Japanese flounder NNV (BAB03598); Striped jack NNV (NP_599247); Atlantic halibut NNV(Q9DIC5); *Mr*NV (West Indies) (AAO60068); *Mr*NV (Chinese) (AAQ54758). NNV represents nervous necrosis virus. The running total number of amino acids is shown on the right. The degree of similarity is illustrated underneath the alignments with a series of consensus symbols. '*' represents residues in that column that are identical in all sequences in the alignment. '.' represents semi conserved substitutions. ':' represents conserved substitutions. Gaps are represented by '—' symbol.

(http://www.ncbi.nlm.nih.gov/BLAST) and ClustalW (http://www.ebi.ac.uk/cgi-bin/clustalW).

3. Results and discussion

Until recently, disease detection of *Mr*NV was based on histological signs and transmission electron microscopy (TEM). A more sensitive detection method by sandwich enzyme linked immunosorbent assay (S-ELISA) was developed by Romestand and Bonami (2003). It was found to have high sensitivity when compared to TEM. However, the availability of antibodies is a major drawback to use this detection method. Much more sensitive and easier genome-based detection methods by dot-blot hybridization, in situ hybridization and RT-PCR for *Mr*NV have recently been reported (Widada et al., 2003). Dot-blot and RT-PCR have also been developed for detection of XSV (Widada et al., 2004). In the present study, primers specific to RNA-1 of *Mr*NV as reported by Widada et al. (2003) were used (i.e., 1A775 and 1B690) for RT-PCR. This set of primers was designed based on sequence data of the *Mr*NV genome (GenBank accession no. AY222839) and was chosen because it had been shown to successfully detect *Mr*NV isolates from both the West Indies and China with a sensitivity of 35 fg of RNA template. Using this set of primers reported to amplify a 0.85-kb PCR product, two PCR bands of 850 bp and 750 bp were visualized by agarose gel electrophoresis. The annealing temperature was raised to 58 °C to eliminate the nonspecific 750-bp PCR product, but this modification was not successful. The expected 850-bp PCR product was extracted from the gel and purified. The purified product was then reamplified with the upstream and downstream primers and the resulting PCR product was sequenced (Fig. 1).

The nucleotide sequence obtained from the 850-bp PCR product and its corresponding deduced amino acid sequence are shown in Fig. 2. BLAST analysis of a 738-bp portion of the nucleotide sequence of our *Mr*NV Indian isolate showed 98% and 95% identity to nucleotide sequences of *M. rosenbergii* nodavirus, RNA segment-1 at GenBank (i.e., accession no. AY222839 and AY231436, respectively). These comparisons confirmed that the PL of Indian *M. rosenbergii* were infected with *Mr*NV. The sequence corresponded to the RNA directed RNA polymerase gene of *Mr*NV. Full-length sequencing of Indian *Mr*NV is needed for virus strain identification and sequence comparison with other strains isolated from the West Indies and China.

BLAST analysis of 246 deduced amino acid residues of Indian *Mr*NV showed very high sequence identity (99%) to those of *M. rosenbergii* nodavirus at GenBank (accession no. AAO60068, AAQ54758). Sequence identity in the range of 33–35% was also observed for the other fish nodaviruses.

ClustalW analysis of 246 deduced amino acid residues of Indian *Mr*NV with West Indian and Chinese *Mr*NV and other fish nodavirus showed identical, conserved and semiconserved amino acids at various positions (Fig. 3). A total of 60 amino acid residues were found to be identical in all sequences. This sequence conservation is indicative that these viruses are closely related and may have a common ancestor. More detailed genomic analysis and full-length sequencing of Indian *Mr*NV are necessary to study the etiology and epidemiology of this disease. Further investigations are also required to find out whether XSV particles are also associated in WTD cases in India.

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