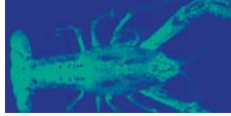
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Cloning, expression and sequence analysis of *Macrobrachium rosenbergii* nodavirus genes: Indian isolate

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Abstract

RNA-dependent RNA polymerase (RdRp), B2 and capsid genes of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) of Indian isolate were polymerase chain reaction amplified, cloned and sequenced. Expression of the *Mr*NV fusion recombinant proteins of RdRp (44.5 kDa), B2 (32.2 kDa) and capsid (58.4 kDa) was confirmed by Western blot analysis using anti-His mouse monoclonal antibodies. Polyclonal antibodies specific to purified recombinant *Mr*NV capsid protein showed specificity against the capsid protein by Western blot. The protein sequence analysis of the partial RdRp gene of *Mr*NV revealed the signature sequence along with the conserved core residues of the catalytic domain and indicated the presence of active sites, metal ion-binding site and nucleic acid-binding site residues. The Indian isolate of *Mr*NV showed high RdRp and capsid gene sequence homology with the other *Mr*NV geographical isolates. However, the Belize isolate was found to be the most distinct among the different geographical prawn nodavirus isolates due to the host specificity. Secondary structure prediction analysis of the *Mr*NV capsid predicted it to be a DNA-binding protein consisting of α helix (22.91%), extended strand (24.80%), β turn (5.39%) and random coil (46.90%) regions.

Keywords: *Macrobrachium rosenbergii* nodavirus, RNA-dependent RNA polymerase (RdRp), B2, capsid, recombinant proteins

Introduction

Incidence of white tail disease (WTD) in *Macrobrachium rosenbergii* has been reported from French West Indies (Arcier, Herman, Lightner, Redman, Mari & Bonami 1999), China (Qian, Shi, Zhang, Cao, Liu, Li, Xie, Cambournac & Bonami 2003), India (Sahul Hameed, Yoganandhan, Sri Widada & Bonami 2004; Shekhar, Azad & Jithendran 2006), Taiwan (Hsieh, Wu, Tung, Tu, Lo, Chang, Chen, Hsieh & Tsai 2006), Thailand (Yoganandhan, Leartvibhas, Sriwongpuk & Limsuwan 2006) and very recently from Australia (Owens, La Fauce, Juntunen, Hayakijkosol & Zeng 2009). The causative agents of the disease have been identified as large virus *M. rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV). *Macrobrachium rosenbergii* nodavirus is an icosahedral, non-enveloped virus of 26–27 nm size. It contains two pieces of ssRNA, of 3.0 kb (RNA-1) and 1.2 kb (RNA-2) (Qian *et al.* 2003). RNA-1 of *Mr*NV is suggested to encode for RNA-dependent RNA polymerase (RdRp), whereas the capsid, which is composed of a single polypeptide of 43 kDa, is encoded by RNA-2 of *Mr*NV (Bonami, Shi, Qian & Sri Widada 2005). In addition, a subgenomic RNA transcribed from the 3′ end of RNA1, termed RNA3, encoding a protein called B2 has been reported in betanodaviruses (Sommerset & Nerland 2004), which is suggested to play a role as an RNA interference antagonist (Fenner, Thiagarajan, Chua & Kwang 2006). The sequence of B2 gene has been reported for *Mr*NV (Sri Widada, Durand, Cambournac, Qian, Shi, Dejonghe, Richard, & Bonami 2003) indicating the presence of putative B2 gene in *Mr*NV.

The smaller virus, XSV is an icosahedral, non-enveloped virus of 14–16 nm size and possesses a linear ssRNA genome of 0.9 kb. Because XSV does not possess gene encoding for RdRp (Sri Widada & Bonami 2004), it is suggested to be a satellite virus, which is

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dependent on *Mr*NV for its replication (Qian *et al*. 2003; Bonami *et al*. 2005). However, the exact relationship between the two viruses is still not clear (Zhang, Wang , Yuan, Li, Zhang, Bonami & Shi 2006).

In the present study cloning, expression of viral recombinant proteins and sequence analysis have been carried out to characterize the *Mr*NV of Indian isolate. The sequence analysis between different viral geographical isolates, indicated host-specific sequence variation in the viral capsid.

Materials and methods

Amplification and cloning of *Mr*NV RdRp, B2 and capsid genes

The polymerase chain reaction (PCR) product of *Mr*NV RdRp gene (859 bp) was obtained in our earlier study (Shekhar *et al*. 2006) using the upstream primer 5′CCACGTTCTTAGTGGATCCT3′ and the downstream primer 5′CGTCCGCCTGGTAGTTCC3′ specific to RNA-1 as reported by Sri Widada *et al* (2003). The PCR product was sequenced and the sequence has now been assigned GenBank accession number DQ146969. This PCR product in the present study was reamplified with a new set of internal primers containing restriction sites for cloning. The upstream primer 5′CGGCCATGGAAGTCCGCCGA3′ containing *Nco*I restriction site and the downstream primer 5′ GCCAAGCTTTTACCACGTTCTTAG 3′ containing *Hind*III restriction site were designed to obtain a 738 bp PCR product (The restriction sites are underlined in the primer sequences). 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 extention at 72 °C. The PCR product was gel extracted using a QIAquick gel extraction kit (Qiagen, Hilden, Germany) and restriction digestion was carried out with *Nco*I and *Hind*III restriction enzymes. The PCR product was then ligated to pET32a (+) vector (Novagen, Darmstadt, Germany) linearized with *Nco*I and *Hind*III restriction enzymes. The ligated mixture was transformed into competent DH5α cells. Plasmids were isolated from the transformed colonies followed by restriction enzyme analysis with *Nco*I and *Hind*III restriction enzymes to confirm the clones for the presence of the insert.

Polymerase chain reaction amplification for *Mr*NV B2 gene was carried out by designing the primers based on the GenBank accession number NC_005094. Amplification to obtain 402 bp PCR product was carried out using the upstream primer 5′ATGCAGTGGACGAACGTCAAT3′ and the downstream primer 5′TTACCACGTTATGAGGTCGC3′.

Full-length PCR amplification for *Mr*NV capsid gene was carried out by designing the primers specific to RNA-2, based on the GenBank accession number NC_005095. Amplification to obtain 1116 bp PCR product was carried out using the upstream primer 5′ATGGCTAGAGGTAAACAAAATTCTAA3′ and the downstream primer 5′CTAGTTATTGCCGACGATAGCTCTGA3′. The primers for *Mr*NV B2 and capsid genes were modified to contain *Nco*I restriction site in the upstream primer and *Hind*III restriction site in the downstream primer for cloning. The PCR cycle conditions and the cloning procedures for *Mr*NV B2 and capsid genes were same as followed for RdRp gene of *Mr*NV.

Dot blot hybridization

The 859 bp PCR product of *Mr*NV RdRp gene was used for generating probe by digoxigenin (DIG) random primed DNA labelling using a DIG DNA labelling and detection kit (Roche, Mannheim, Germany) following the manufacturer's protocol. One microlitre of recombinant plasmids-containing RdRp gene was isolated from transformed cells and spotted on nylon membrane for dot blot analysis. One microlitre of 859 PCR product of *Mr*NV and pET32a (+) plasmid DNA were also spotted on the same nylon membrane as a positive and negative control respectively. The DNA spots on the membrane were fixed by cross linking with UV light. Prehybridization, hybridization and detection with NBT/BCIP for the dot blot experiments were performed as per the protocol described in the kit.

Expression and purification of recombinant *Mr*NV RdRp, B2 and capsid proteins

Plasmids isolated from the positive clones of DH5α cells containing the gene inserts for *Mr*NV RdRp, B2 and capsid were transformed into competent BL21(DE3) pLysS cells. The positive clones were initially screened by colony PCR followed by *Nco*I and *Hind*III restriction enzyme analysis to confirm the presence of the viral gene inserts in the vector DNA. The positive colonies were grown overnight in Luria Bertani (LB) broth containing 100 μg mL^{-1} ampicillin. The overnight culture was inoculated (1:10) a dilution of fresh LB broth containing

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100 μg mL $^{-1}$ ampicillin and incubated with shaking till the optical density (OD_{600}) reached 0.4 to obtain the cells at mid log phase. Isopropyl β-D-thiogalactopyranoside (IPTG) was added at 1 mM final concentration and the cells were harvested 4 h post IPTG induction. The harvested cells were lysed with a bacterial cell lysis reagent (Bangalore Genei, Bangalore, India) by incubating the cell suspension at room temperature with shaking for 20 min followed by sonication on ice with three 5 s pulses. Expression of recombinant protein was analysed by SDS-PAGE, which included uninduced bacterial culture as a negative control.

pET32a (+) vector has six histidine residues, which enable to express and purify recombinant fusion protein carrying histidine tag (His.Tag). Purification of the *Mr*NV recombinant proteins was carried out using ProBond purification system (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, IPTG-induced bacterial cells were harvested from 50 mL of culture. The cell pellet was resuspended in 8 mL of guanidium lysis buffer (6 M guanidine HCL, 20 mM sodium phosphate, pH 7.8, 500 mM NaCl) with gentle rocking at room temperature for 10 min. The bacterial cell suspension was sonicated on ice with three 5 s pulses followed by centrifugation at 11 000 *g* for 15 min. The recombinant proteins were purified by passing the supernatant of the bacterial lysate through a ProBond resin column.

Polyclonal antibody production

The purified recombinant *Mr*NV capsid protein (58.4 kDa) protein was used for immunization in New Zealand white rabbit for raising polyclonal hyper immune serum. The rabbits were first immunized with 300 μg of recombinant protein emulsified with Freund's complete adjuvant, followed by two booster doses of 200 μg each of protein emulsified with Freund's incomplete adjuvant at 30 and 45 days. Blood was collected 20 days after the last booster dose and the serum was separated. The immunoglobulin G fraction was purified by protein A affinity chromatography.

Western blot

Western blot analyses of *Mr*NV RdRp, B2 and capsid recombinant proteins were carried out by the standard procedure using a semidry western blot apparatus (Hoeffer, Holliston, MA, USA). The recombinant fusion proteins of *Mr*NV (RdRp, B2 and capsid) expressed from the pET32a (+) expression vector were detected using the Western Breeze chromogenic western blot immunodetection kit, with alkaline phosphatase-conjugated anti-His mouse monoclonal antibodies (1:10 000 dilution) (Invitrogen). The membranes were developed using BCIP/NBT as a substrate. Western blot was also carried out to check the specificity of the polyclonal antibodies raised against recombinant *Mr*NV capsid protein. The membranes transferred with *Mr*NV capsid protein were incubated with anti *Mr*NV capsid primary antibodies (1:20 000 dilution) followed by horseradish peroxidase (HRP)-conjugated mouse anti-rabbit secondary antibody (Bangalore Genei) at 1:20 000 dilution. The membranes were developed using TMB as a substrate.

Sequence analysis

Nucleotide and amino acid sequence analysis and phylogenetic trees were constructed using Advanced GENEBEE CLUSTALW 1.83 (http://www.genebee.msu.ru/clustal/advanced.html). PREDICTPROTEIN (http://www.predictprotein.org) and SOPMA, a web-based software, was used for the prediction of protein secondary structure (http://npsa-pbil.ibcp.fr). Conserved domain database search was carried out using RPS – BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi).

Results and discussion

Positive clones of the transformed DH5α cells containing recombinant plasmids of pET32a (+) with *Mr*NV gene inserts, on PCR amplification revealed amplified products in the expected size for RdRp (738 bp), B2 (402 bp) and capsid genes (1116 bp) as shown in Fig. 1. These clones were further confirmed by *Nco*I and *Hind*III restriction enzyme analysis. The recombinant plasmids released the respective gene inserts of *Mr*NV as shown in Fig. 2. Dot blot hybridization using DIG DNA labelling and detection kit, could successfully detect positive clones containing recombinant plasmids of pET32a (+) with *Mr*NV RdRp gene insert (Fig. 3).

In the present study, *Mr*NV RdRp, B2 and capsid genes have been expressed as recombinant fusion proteins in *Escherichia coli*. Expression of *Mr*NV recombinant proteins of RdRp (44.5 kDa), capsid

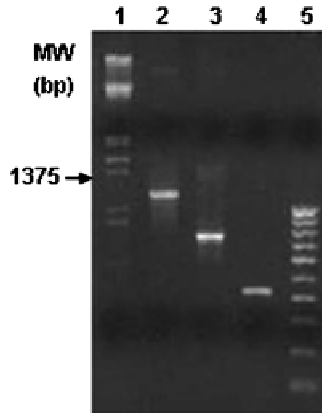

Figure 1 PCR-amplified products of *Mr*NV capsid, RdRp and B2 genes. Lane 1. λ DNA *Eco*RI/*Hind*III molecular weight marker. Lane 2. *Mr*NV capsid (1116 bp). Lane 3. *Mr*NV RdRp (738 bp). Lane 4. *Mr*NV B2 (402 bp). Lane 5. 100 bp molecular weight marker. PCR, polymerase chain reaction; *Mr*NV, *Macrobrachium rosenbergii* nodavirus; RdRp, RNA-dependent RNA polymerase.

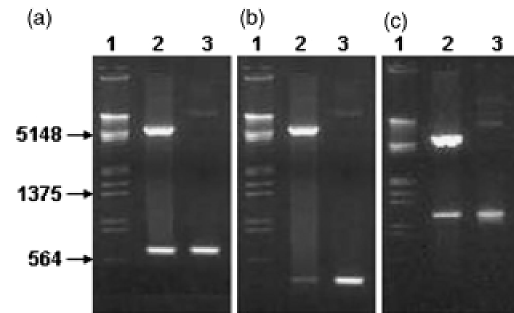

Figure 2 *Nco*I and *Hind*III restriction digestion of pET32a (+) recombinant plasmids containing *Mr*NV gene inserts. (A) Lane 1. λ DNA *Eco*RI/*Hind*III molecular weight marker. Lane 2. *Mr*NV gene insert RdRp (738 bp). Lane 3. PCR product *Mr*NV RdRp (738 bp). (B) Lane 1. λ DNA *Eco*RI/*Hind*III molecular weight marker. Lane 2. *Mr*NV gene insert B2 (402 bp). Lane 3. PCR product *Mr*NV B2 (402 bp). (C) Lane 1. λ DNA *Eco*RI/*Hind*III molecular weight marker. Lane 2. *Mr*NV gene insert capsid (1116 bp). Lane 3. PCR product *Mr*NV capsid (1116 bp). PCR, polymerase chain reaction; *Mr*NV, *Macrobrachium rosenbergii* nodavirus; RdRp, RNA-dependent RNA polymerase.

(58.4 kDa) and B2 (32.2 kDa) genes was analysed by SDS-PAGE, which included uninduced bacterial culture as negative controls Purification of recombinant proteins carried out using affinity column revealed a single protein band in the expected size range

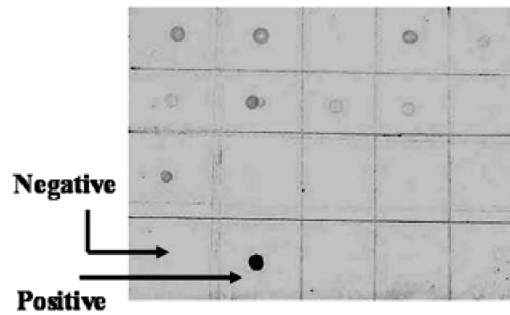

Figure 3 Screening of recombinant plasmids containing *Mr*NV RdRp gene by dot blot hybridization. The PCR amplified product (859 bp) of MrNV used as a positive control and pET32a (+) plasmid DNA as a negative control are indicated. PCR, polymerase chain reaction; *Mr*NV, *Macrobrachium rosenbergii* nodavirus; RdRp, RNA-dependent RNA polymerase.

(Fig. 4a–c). Western blot using anti-His mouse monoclonal antibodies confirmed the expression of viral recombinant proteins, as shown in Fig. 5. The polyclonal antibodies raised against *Mr*NV capsid revealed specific immunoreactivity with the expressed and the purified recombinant protein (Fig. 6)

Viral RdRp plays an important role in RNA genome replication, which is an essential step in the pathogenesis of many RNA viruses. Not much information is available regarding the mode of infection and virus replication in *M. rosenbergii* infected with *Mr*NV. *Macrobrachium rosenbergii* nodavirus infection in *M. rosenbergii* was shown to be located in striated muscle tissues by *in situ* hybridization technique (Sri Widada *et al.* 2003) and in connective tissue by transmission electron microscopy (Qian *et al.* 2003). To our knowledge, a sandwich enzyme-linked immunosorbent assay (Romestand & Bonami 2003) and monoclonal antibodies-based triple antibody sandwich enzyme-linked immunosorbent assay (Qian, Liu, Jianxiang &Yu 2006) are the only immuno-based techniques, which have been developed to diagnose WTD. In both the techniques, antibodies were raised against purified suspension of virus. Expression of recombinant RdRp, B2 and capsid proteins of *Mr*NV in *E. coli* system is an easy and inexpensive technique for producing specific antigen, which can be subsequently purified and used for raising antiserum. With the availability of antiserum against *Mr*NV proteins, it will be possible to carry out histopathological investigations for in depth examination of *Mr*NV infection in different tissues of *M. rosenbergii*. The use of antiserum and DIG probe to study shrimp viral pathogen-

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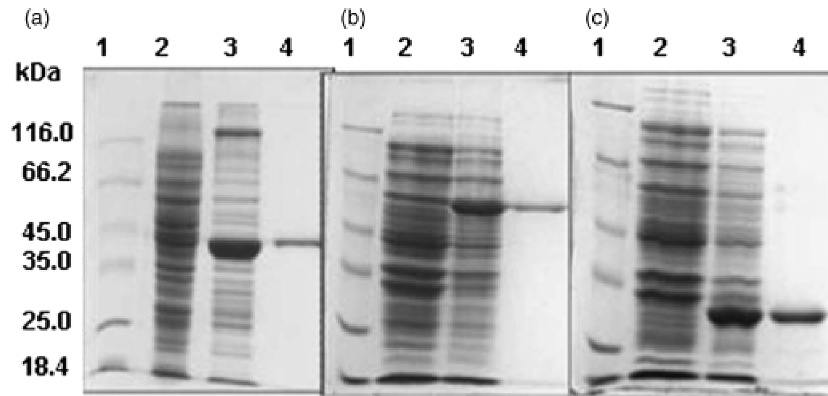

Figure 4 Expression of *Mr*NV RdRp, capsid and B2 recombinant proteins in *Escherichia coli* strain BL21(DE3)pLysS. (a) Lane 1. Protein molecular weight marker. Lane 2. Uninduced cells (negative control). Lane 3. IPTG-induced cells showing protein expression for *Mr*NV RdRp. Lane 4. Purified *Mr*NV RdRp recombinant protein (44.55 kDa). (b) Lane 1. Protein molecular weight marker. Lane 2. Uninduced cells (negative control). Lane 3. IPTG-induced cells showing protein expression for *Mr*NV capsid. Lane 4. Purified *Mr*NV capsid recombinant protein (58.4 kDa). (c) Lane 1. Protein molecular weight marker. Lane 2. Uninduced cells (negative control). Lane 3. IPTG-induced cells showing protein expression for *Mr*NV B2. Lane 4. Purified *Mr*NV B2 recombinant protein (32.2 kDa). *Mr*NV, *Macrobrachium rosenbergii* nodavirus; RdRp, RNA-dependent RNA polymerase; IPTG, isopropyl β-D- thiogalacto-pyranoside.

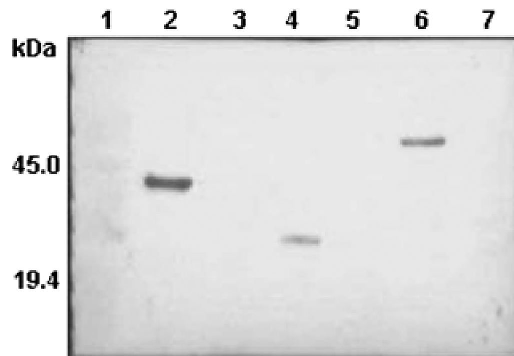

Figure 5 Western blot analysis of *Mr*NV RdRp, B2 and capsid recombinant proteins using alkaline phosphatase-conjugated anti-His mouse monoclonal antibodies Lane 1: Coloured protein marker. Lane 2: *Mr*NV RdRp IPTG-induced BL21(DE3) pLysS cells. Lane 3: *Mr*NV RdRp uninduced BL21(DE3) pLysS cells. Lane 4: *Mr*NV B2 IPTG-induced BL21(DE3) pLysS cells. Lane 5: *Mr*NV B2 uninduced BL21(DE3) pLysS cells. Lane 6: *Mr*NV capsid IPTG-induced BL21(DE3) pLysS cells. Lane 7: *Mr*NV capsid uninduced BL21(DE3) pLysS. *Mr*NV, *Macrobrachium rosenbergii* nodavirus; RdRp, RNA-dependent RNA polymerase; IPTG, isopropyl β-D- thiogalacto-pyranoside.

Figure 6 Western blot analysis of the polyclonal antibodies raised against recombinant *Mr*NV capsid protein. Lane1. *Mr*NV capsid purified protein. Lane 3. *Mr*NV capsid IPTG-induced BL21(DE3) pLysS cells. Lane 3. *Mr*NV capsid uninduced BL21(DE3) pLysS cells. *Mr*NV, *Macrobrachium rosenbergii* nodavirus.

esis and tissue tropism has been reported. For example, a DIG-labelled white spot syndrome virus (WSSV)-specific probe was used to detect the virus in haemocytes from WSSV-infected crayfish (Jiravanichpaisal, Sricharoen, Söderhäll & Söderhäll 2006). Digoxigenin-labelled probe directed against RdRp of virus involved in monodon slow growth syndrome in cultured black tiger shrimp (*Penaeus monodon*) has been reported for use in pathological investigations by *in situ* hybridization (Sritunyalucksana, Apisawetakan , Boon-nat, Withyachumnarnkul & Flegel 2006). In case of WTD, DIG-labelled DNA probes based on *Mr*NV RNA-2 as DNA template, have been

successful in the detection of *Mr*NV infection in tissues of WTD-diseased prawns by *in situ* hybridization (Sri Widada *et al*. 2003; Hsieh *et al*. 2006; Wang, Chang, Wen , Shih & Chen 2008). Very limited reports are available on tissue tropism associated with *Mr*NV pathogenesis. Arcier *et al*. (1999) by histopathological examination reported apparent viral inclusions, but much less conspicuous, in connective tissue of the subcutis and rarely present in the gills in the infected postlarvae of *M. rosenbergii* prawn. The use of *in situ* hybridization technique for histopathological studies by Sri Widada *et al*. (2003) revealed no positive reactions for the presence of *Mr*NV viral RNA in gill and hepatopancreatic tissues of *M. rosenbergii*. Basophilic cytoplasmic inclusion bodies in the musculature and hepatopancreas has been reported by Hsieh *et al*. (2006). The use of specific antibodies and the DIG probe will enable in exploring cellular localization of viral proteins. In the present study, the polyclonal antibodies raised against purified *Mr*NV capsid protein was successful in locating the viral inclusion bodies in *Mr*NV-infected muscle tissues by immunostaining. The viral antigens were detected using HRP-conjugated secondary antibody and 3-3′ diaminobenzidine tetrahydrochloride as substrate. The positive staining in infected prawns confirmed the presence of the virus and the specificity of the polyclonal antibody produced against *Mr*NV capsid protein (Fig. 7)

The PCR products of *Mr*NV B2 (402 bp) and capsid (1116 bp) genes were sequenced and the sequence information of these genes has been deposited in the GenBank with accession no.s GU300103 and GU300102 respectively. The nucleotide and amino acid sequence of RdRp for *Mr*NV Indian isolate (GenBank accession no. DQ146969) were compared with Taiwanese (GenBank accession no. DQ459205) and Chinese (GenBank accession no. AY231436) *Mr*NV isolates. The nucleotide and amino acid sequence of *Mr*NV capsid gene were compared with Thai (GenBank accession no. EU150126) and *Pv*NV Belize (GenBank accession no. EF137180) isolates in addition to Taiwanese (GenBank accession no. DQ521575) and Chinese (GenBank accession no. AY231437) *Mr*NV isolates. Presently, the sequence information for RdRp gene of *Mr*NV Thai and *Pv*NV Belize isolates are not available in the GenBank database.

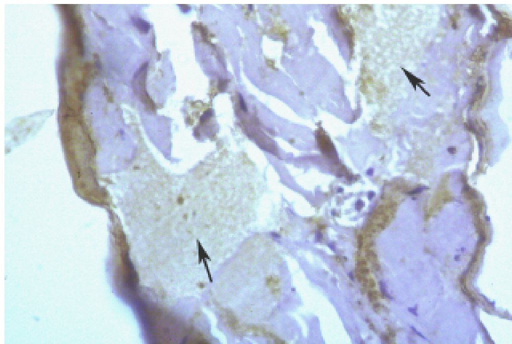

Figure 7 Immunostaining of *Mr*NV-infected muscles of *Macrobrachium rosenbergii* using polyclonal antibodies raised against *Mr*NV capsid protein (× 400). *Mr*NV, *Macrobrachium rosenbergii* nodavirus.

Currently, two genera have been identified in the family *Nodaviridae*, the alphanodaviruses infecting insects and betanodaviruses infecting fish (Ball, Hendry, Johnson, Rueckert & Scotti 2000). Sri Widada *et al*. (2003) reported complete sequence of RNA-1 of 3202 bp (Gen Bank accession no. AY222839) encoding an open reading frame (ORF) of 1045 amino acids for RdRp protein. Comparison of the RdRp gene sequence of *Mr*NV indicated its proximity to alphanodaviruses (Bonami *et al*. 2005). In the present study, the partial nucleotide sequence of the RdRp revealed that the Indian isolate of *Mr*NV (859 bp) did not differ much in nucleotide identity from the other two isolates of Taiwan (98%) and China (96%) (Table 1). At the amino acid level, the Chinese isolate differed from the Taiwanese and Indian isolate with respect to one amino acid replacement (Arg93Gln) as shown in Fig. 8. However, the full-length gene sequence analysis of RdRp of Indian isolate of *Mr*NV would be required to know its complete sequence homology to other *Mr*NV geographical isolates.

The protein sequence analysis of the ORF sequence of *Mr*NV RdRp used for protein expression (738 bp) of *Mr*NV (Indian isolate) revealed the signature sequence along with the conserved core residues, which represent the catalytic domain of RdRp (Fig. 9). This observation is based on the reports that have identified conserved sequence motifs in RdRp sequence (Poch, Sauvaget, Delarue & Tordo 1989). These motifs define the signature sequence of RdRp as $DX_3(FYWLCA)X_{0-1}DX_n(STM)GX_3TX_3(NE)X_n(GS)DD$ (Koonin & Dolja 1993). It is suggested that the presence of signature sequence identifies the protein as a catalytic subunit of viral RNA replicase and defines it as heart of the polymerase domain (Johnson, Johnson, Dasgupta, Gratsch, & Ball 2001). The motifs of the signature sequence are reported to be well conserved in RdRp sequences of both insect and fish nodaviruses, even though the overall amino

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Table 1 Nucleotide and protein sequence per cent homology of RdRp and capsid genes of *Mr*NV Indian isolate with other geographical isolates

Virus isolate	Host	Gene	GenBank accession no. (nucleotide)	GenBank accession no. (protein)	Nucleotide sequence identity (%)	Protein sequence identity (%)
Thailand	*Macrobrachium rosenbergii*	RdRp	–	–	–	–
		capsid	EU150126	ABW38168	99	99
Taiwan	*Macrobrachium rosenbergii*	RdRp	DQ459205	ABE69181	98	100
		capsid	DQ521575	ABG25924	96	97
China	*Macrobrachium rosenbergii*	RdRp	AY231436	AAQ54758	96	99
		capsid	AY231437	AAQ54759	93	95
Belize	*Litopenaeus vannamei*	RdRp	–	–	–	–
		capsid	EF137180	ABO33432	52	Not significant

*Mr*NV, *Macrobrachium rosenbergii* nodavirus; RdRp, RNA-dependent RNA polymerase.

Figure 8 CLUSTALW multiple amino acid sequence alignment of *Mr*NV RdRP gene. The information about residues match is shown below each block of residues. '*' means that the residues in that column are identical in all sequences in the alignment, ':' means that conserved substitutions have been observed, '.' means that semi-conserved substitutions are observed. *Mr*NV, *Macrobrachium rosenbergii* nodavirus; RdRp, RNA-dependent RNA polymerase.

acid sequence similarity of RdRp between them is less (Nagai & Nishizawa 1999; Sommerset & Nerland 2004). Our observation for the presence of similar signature sequence motifs and the associated core residues identified in *Mr*NV sequence in this study strongly suggests that the ORF encodes the viral part of RdRp. Moreover, based on the conserved domain database search, *Mr*NV RdRp protein sequence of Indian isolate (GenBank accession no. AAZ78232) showed the presence of active sites residues (D123,F124,S125,N126,F127,D128,S185,G186,F226,D228, D229,L265,A266), metal ion-binding site residues (D123,F124, D228,D229) and nucleic acid-binding site residue (G186).

The full-length amino acid sequence of the capsid gene of Indian isolate of *Mr*NV (371 amino acid) was predicted to bind to DNA consisting of α helix (22.91%), extended strand (24.80%), β turn (5.39%) and random coil (46.90%) regions using the structure prediction tools (Fig. 10).

```
  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 tgtgaccttaattcagttctaaataactttactcaatacgcagca
    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
```

Figure 9 ORF sequence of *Mr*NV RdRp used for protein expression. 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. The core residues of the RdRp signature sequence are boxed. ORF, open reading frame; *Mr*NV, *Macrobrachium rosenbergii* nodavirus; RdRp, RNA-dependent RNA polymerase.

The full-length nucleotide sequence of the capsid gene of Indian isolate of *Mr*NV (1116 bp) showed high nucleotide identity of when compared with Thai (99%), Taiwanese (96%) and Chinese (93%) isolates. Interestingly, Belize *Pv*NV isolate was very distinct as the nucleotide sequence of this isolate revealed no significant similarity on comparison with other geographical isolates of *Mr*NV. Even at the amino acid

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```
        10        20        30        40        50        60        70
         |         |         |         |         |         |         |
MARGKQNSNQTQNNSNANGKRRKRNRRNRNPQTVPNFNPIVAKPAVAPLQTNIRSARSDVNAITVLNGSD
eetcccccccccccccttccccccccccccccccccccccccccchhhhhcccccceeeeecccc
FLTTVKVRGSNNLTDSKSRILVKQPISASSFLGTRISGLSQFWERYRWHKAAVRYVPAVPNTLACQLIGY
eeeeeeecccccccttceeeeecccccccccccccthhhhhhhhhhhhhheccccchhhhheeee
IDTDPLDDPNVILDVDQLLRQATSQVGARQWNFSDTTTIPLIVRRDDQLYYTGQDKENVRFSQQGVFYLL
ecccccccceeechhhhhhhhhhhcccccccccccceeeeecccceeeecccccceeechtteeeee
QVTTLLNISGEAITNDLISRSLYLDWVCGFSMPQINPTPVEISQLTYDADTIGNWVPPTELKQTYTQDIT
ehhheeccttchhhhhhhhhheehheecccccccccceeeeeeeechhhhcccccccchhhhhht
GLKPNSKFIIVPYMDRVSSEVLQKCTITCNEVDAVGSISYFDTNAIKCDGYISFQANSIGEATFTLVTDY
tccttceeeeeechhhhhhhhhhceecccchhhhheeeeecttcccccceeeeehcccccceeeeeecc
QGAVDPKPYQYRIIRAIVGNN
ttcccccccceeeeeeeectt
```

Figure 10 Secondary structure prediction of *Mr*NV (Indian isolate) capsid protein using SOPMA. h, α helix; e, extended strand, t, β turn, c, random coil. *Mr*NV, *Macrobrachium rosenbergii* nodavirus.

```
ADB77879_India          MARGKQNSNQTQNNS--NANGKRRKRNRRNRNPQTVPNFN--PIVAKPAVAPLQTNIRSA 56
ABW38168_Thailand       MARGKQNSNQTQNNS--NANGKRRKRNRRNRNPQTVPNFN--PIVAKPTVAPLQTNIRSA 56
ABG25924_Taiwan         MARGKQNSNQIQNNS--NANGKRRKRNRRNRNPQTVPNFN--PIVAKPTVAPLQTNIRSA 56
AAQ54759_China          MARGKQNSNQAQNNN--NANGKRRKRSRRNRNPQTIPNFN--PIVAKPTVAPLQTNIRSA 56
ABO33432_Belize         --KRKPNSNQNNNNNRGNGNGLRRVRGGRVSRRRVVINQSNQSMPVTSNGAPLQALTSYS 58
                          : * **** :**.  *.** ** *. *  . :.: * .  .: ...  ****:    :

ADB77879_India          RSDVNAITVLN-GSDFLTTVKVRGSNNLTDSKSRILVKQPISASSFLGTRISGLSQFWER 115
ABW38168_Thailand       RSDVNAITVLN-GSDFLTTVKVRGSNNLTDSKSRILVKQPISASSFLGTRISGLSQFWER 115
ABG25924_Taiwan         RSDVNAITVLN-GSDFLTTVKVRGSNNLIDSKSRILVKQPISASSFLGTRISGLSQFWER 115
AAQ54759_China          RSDVNAITVLN-GSDFLTTVKVRGSNNLTDSKSRILVKQPISASSFLGTRISGLSQFWER 115
ABO33432_Belize         RPNVNKISRLGPDSDFLTSVVAKASTSIVTPADRILVKQPLSASSFPGTRITGLSSYWER 118
                        *.:** *: *. .*****:* .:.*..:  . .*******:***** ****:***.:***

ADB77879_India          YRWHKAAVRYVPAVPNTLACQLIGYIDTDPLDDPNVILDVDQLLRQATSQVGARQWNFSD 175
ABW38168_Thailand       YRWHKAAVRYVPAVPNTLACQLIGYIDTDPLDDPNVILDVDQLLRQATSQVGARQWNFSD 175
ABG25924_Taiwan         YRWHKAAVRYVPAVPNTLACQLIGYIDTDPLDDPNVILDVDQLLRQATSQVGARQWNFSD 175
AAQ54759_China          YRWHKAAVRYVPAVPNTLACQLIGYIDTDPLDDPNVILDVDQLLRQATSQVGARQWNFSD 175
ABO33432_Belize         YKWLSAVARYVPAVPNTVACQFVMYIDTDPLDDPSNISDDNQIVRQAVSQAGSNQFNFNT 178
                        *:* .*..*********:***:: **********. * * :*::***.**.*:.*:**.

ADB77879_India          TTTIPLIVRRDDQLYYTGQDKENVRFSQQGVFYLLQVTTLLNISGEAITNDLISRSLYLD 235
ABW38168_Thailand       TTTIPLIVRRDDQLYYTGQDKENVRFSQQGVFYLLQVTTLLNISGEAITNDLISGSLYLD 235
ABG25924_Taiwan         TTTIPLIVRRDDQLYYTGQDKENVRFSQQGVFYLLQVTTLLNISGEAITNDLISGSLYLD 235
AAQ54759_China          TTTIPLIVRRDDQLYYTGQDKENVRFSQQGVFYLLQVTTLLNISGEAITNDLISGSLYLD 235
ABO33432_Belize         SKTVPLIVRADNQYYYTGVDKQNLRFSLQGILYIIQVTDLINFNGELITQDLTCGSLFLD 238
                        :.*:***** *:* **** **:*:*** **::*::*** *:*:.** **:** . **:**

ADB77879_India          WVCGFSMPQINPTPVEISQLTYDADTIGNWVPPTELKQTYTQDITGLKPNSKFIIVPYMD 295
ABW38168_Thailand       WVCGFSMPQINPTPVEISQLTYDADTIGNWVPPTELKQTYTQDITGLKPNSKFIIVPYMD 295
ABG25924_Taiwan         WVCGFSMPQINPSPVEVSQLTYNADTIGNWVPPTELKQTYTQDITGLKPNSKFIIIPYMD 295
AAQ54759_China          WVCGFSMPQINPTPVEISQLTYNADTIGDWVPPTELNQTYTQDITGLKPNSKFIIVPYMD 295
ABO33432_Belize         WLVNFSIPQINPT----SLTDVRVDKAVNFIKPEVSGVAEIQTVTGLSPSTSYLLTP--- 291
                        *: .**:*****:    *     .*.  ::: *     :  * :***.*.:.::: *

ADB77879_India          RVSSEVLQKCTITCNEVDAVGSISYFDTNAIKCDGYISFQANSIGEATFTLVTDYQGAVD 355
ABW38168_Thailand       RVSSEVLQKCTITCNEVDAVGSISYFDTNAIKCDGYISFQANSIGEATFTLVTDYQGAVD 355
ABG25924_Taiwan         RVSSEVLQKCTITCNEVDAVGSISYSDTSAIKCDGYILFQANSIGEATFTLVTDYQGAVD 355
AAQ54759_China          RTSSEVLQKCTITCNEVDAVGSISYLDTNDIKCNGYITFQANNIGEATFTLVTDYQGVTE 355
ABO33432_Belize         -----------------------AFLEQN---------FQS----EAGIYILSA------ 309
                                               :: : .         **:    ** : :::

ADB77879_India          PKPYQYRIIRAIVGNN 371
ABW38168_Thailand       PKPYQYRIIRAIVGNN 371
ABG25924_Taiwan         PKPYQYRIIRAIVGNN 371
AAQ54759_China          PKPYQYRIIRAIVGNN 371
ABO33432_Belize         ----------------
```

Figure 11 CLUSTALW multiple amino acid sequence alignment of *Mr*NV capsid gene. The information about residues match is shown below each block of residues. '*' means that the residues in that column are identical in all sequences in the alignment, ':' means that conserved substitutions have been observed, '.' means that semi-conserved substitutions are observed.

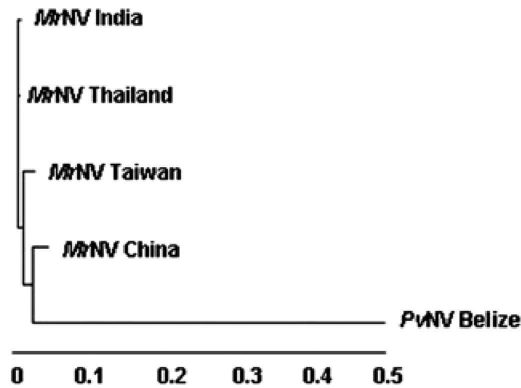

Figure 12 Phylogenetic analysis of different viral geographical isolates based on the sequence comparison of capsid gene.

level, the Belize isolate with 309 amino acid sequence revealed an identity of only 52% with the Indian isolate, whereas on comparison the Indian isolate of *Mr*NV showed high sequence identity with the Thai (99%), Taiwanese (97%) and Chinese (95%) isolates (Table 1 and Fig. 11). Phylogenetic analysis based on the sequence comparison of capsid gene revealed the Belize isolate to be the most distinct between the geographical isolates (Fig. 12). The distinct nature of the Belize isolate may be due to the host specificity of the virus. The Belize *Mr*NV isolate differs from all the other four geographical *Mr*NV isolates as the virus is reported to be isolated from *Litopenaeus vannamei*, whereas the other geographical *Mr*NV isolates have been isolated from *M. rosenbergii*.

Similar observations in sequence variability originating due to host specificity have been reported in other shrimp viruses. For example, distinct Taura syndrome virus (TSV) isolates were found in *Metapenaeus ensis*, *P. monodon* and *L. vannamei*. Nucleotide sequence analysis of these three isolates revealed differences in the TSV structural protein (capsid protein precursor) gene ORF 2 (Chang, Peng, Yu, Liu, Wang, Lo & Kou 2004). In case of WSSV, variations in tandem repeats in ORF 94 of the WSSV genome have been reported to occur upon passaging through different hosts. The differences in tandem repeats number appear to have resulted from host selection rather than geographical isolation (Waikhom, Riji John, George & Prince Jeyaseelan 2006).

Alterations in the viral surface structure can change the host–virus interactions. The emergence of host range variants due to mutation have been extensively reported for canine parvovirus (Chang, Sgro & Parrish 1992; Govindasamy, Hueffer, Parrish & McKenna 2003). In marine systems, the capsid alterations are reported to affect the phytoplankton–virus (host–parasite) interactions (Nagasaki, Shirai, Takao, Mizumoto, Nishida &Tomaru 2005). Recently, natural infections of WTD have been reported in hatchery-reared postlarvae of other marine shrimps such as *P. monodon* and *Penaeus indicus* (Ravi, Nazeer Basha, Sarathi, Idalia, Sri Widada, Bonami & Sahul Hameed 2009). In addition, the aquatic insects have been reported as potential natural carriers of *Mr*NV and XSV (Sudhakaran, Haribabu, Kumar, Sarathi, Ahmed, Babu, Venkatesan & Hameed 2008). It would be interesting to compare the sequences of *Mr*NV isolated from these different natural hosts. Further analysis of the conserved and non-conserved sites between the *Mr*NV isolates with different host specificity may indicate the reasons for the shift between hosts and adaptability of the virus to different host.

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