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Full Length Research Paper

Reverse transcriptase polymerase chain reaction is a sensitive diagnostic tool for detection of nodavirus infection in *Macrobrachium rosenbergii*

D. P. Behera*, K. Devdas and L. Nayak

Department of Marine Sciences, Berahmpur University, Bhanja Bihar, Berhampur, Orissa-760007,India.

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Macrobrachium rosenbergii **is the most important cultured Palaemonid in the world. It is farmed on a large scale in many countries including India, once upon a time this species was considered as disease free animal but towards later part of 2001, the hatchery and nursery pond of these animals suffered a massive loss due to a new viral disease named white tail disease. Till date different diagnosis has been established for detection of this disease but reverse transcriptase polymerase chain reaction (RT-PCR) techniques is the most sensitive diagnostic tool among those present available for detection of MrNv (*Macrobrachium rosenbergii* nodavirus). The designed primer fair (1F/1R) in 1st step PCR was carried out using 1 µl e DNA like wise in 2nd step RT-PCR product the nested primer 2nd 1 µl of 1st RT-PCR product were utilized. At the end of 8 µl of each step PCR product was analyzed by electrophoresis on 1% agarose gel along with sample. The confirm positive and negative sample were run in the gel, the gel electrophoresis of RT-PCR product positive control (Lane-3) is expected amplified product of about 489 bp size of noda virus presence in sample.**

Key words: Reverse transcriptase, diagnostic tool, nodavirus, *Macrobrachium.*

INTRODUCTION

The giant freshwater prawn, *Macrobrachium rosenbergii*, popularly known as scampi, an economically important, farmed crustacean species cultured in different part of world including India. This species is commercially important for food due to its faster growth rate in tropical and sub tropical region. During past decades the fresh water prawn (*M. rosernbergii*) was considered as disease free animal, but towards later part of the year 2001 the hatchery and nursery ponds suffered a massive loss due to new viral disease named as white tail disease. It is a new serious epizootic disease caused by *M. rosenbergii* nodovirus (*Mr*NV) which affects post larvae and early juveniles causing mortality up to 100%. The affected animals' shows a sign of white colorations in tail region of species or white muscle before death. Only three viral diseases have been reported in this species till now (Anderson et al., 1990; Bonami, 2002), one is affecting the hepato pancreas and the others affecting muscle tissue of the species. White tail disease (WTD) of *M. rosenbergii* was first observed in Guadeloupe Island in 1995, Artinique Island of West Indies (Arcier et al., 1999) then Taiwan, Tung et al. (1999) and after on peoples of Republic of China (Qian et al., 2003). The causative agent of WTD has been identified as *M. rosenbengi*i nodovirus (MrNv) associated with extra small virus (XSV) (Qian et al., 2003). It is a small icosahedra non-evolved virus, 26 to 27 mm in diameter that has been identified in cytoplasm of connective cell and has been placed in *Nodaviridae* family based on its characteristic and genome sequence (Garzans and Charpentier, 1992; Van Regenmortel et al., 2000; Romestand and Bonami, 2003). Sri et al. (2003) developed 2 genome based detection method for the identification of XSV,

*Corresponding author. E-mail: durga.marine@gmail.com.

Abbreviations: RT-PCR, Reverse transcriptase polymerase chain reaction; **DNA,** deoxy ribose nucleic acid; ***Mr*NV,** *Macrobrachium rosenbergii* nodovirus ; **WTD,** White tail disease; **XSV,** extra small virus; **S.Elisa,** sandwich enzymes linked immunosorbent assay; **CIFA,** central institute of fresh water aquaculture; **PL,** post-larvae; **RNA,** ribose nucleic acid; **DEPC,** diethyl pyrocarbonated; **OD,** optical density.

Table 1. Pairs of primer used to detect *M. rosenbergi* nodavirus (*Mr*Nv) by reverse transcriptase-polymerase chain reaction (RT-PCR) technique.

Pair	Name	Size(bp)	Sequence	Orientation
1(Bonami)[a]	1A 775	850	CCACGTTCTTAGTGGATCCT	Upstream primer
	1B 690		CGTCCGCCTGGTAGTTCC	Down steam primer
2(RNA-1)	MrNv1F	590	TCCAACACCTCGCATAGC	Upstream primer
	MrNv1R		CACTCTTAACCCCCACTCC	Downstream primer
3(RNA-2)	MrNv2F	681	GATACAGATCCACTAGATGACC	Upstream primer
	MrNv2R		GACGATAGCTCTGATAATCC	Downstream primer
4(RNA-2)	MrNv2aF	425	GCGTTATAGATGGCACAAGG	Upstream primer
	MrNv2aR		AGCTGTGAAACTTCAACTGG	Downstream primer

[a]Primers from Sri et al. (2003).

namely dot-blot hybridization and single step RT-PCR. The dot-blot hybridization constitutes an easy technique was establish its sensitivity and hybridization was first carried out using purified viral RNA (Widad et al., 2004). A sandwich enzymes linked immunosorbent assay (S. Elisa) was developed using antibody raised against *Mr*NV diagnosis (Romestand and Bonami, 2003). As this disease is persist in Andhra Pradesh and Tamil Nadu, an attempt has been under take to collect sample from hatchery to detect the presence of virus by applying reverse transcriptase polymerase chain reaction (RT-PCR) based technique of modified version of PCR used to amplify DNA copies of RNA molecules standardize at CIFA for best result. The prime objective of study is though all the field samples studied were negative, the positive samples supplied with the kit showed amplification. Hence this kit may find application in WTD investigation in field samples.

MATERIALS AND METHODS

Sample collection

The larvae, post-larvae (PL) and adult prawn were collected from Sripa Aqua marine Pvt. Ltd a Scampi hatchery, Reba village of Nellore Andhra Pradesh. The post-larvae were washed in sterile saline solution then transferred to sterile tubes and brought to laboratory on dry ice which was stored at -30°C till analysis.

Extraction of total RNA

For extraction of total RNA, about 100 mg of tissue was taken in a sterile paste and mortal and homogenized in a 1 ml of TN buffer (50 mM Tris-Hcl, 5.25 m guanidinium thiocyanate ,pH 6.4). To this 1 ml of tri-reagent (Sigma) was added. The homogenized samples was transferred to a sterile tube and kept at 5 min. After 5 min of incubation at room temperature 0.2 ml of chloroform was added and mixed thoroughly which allowed stand for 15 min at room temperature. Then tube was centrifuged at 11,000 × g for 15 min at 4°C. The upper aqueous phase was transferred to a new tube to which 1 ml of iso-proponol was added and centrifuged at 11,000 × g for 10 min at 4°C. The RNA pellet was washed with 75% ethanol in DEPC (diethyl pyrocarbonated) water and finally dissolved in 0.1% DEPC treated water. This method was previously described by Sahul et al. (2004). The quality and quantity of RNA was checked taking absorbance ratio of optical density OD260/nm and OD280/ nm in UV/visible spectrophotometer.

RT-PCR

The reverse transcriptase polymerase chain reaction was carried out using CIFA developed RT-PCR kit has the detection limit of quantifying 4 viral particles. One primer pair (Pair 1) used for detection of *Mr*Nv was published by Sri et al. (2003) while other pair (Pair 2) designed from the sequence of Gene bank record AY222839 and two other (pair 3 and 4) from record AY222840. The primer sequence and amplicon sizes are given in Table 1. Reaction were performed in 20 µl of RT buffer containing 0.5 µg of RNA template, using following steps: RT at 52°C for 30 min; 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 of 10 min at 68°C. The 1st step PCR was carried out using 1 µl of DNA and primer pair (1F/1R) like wise in 2nd step RT-PCR (Nested) the primer pair 2nd and 1 µl of 1st step PCR product was utilized and the primers designed by Sri et al. (2004) were used. The RT-PCR products were analyzed by electrophoresis on 1% Agarose gel.

RESULTS

Three sample one each for Larvae sample 1, post-larvae sample 2 and adult stage sample 3 were taken for total RNA isolation, the quality and quantity of RNA samples were checked by taking absorbance at 260 and 280 nm. OD_{260} of the sample were showed about 330,410 and 210 µg of RNA per ml and the ratio of $OD_{260/280}$ were 1.87, 1.92 and 2.01 in larvae, post larvae and adult samples respectively. The samples were further tested by RT-PCR for the presence of virus *M. resenbergii* nodavirus using nested primer, the gel electrophoresis of RT-PCR

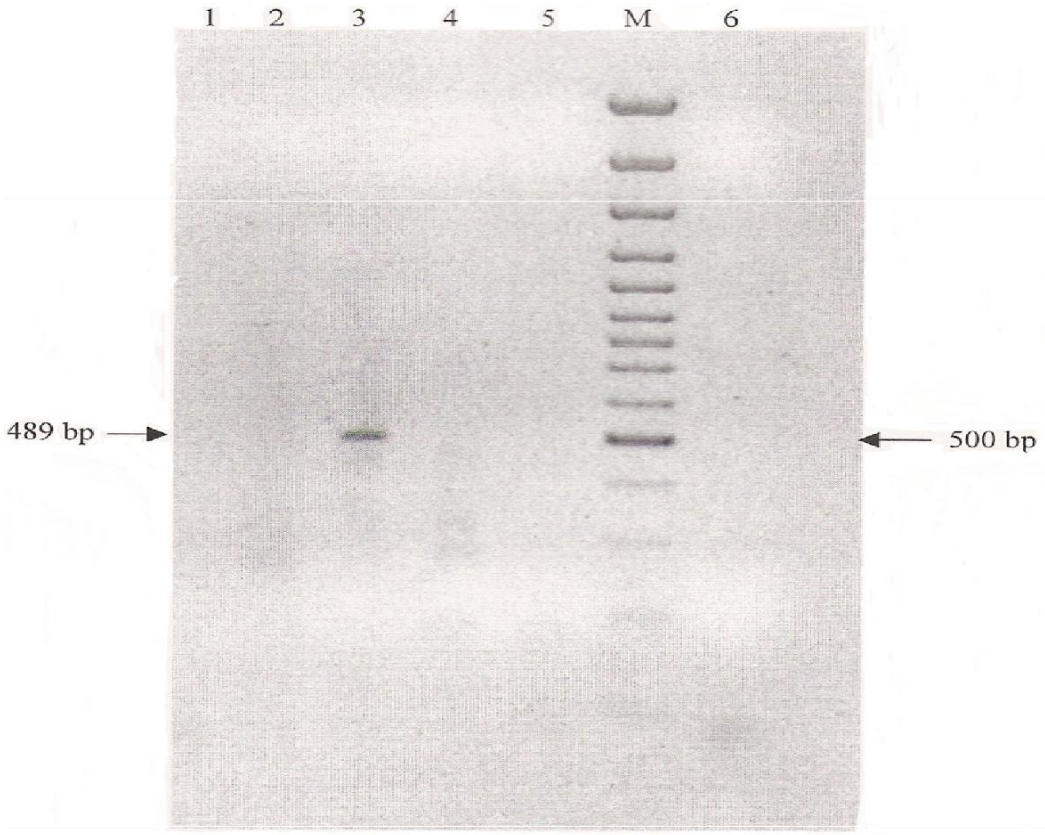

Figure 1. Nested –PCR of the sample collected from Nellore, Andhra Pradesh analyzed on 1% Agarose gel for *M. rosenbergii*. 1. Reagent control, 2. Control RNA, 3. Positive RNA, 4.Sampl No-1, 5. Sample No-2, M-Marker, 6. Sample No- 3.

product (Figure 1) showed that the samples were negative (Lanes 4, 5 and 6) while the positive control Lane 3 showed as expected amplified product of about 489 bp size.

DISCUSSION

The main clinical sign of WTD (that is, whitish colouration in the abdominal and tail muscles) is not specific to WTD but is also characteristic for other diseases and stress that are not always lethal. However, WTD due to *Mr*Nv cause high mortality in scampy hatcheries. The RT-PCR based diagnostic tool to diagnose *Mr*Nv is the most sensitive method to detect as low as four viral particles (Sahoo et al., 2005; Sri et al., 2003). In our present observation the results shows that OD_{260} of the sample have 330, 410 and 210 µg of RNA per ml and the ratio of $OD_{260/280}$ were 1.87, 1.92 and 2.01 in larvae, post larvae and adult sample respectively again the samples were further tested by Nested primer, the gel electrophoresis of RT-PCR product shows an expected amplified product of 489 bp in 1% agarose gel. This result is in agreement with the result of Sahoo et al. (2005) of expected product size 313 and 176 bp on 1% agarose gel. The genome of MrNV is composed of two segment of linear single-standards RNA (ss RNA), of 2.9 and 1.3 kb two viruses were found associated with WTD-diseased animals, both developing in the cytoplasm of target cells. The viruses were isolated from infected post-larvae and their purification was achieved using a combination of centrifugation steps at low and high speed (Bonami et al., 2005). An 850 bp amplified product could be obtained by RT-PCR where 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 (Shekhar et al., 2006). Though results obtained from our field samples were negative (Lanes 4, 5 and 6) while the positive control (Lane 3) showed expected amplified product about 489 bp size which is very similar to the result of Shekhar et al. (2006).

Conclusion

In the present study it shows that even all the field samples were negative, the positive samples supplied

with kit showed amplification. Hence this RT-PCR based diagnostic tool is the most sensitive method for detection of this *M. rosenbergii* nodavirus among all the published protocol. Therefore, the disease management strategies are aimed at improving the disease resistance of the host, reducing pathogen level and detection of pathogen. Early detection of viral pathogen would help in taking proper management steps to control the incident of white tail disease in *M. rosenbergii.*

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