

COMPARISON OF SENSITIVITY OF ELISA, DOT-ELISA AND LATEX AGGLUTINATION TEST WITH PCR FOR DETECTION OF MrNV IN GIANT FRESH WATER PRAWN, *Macrobrachium rosenbergii*

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ABSTRACT

A comparative analysis was done to evaluate the efficacy and specificity of developed tests of ELISA, Dot ELISA, and latex agglutination test to PCR. Artificial infection trials of challenged prawns were screened for infectivity on first, second and third day of post infection through developed tests and PCR method. On first day of post infection prawns were tested WTD positive in PCR whereas developed immunodiagnosics tests could detect the infection from second day. The obtained result showed that sensitivity of the developed testswere comparatively lower to PCR on first day but were similar from second day of post challenge. In one another trial a total 220 prawns were tested for WTD infection. Among total 220 infected prawns, a total of 206 were tested PCR positive, however in ELISA, Dot ELISA, and latex agglutination test a total 198 numbers were tested positive. Result of this experiment showed that the developed tests are comparable to PCR test with 96 % specificity and are useful for screening the prawns for the presence or absence of WTD.

KEYWORD: Key words:ELISA, dot-ELISA, latex agglutination, PCR, MrNV, *M. rosenbergii*

INTRODUCTION

Noda virus and extra small virus are causative agents of white tail disease of *Macrobrachium rosenbergii*. WTD outbreaks were reported from different parts of the world viz. West Indies (Arceret *al* 1999), Taiwan (Tung *et al* 1999), China (Qianet *al* 2003) India (SahulHameedet *al* 2004). These viruses are of economic importance worldwide and a potential health hazard in *Macrobrachium rosenbergii* hatcheries and nursery ponds (Sudhakaranet *al* 2006). Efforts to control the disease have been difficult because of the lack of effective vaccines and the lack of diagnostic assays that can identify infected animals before the appearance of clinical disease. This has been a major problem in the control of WTD.

There are different immunodiagnosics, molecular diagnostics and multiplex technologies are developed for the detection. Such methods include PCR, agglutination (slide/latex); fluorescent antibody test (FAT/IFAT); immunohistochemistry (IHC); enzyme linked immunosorbent assay (ELISA); and blot (dot-blot/dip-stick/western blot) (Miahleet *al.*, 1995;Adams 2004). Molecular technologies are also widely used for the detection of fish pathogens (Adams and Thompson, 2006;Wilson and Carson, 2003). They have been successfully utilized for the detection and identification of low levels of aquatic pathogens. The DNA-based methods such as polymerase chain reaction (PCR) are extremely sensitive. Real-time PCR (closed tube to reduce contamination) and Nucleic

Acid Sequence Based Amplification (NASBA) are alternatives that reduce this risk and offer high sample throughput (Starkey, *et al.*, 2004). Some of the most common PCR-based technologies used for the detection of pathogens are nested PCR, random amplification of polymorphic DNA (RAPD), reverse transcriptase-PCR (RT-PCR), reverse cross blot PCR (rcb-PCR) and RT-PCR enzyme hybridisation assay (Wilson and Carson, 2003). *In situ* hybridisation is also widely used in the detection of shrimp viruses (Lightner and Redman, 1998). These techniques have immense advantage of being very accurate and sensitive but these are time consuming, costly, require sophisticated equipments, which are practically not feasible to operate under farm condition. So the urgent need for reliable diagnostic tool allowing viral detection in large numbers of samples has led us to develop rapid immunodiagnostic tests, which are simple to use, sensitive, inexpensive and can be used on farm condition may have immense future. Hence, in the present study, a comparative analysis was carried out to evaluate the specificity and sensitivity of developed immunodiagnostic tests which are rapid, cost effective and based on the reagents easily available in the country itself.

MATERIALS AND METHODS

The White tail disease infected *Macrobrachium rosenbergii* PL (0.02±0.08 g) samples were collected from Nellore, West Godavari and East Godavari districts of Andhra Pradesh. The specimens were transported in dry ice to the laboratory and stored at -80°C till purification of the virus. The live animals at the site of collection were off feed, lethargic, empty gut with opaque muscle. All the infected PL aggregated to the periphery of the cement tank and died within 7-20 days after the onset of the disease. The infected specimens were collected and observed for the presence of clinical signs of white tail disease viz. opaqueness of the muscle and loose cuticle.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from infected and normal post-larvae using Trizol reagent (Invitrogen Life Technologies, Cergy-Pontoise, France) as described by the manufacturer and stored at -80°C until use. RT-PCR was carried out with one step RT PCR test (Abgene, Rochester, USA). Reactions were performed in 50 µl RT-PCR buffer containing 20 pmol of each primer and RNA template. Conditions of the reaction were reverse transcription (RT) at 52°C for 30 min, denaturation at 94°C for 2 min followed by 30 cycle denaturation at 94°C for 40 s and annealing at 55°C for 40 s followed by 30 cycles of elongation at 68°C for 1 min and an additional elongation step at 68°C for 10 min.

The RT-PCR products were analyzed by electrophoresis in 2 % agarose gel stained with ethidium bromide. The primers used for MrNV: Forward: 5'-GAT ACA GAT CCA CTA GAT GAC C-3' Reverse: 5'-GAC GAT AGC TCT GAT AAT CC-3' and for XSV Forward: 5'-GGAGAACCATGAGATCACG-3' Reverse: 5'-CTGCTCATTACTGTTCGGAGTC-3'

RT-PCR was carried out with reported primers for nodavirus amplification i.e. 5' GCG TTATAG ATG GCACAA GG 3' forward and 5' AGC TGT GAA ACTTCC ACT GG 3' reverse (SahulHameed *et al.* 2004a).

ELISA for detection of MrNV

An indirect ELISA was performed for the detection of MrNV using penicillinase anti MrNV rabbit Ig G conjugate. 100 µl of the infected PLs tissue extract is coated on ELISA plates in carbonate-bicarbonate buffer, pH 9.6 and incubated at 4°C. After three cycles of washing with PBS-Tween 20 (PBS-T), two hundred microliters of 1:500 dilution of anti MrNV rabbit IgG was added to each well and incubated at 37°C for 2 hr. The plates were washed three times, with distilled water containing Tween-20. Then, 1:1000 dilution of anti-rabbit goat Ig G was added to each

well and incubated for 2 hr. Again the plates were washed three times with distilled water containing Tween-20. Finally, 200 µl of bromothymol blue containing penicillin G substrate was added to each well. After incubation at room temperature for 30 min, results were scored visually by considering yellow color as positive and blue color as negative.

Dot-ELISA for detection of MrNV

Ten µl of infected tissue homogenate or 1:100 dilution of purified MrNV was applied as a small dot on nitrocellulose dip sticks (M/s. Advanced Micro Devices Pvt. Ltd., Ambala Cantt.) with a microsyringe. These sticks were air-dried and were treated with blocking solution (containing 5% skim milk powder in tris buffer) at 37°C for one hour. The sticks were washed with TBS-T thrice, shaking 5 min at each wash. Washed sticks were dipped in 1:200 dilution of anti MrNV rabbit IgG HRPO Conjugate. After one hour of incubation at 37°C, the sticks were washed with TBS. Finally, the substrate was added for the colour development with five percent diaminobenzidine tetra-hydrochloride (DAB). After 5 min the sticks were washed with distilled water and dried in folds of tissue paper and results were recorded as a brown spot in case of positive reaction and a negative reaction gives no spot.

Latex Agglutination Test

After optimisation of the volume of latex reagent and antigen concentration, latex agglutination test was performed for the detection of antigen of MrNV using latex reagent sensitized with anti MrNV rabbit IgG. 40 µl of the reagent and 40 µl of infected tissue homogenate were transferred on to a clean glass slide and mixed with a toothpick. The mixture was observed for visible clumps within 1-3 min. The good visible agglutination reactions were scored as 4+. Suitable negative controls were also included.

RESULTS AND DISCUSSION

A comparative analysis was done to evaluate the efficacy and specificity of

developed tests of ELISA, Dot ELISA, and Latex agglutination test to PCR. Artificial infection trials of challenged prawns were screened for infectivity on first, second and third day of post infection through developed tests and RT-PCR method. On first day of post infection prawn were tested WTD positive in RT-PCR whereas developed immunodiagnosics tests could detect the infection from second day.

The obtained result showed that sensitivity of the developed tests are comparatively lower to RT-PCR on first day but shown similar from second day of post challenge. In one another trial a total 220 prawns were artificially infected and tested for WTD infection. Among total 220 infected prawns, a total of 206 were tested PCR positive, however in ELISA, Dot ELISA, and Latex agglutination test a total 198 numbers were tested positive. Result of this experiment showed that the developed tests are comparable to PCR test with 96 % specificity. RNA was isolated from whole PLs tissue homogenate by Trizol extraction method and 0.5µg/µl RNA was obtained (Plate-8). A positive RT-PCR reaction for MrNV and XSV showed an amplified band of 600 and 410 bp respectively (Fig-1).

Antibodies against MrNV have been shown to be reliable indicators of previous infection in *Macrobrachium rosenbergii* both in challenge tests and in disease outbreaks. However, in comparative evaluations of kits for use in WTD diagnosis, other researchers have not included sample numbers of the order suggested by OIE (O.I.E., 2004a). In this study, we compared PCR versus Immuno based diagnostics i.e., ELISA, Dot Elisa and Latex Agglutination test for WTD diagnosis, based on MrNV antigens from capsid protein, for sensitivity and specificity on well-characterized stored samples from artificially infected and natural outbreaks of giant freshwater prawn.

The infected PLs whole tissue homogenate was tested for the presence of MrNV by ELISA, Dot-ELISA and latex agglutination tests developed. The results

for the presence or absence of MrNV are shown in Table 1. These results were compared with the PCR which shows that ELISA, DOT- ELISA and Latex detection test was 96% sensitive. ELISA and Dot ELISA could detect the MrNV in tissue homogenate of infected PL from day 3 post infections, latex agglutination test from day 2 and PCR from day one. The results of this comparison indicated that ELISA is 96% sensitive and specific. Similarly Dot ELISA was also 96% sensitive and specific. The results of this comparison indicate that all the three methodologies gave similar qualitative results. However, each method has its own advantages and disadvantages. ELISA requires expensive instrumentation and expertise and so not amendable to field testing. On the other hand Dot ELISA is simpler, rapid and sensitive and the reactions can easily be read visually without the help of equipment. So the test can be done by less experienced persons also under the field conditions.

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Table 1:Comparative efficacy of ELISA, Dot-ELISA, Latex Agglutination test and PCR in detection of *MrNV*

(n=220)	
Test	Number of samples found positive for <i>MrNV</i>
ELISA	198
Dot-ELISA	198
Latex Agglutinin	198
PCR	206

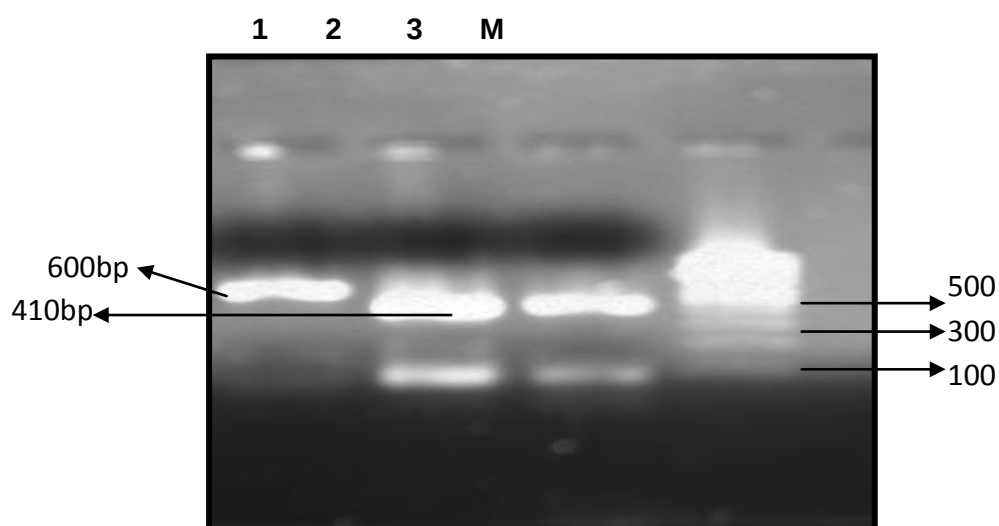


Fig.: 1: Amplification of the RT-PCR products of *MrNV* and *XSV* from PL tissue extract
Lane1: *MrNV*-600bp, lane2 and 3: *XSV*-410bp and lane 4: 100bp ladder

[MS received: December 22, 2016]

[MS accepted: December 29, 2016]