

Evaluation of different RTPCR assays for diagnosis of carrier infection of nodavirus (MrNV) and extra small virus (XSV) in *Macrobrachium rosenbergii* in Egypt

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Abstract: A total number of 500 specimens of *Macrobrachium rosenbergii* were collected during (2011) from Alexandria (Maruit). Each five specimens were pooled for extraction of RNA and carrying out of RTPCR analysis. These specimens were transferred to the laboratory of Central Laboratory for Aquaculture Research (CLAR), Abbassa, Sharkia, Egypt for evaluation of different reverse transcriptase polymerase chain reaction (rt-PCR) assays for diagnosis of *Macrobrachium rosenbergii* nodavirus (MrNV), and extra small virus (XSV). Multiplex reverse transcriptase Polymerase chain reaction (MRT-PCR) assay for simultaneous detection of MrNV and XSV were classified into one-step MRTPCR and two steps MRTPCR. Results of one-step multiplex (MRTPCR) using (BioRT One step RT-PCR kit) and primer for MrNV virus (product size 681 bp) were negative. Also, the results of One-step multiplex (MRTPCR) using primer for XSV (product size 500 bp) were negative. In addition, results of two steps multiplex MRTPCR using (GoScript™ Reverse Transcription System kits), primer for MrNV (product size 681 bp) and primer Oligo(dT)15 for obtaining first CDNA were negative. Where, percentage of infections of two steps multiplex RTPCR using primer for XSV (product size 500 bp) and primer Oligo(dT)15 for obtaining first CDNA were (4%). Percentage of infections of nested (nRTPCR) for detection of MrNV virus using (AccessQuick™ RT-PCR kit), primer amplification (product size 205 bp) were 9%. Nested (nRTPCR) for detection of XSV virus using (AccessQuick™ RT-PCR kit), primer amplification (product size 236 bp) were 7%.

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1. Introduction:

During past decades the freshwater prawn (*M. rosenbergii*) was considered as diseases 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 (**Behera et al, 2011**). Polymerase chain reaction technique has been considered the most rapid and sensitive technique and it take approximately 3 hr to complete the diagnosis of the disease (**Flegel, 2006**). RT-PCR technique is the most sensitive and versatile diagnostic method available for detection of MrNV and XSV. This technique was applied for routine health monitoring, early virus detection, studying virus–host interaction, and detection of carriers and screening of broodstock (**Sri Widada et al., 2003**). To avoid the necessity of carrying out two separate RT-PCR reactions, a modified method for simultaneous detection of MrNV and XSV in a single-tube, one-step multiplex RT-PCR assay can be performed and diagnosed the viral infection of MrNV and XSV (2.5 fg) of total RNA in tissue (**Yoganandhan et al, 2005**). While, nested RT-PCR (Nrt-PCR) is more sensitive and useful for screening seed and broodstock than multiplex RT-PCR. The detection sensitivity of the nRT-PCR is ~1000-fold

greater than the one-step RT-PCR (**Sudhakaran et al, 2006**).

The present study was planned to evaluate the different RTPCR (one step multiplex RTPCR, two step multiplex RTPCR and nested RTPCR) assays for diagnosis of the carrier state of *Macrobrachium rosenbergii* nodavirus (MrNV), and extra small virus (XSV) in Egypt

2. Material and methods

1- Sampling:

A total number of 500 juvenile specimens of freshwater prawn (*Macrobrachium rosenbergii*) were collected during (2011) from Maryout hatchery, Alexandria province. Each five specimens were pooled for extraction of RNA and carrying out of RTPCR analysis. The collected specimens were transferred to the laboratory of Central Laboratory for Aquaculture Research Abbassa (CLAR) Sharkia, Egypt.

2- Reverse transcriptase Polymerase chain reaction (rt-PCR)

a- RNA extraction

RNA extraction were carried out according to **Sahul Hameed et al, (2004)**.

100 mg from juvenile of *Macrobrachium rosenbergii* (each specimen represent 5-pooled prawn) were homogenated in TN buffer (20 mM Tris/HCl, 0.4 M NaCl, pH 7.4). Homogenates were centrifuged at 12,000 *g* for 15 minutes. Supernatant was mixed with one ml boizol thoroughly and it was incubated for 5 minutes at room temperature. 200µl chloroform was mixed to the sample, centrifuged at 12,000 *g* for 15 minutes. The aqueous phase was collected and it was transferred to a fresh tube, and RNA was precipitated by mixing with isopropanol. The samples incubated for 10 minutes at room temperature and centrifuged at 12,000 *g* for 10 minutes at 4°C. RNA pellets were dissolved in TE buffer (10 mM Tris/HCl, 1 mM EDTA [ethylene diamine tetra-acetic acid], and pH 7.5) after washing with 75% ethyl alcohol. Extracted RNA were quantified and qualified by determining absorbance at 260 nm using a spectrophotometer against distilled water blank to determine the concentrations of RNA.

b- Multiplex reverse transcriptase Polymerase chain reaction (Mrt-PCR) assay for simultaneous detection of MrNV and XSV

This protocol was carried out according to (Yoganandhan *et al*, 2005 and OIE, 2009) with some modifications.

1- One step multiplex rt-PCR using (BioRT One step RT-PCR kit)

Procedures of PCR were performed in a 25µl reaction mixture. To avoid the necessity of carrying out two separate RT-PCR reactions, a modified method for simultaneous detection of MrNV and XSV in a single-tube, one-step multiplex RT-PCR assay was performed. Procedures were performed using primer sequences for MrNV (annealing temperature 55°C; product size 681 bp): Forward: 5'-GAT-ACA-GAT-CCA-CTA-GAT-GAC-C-3' and Reverse: 5'-GAC-GAT-AGC-TCT-GAT-GAT-AAT-CC-3'. Moreover, PCR primer sequences for XSV (annealing temperature 55°C; product size 500 bp): Forward: 5'-GGA-GAA-CCA-TGA-GAT-CAC-G-3' and Reverse: 5'-CTG-CTC-ATT-ACT-GTT-CGG-AGT-C-3'. Reaction was performed according to BioRT One step RT-PCR kit

PCR profile (thermal cycles): RT at 52°C for 30 minutes; denaturation at 95°C for 2 minutes, thirty cycles of amplification were performed with the following protocol: denaturation at 94°C for 40 seconds, annealing at 55°C for 40 seconds and elongation at 68°C for 1 minute. Ending with an additional elongation step 10 minutes at 68°C

2- Two step multiplex PCR((Mrt-PCR) using GoScript™ Reverse Transcription System kits (Promega USA)

Procedure was used to convert RNA by using Primer Oligo (dT)15 into first-strand cDNA. RNA and primers [Oligo(dT)15 (0.5µg/reaction)] mixtures were incubated at 70°C for 5 minutes and Stored on ice until

reverse transcription mix is added. The reverse transcription reaction mixture was prepared by volume of 15µl for each cDNA reaction (GoScript™ 5X Reaction Buffer, MgCl₂, PCR Nucleotide Mix, Recombinant RNasin® Ribonuclease Inhibitor, GoScript™ Reverse Transcriptase and Nuclease-Free Water). Procedure was performed according to GoScript™ Reverse Transcription System kits.

PCR profile (thermal cycles): Anneal temperature 25°C for 5 minutes. Extension temperature 42°C for one hour (RNA converted to cDNA by Reverse transcriptase). Inactivation temperature 70°C for 15 minutes (for inactivation of Reverse transcriptase).

The mix (cDNA) was added to 20µl GoTaq® Green Master Mix kit the reaction mixture contained the following: (GoTaq® Green Master Mix, cDNA, primer sequences for MrNV (product size 681 bp): Forward: 5'-GAT-ACA-GAT-CCA-CTA-GAT-GAC-C-3' and Reverse: 5'-GAC-GAT-AGC-TCT-GAT-AAT-CC-3'. Primer sequences for XSV (product size 500 bp): Forward: 5'-GGA-GAA-CCA-TGA-GAT-CAC-G-3' and Reverse: 5'-CTG-CTC-ATT-ACT-GTT-CGG-AGT-C-3' and nuclease-free water was added to final volume 25 µl).

PCR profile (thermal cycles): (Initial denaturation at 95°C for 5 minutes, thirty cycles of amplification were performed with the following protocol: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 1 minute, ending with an additional elongation step for 10 minutes at 72°C).

c- Nested RTPCR for detection of Mrnv using AccessQuick™ RTPCR kit:

This protocol was carried out according to (Sudhakaran *et al*, 2006 and OIE, 2009) with some modifications. Procedures were performed in two-step:

- First step using external primer: (AccessQuick™ Master Mix 2X, external primer sequences for MrNV Forward: 5'-GCG-TTA-TAG-ATG-GCA-CAA-GG-3', Reverse: 5'-AGC-TGT-GAA-ACT-TCC-ACT-GG-3', RNA template, AMV reverse transcriptase and nuclease-free water)

PCR profile (thermal cycles): Reaction tubes were incubated at 45°C for 45 minutes. Initial denaturation at 95°C for 2 minutes, thirty cycles of amplification were performed with the following protocol: Denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute elongation at 72°C for 1 minute and ending with an additional elongation step for 10 minutes at 72°C.

- Second step using internal primer: 5 µl from the first step was added to 20µl GoTaq® Green Master Mix, MrNV internal primer (product size 205 bp) Forward: 5'-ACA-TTG-GCG-GTT-GGG-TCA-TA-3', Reverse: 5'-GTG-TAG-TCA-CTT-GCA-AGA-GG-3' and nuclease-free water.

PCR profile (thermal cycles): (Initial denaturation at 95°C for 2 minutes, thirty cycles of amplification were performed with the following protocol: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 1 minute, ending with an additional elongation step for 10 minutes at 72°C).

d- Nested RTPCR for detection of XSV using AccessQuick™ RTPCR kit:

This protocol was carried out according to (Sudhakaran *et al*, 2006 and OIE, 2009) with some modifications. Procedures were performed in two-step:

First step using external primer: (AccessQuick™ Master Mix 2X, external primer sequences for XSV Forward: 5'-CGC-GGA-TCC-GAT-GAA-TAA-GCG-CAT-TAA-TAA-3', Reverse: 5'-CCG-GAA-TTC-CGT-TAC-TGT-TCG-GAG-TCC-CAA-3', RNA template, AMV reverse transcriptase and nuclease-free water)

PCR profile (thermal cycles): Reaction tubes were incubated at 45°C for 45 minutes. Initial denaturation at 95°C for 2 minutes, thirty cycles of amplification were performed with the following protocol: Denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute elongation at 72°C for 1 minute and ending with an additional elongation step for 10 minutes at 72°C.

- Second step using internal primer: 5 µl from the first step was added to 20µl GoTaq® Green Master Mix, XSV internal primer (product size 236 bp) Forward: 5'-ACA-TTG-GCG-GTT-GGG-TCA-TA-3', Reverse: 5'-GTG-TAG-TCA-CTT-GCA-AGA-GG-3' and nuclease-free water.

PCR profile (thermal cycles): (Initial denaturation at 95°C for 2 minutes, thirty cycles of amplification were performed with the following protocol: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 1 minute, ending with an additional elongation step for 10 minutes at 72°C).

3-Electrophoresis

The protocol of electrophoresis was carried out according to Sambrook *et al.*, (1989). The RT-PCR products were analyzed by electrophoresis on a 1% agarose gel stain with ethidium bromide and (50-1500bp) DNA ladder marker and using an ultraviolet transilluminator.

3. Results

The results were classified according to the different protocols of RTPCR.

The results of one-step multiplex RTPCR using (BioRT One step RT-PCR kit) were negative result for both MrNV and XSV virus as showed in figure (1).

Nested RTPCR (AccessQuick™ RT-PCR kit) results were 9% for MrNV by using of external and internal primers. While, the results of one-step multiplex RTPCR and two step multiplex RTPCR using (GoScript™ Reverse Transcription System kits, Promega USA) were negative (table 1) and Figure (3).

Nested RTPCR (AccessQuick™ RT-PCR kit) results were 7% for XSV by using of external and internal primers. The results of two-steps multiplex RTPCR using poly-T primer and (GoScript™ Reverse Transcription System kits, Promega USA) were 4%. While, one-step multiplex RTPCR was negative result. (Fig 2) and (table 2).

Table (1) Showing the percentage of infections of (MrNV) by using one-step multiplex RTPCR (MRT-PCR), Two step multiplex RTPCR, nested RTPCR.

Numbers Technique	Total no. of examined specimens	Positive specimens of MrNV	
		No of infected specimens with (MrNV)	Percentage of infection%
one step multiplex RTPCR	100	0	0.0
Two step multiplex RTPCR	100	0	0.0
nested RTPCR	100	9	9

Table (2) Showing the percentage of infections of (XSV) by using one-step multiplex RTPCR (MRT-PCR), two step multiplex RTPCR, nested RTPCR.

Numbers Technique	Total no. of examined specimens	Positive specimens of XSV	
		No of infected specimens with (XSV)	Percentage of infection%
one step multiplex RTPCR	100	0	0.0
Two step multiplex RTPCR	100	4	4
nested RTPCR	100	7	7



Figure (1) Showing the negative results for the two viruses (MrNV and XSV) by using One step multiplex RTPCR using (BioRT One step RT-PCR kit). Lane (1) molecular weight marker (1500bp), Lane (2) control negative, Lane (3), Lane (4), Lane (5), Lane (6) and Lane (7) prawn specimens gave negative results (no bands for MrNV and XSV)

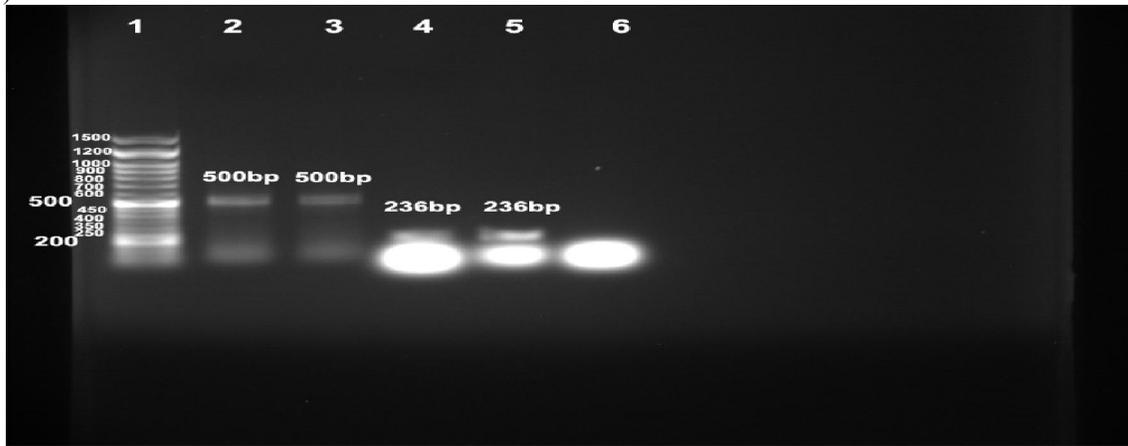


Figure (2) Showing the results of prawn specimens infected with (XSV) by using nested RTPCR and two steps multiplex RTPCR for both virus (MrNV and XSV). Lane (1) molecular weight marker (1500bp). Lane (2) and lane (3) showing prawn specimens results were positive for XSV (500bp) and negative for MrNV by two steps multiplex RTPCR. Lane (4) and Lane (5) showing prawn specimens results were positive for XSV by using nested RTPCR (236bp). Lane (6) results were negative for XSV by two steps multiplex RTPCR.

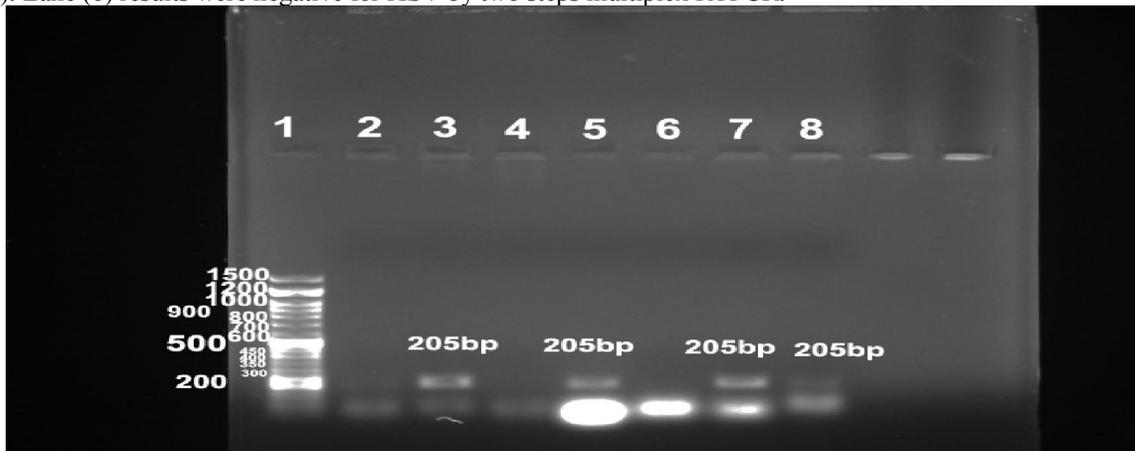


Figure (3) Showing the positive and negative results of prawn specimens infected with (MrNV) by using nested RTPCR. Lane (1) molecular weight marker (1500bp), Lane (4) control negative. Lane (2) and Lane (6) showing prawn specimens negative results with nested RTPCR. Lane (3), lane (5), Lane (7) and lane (8) showing prawn specimens with positive results (205bp) for MrNV by nested RTPCR.

4. Discussion

The present study used RTPCR for diagnosis and detection of MrNV and XSV. RTPCR was the most sensitive technique and it was taken about 6 hours for diagnosis the viral infection. Such findings were analogous with **Sri Widada *et al.*, (2003)** who discussed that the RT-PCR technique is the most sensitive and versatile diagnostic method available for detection of MrNV and XSV. It could be applied for routine health monitoring, early virus detection, studying virus–host interaction, detection of carriers and screening of broodstock, while the detection limits were 3000 virus particles by RT-PCR. In the obtained study, one-step multiplex reverse transcriptase Polymerase chain reaction (MRT-PCR) assay for simultaneous detection of MrNV and XSV was carried out to avoid the necessity of carrying out two separate RT-PCR reactions, a modified method for simultaneous detection of MrNV and XSV in a single-tube. However, the results of the one-step multiplex RTPCR using (BioRT One step RT-PCR kit) were negative by using the RTPCR primer for MrNV (product size 681 bp) and primer for XSV (product size 500 bp). These findings were attributed to the lowering of the detection sensitivity of the multiplex RT-PCR assay especially in carrier state of infection and low load of virus particles. The sensitivity is approximately 25 fg of total RNA. Such obtained results supported the results obtained by **Yoganandhan *et al.*, (2006)** who explained that it is possible that the failure to detect the dual infections due to the fact that single-step RT-PCR protocols were used. Furthermore, primer sequences for MrNV (product size 681 bp) and primer sequences for XSV (product size 500 bp). The higher primer amplified products, the lowering sensitivity to the positive specimens. Primers were chosen depending on the purpose of diagnosis. When RTPCR was used for confirmatory diagnosis of overt disease, as prawn with clear signs with intracytoplasmic inclusions and high mortality, primers yielding larger fragments can be used. When the purpose of the PCR was to screen for MrNV and XSV, as prawn without clearly lesions or apparently healthy prawn, primers yielding smaller fragments should be used (205bp and 236bp) as in nested RTPCR internal primer. Hence, it was cleared that the sensitivity of detection and number of positivity increased as the amplicons size of the primers used decreases. Such results were supported with **Hossain *et al.*, (2001)** who concluded that in the case of screening for carrier state infection as in brooders and post larvae; there is a need to use primers yielding smaller amplicons. The obtained results proved that two step multiplex RTPCR with Primer Oligo(dT)15 gave negative results for MrNV due to lack of polyadenylation of the viral RNA. Those

findings explained with **Dasmahapatra *et al.*, (1985)** and **Kaesberg *et al.*, (1990)** who established that MrNV virus have a bipartite genome of messenger-sense RNAs, which are capped but not polyadenylated. The roles of polyadenylation discussed by **Moore and Proudfoot (2009)** who considered that Nascent pre-mRNA transcripts undergo multiple co-transcriptional/post-transcriptional processing and modification events during their maturation. A poly(A) tail is added post-transcriptionally to the 3' end of almost all eukaryotic mRNAs and plays an important role in mRNA stability, nucleocytoplasmic export, and translation. The obtained study showed that nested RTPCR for detection of Mrnv using external primer and internal primer was used for the amplification (product size 205 bp) gave positive results (9%). In addition, nested RTPCR for detection of XSV using external primer and internal primer was used for the amplification (product size 236 bp) gave positive results (7%). Thus, nested RTPCR were more sensitive than multiplex TRPCR. Such findings were nearly similar to the results obtained from **OIE (2009)** who found that the detection sensitivity of the nRT-PCR is ~1000-fold greater than the one-step RT-PCR. While the sensitivity of the one-step RT-PCR assay is approximately 2.5 fg of total RNA and the detection sensitivity of the multiplex RT-PCR assay is approximately 25 fg of total RNA. Furthermore, our obtained study was in harmony with **Khawsak *et al.*, (2008)** who mentioned that the sensitivity comparison of developed multiplex RT-PCR was 150–1000 times less sensitive than single and nested PCR for detection of the other five viruses yellow-head virus (YHV), Taura syndrome virus (TSV), hepatopancreatic parvovirus (HPV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and monodon baculovirus (MBV). The lower sensitivity of multiplex RTPCR could be resulted from the competition between each primer set in the PCR reaction.

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