SCANNING OF *MACROBRACHIUM ROSENBERGII NODAVIRUS (Mr*NV) IN *MACROBRACHIUM ROSENBERGII* HATCHERIES

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Abstracts

Macrobrachium rosenbergii nodavirus (*Mr*NV) is a pathogen of giant freshwater prawns that causes white tail disease (WTD). It leads to high economic losses in hatcheries and farms of freshwater shrimp culture area across the world. In the present study, two prawns hatcheries: Twantay and Htantabin hatcheries located at Yangon Division were selected as a study sides and to examine the distribution *Macrobrachium rosenbergii* nodavirus *(Mr*NV) in different life stages of *Macrobrachium rosenbergii* using RT-PCR and RT-nested PCR (nRT-PCR). Negative results were record in all life stages of *Macrobrachium rosenbergii* from Htantabin hatchery. However, different life stages broodstocks, post-larvae and juveniles of *M. rosenbergii showed positive* in nRT-PCR at Twantay hatchery. The result in the present study indicated that *Mr*NV has been introduced to Twantay hatchery through the broodstocks.

Keyword: *Macrobrachium rosenbergii* nodavirus *(Mr*NV), RT-PCR, *Macrobrachium rosenbergii*, hatchery, Myanmar

Introduction

Farming of the giant freshwater prawn, *Macrobrachium rosenbergii* de Man 1879 is a rapidly growing industry, due to its high commercial value and inland based production. The global aquaculture production of the giant freshwater prawn has been showing 130,689 t in 2000 to 203,211 t by 2011 (FAO, 2013). However, production of the prawns has gradually declined due to various disease infections in aquaculture (Cheng and Chen, 1998).

The production success depends on disease causing agents environmental factors and sustainable management practices influence in the success of the hatchery operations (Shailender, 2012). The important disease causative agents of infectious diseases in prawns are viruses, bacteria, fungi, protozoans and other parasites. Viral diseases in shrimp aquaculture have resulted in large economic losses and it has been estimated that over the past 15 years global losses due to diseases reached more than US $15 billion (Flegel, 2012; Flegel *et al*., 2008). The pathogen can enter the hatchery system by various pathways, most important through feed, broodstocks, instruments, and water as well as unhygienic handless of workers (Sahul Hameed *et al*., 2004).

Macrobrachium rosenbergii is moderately disease-resistant in comparison to penaeid shrimp. The diseases of cultured prawn include syndromes with infectious and noninfectious. Viruses are responsible for main considerable economic losses worth prawn industries (Ganjoor, 2015). Among the pathogens, prawn viruses are very important and responsible for huge economic losses, particularly in the hatchery and nursery ponds. Reported prawn viruses include *Macrobrachium* hepatopancreatic parvo-like virus (MHPV), *Macrobrachium* muscle virus (MMV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), white spot syndrome virus (WSSV), *Macrobrachium rosenbergii* nodavirus (*Mr*NV), and extra small virus like particle (XSV).

Nowadays, the decline in the production of *M. rosenbergii* was mainly attributed to the emergence of a new disease namely, white tail disease (WTD) or white muscle disease (WMD)

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(Sahul Hameed *et al*., 2004). This disease pathogen is *Macrobrachium rosenbergii* nodavirus (*Mr*NV). This virus is a small, non-enveloped virus and containing the genome of two pieces of RNA. MrNV was first reported in the French West Indies in 1999 (Arcier *et al*., 1999). Very few post-larvae with *Mr*NV survive beyond 15 days in an outbreak, and post-larvae that survive may grow to market size like any other normal post-larvae. Adults are resistant to WTD, but act as carriers.

The disease infects hatchery-reared larvae and post-larvae in addition to nursery-raised early juveniles. *Mr*NV is responsible for large-scale mortalities in hatchery and nursery phases of prawn culture system (Vijayan *et al.*, 2005). This virus might be responsible for high economic losses particularly in hatchery in Myanmar. However, there was no scanning on *Mr*NV infection in prawn hatcheries in Myanmar. Hence, the present study was focused to detect *Mr*NV infection in Twantay and Htantabin hatcheries using RT- PCR and nRT-PCR methods.

Materials and Methods

Study site

Samples were collected from two Prawn Hatcheries in Twantay Township (16˚71' 23" N - 95˚90' 59" E) and Htantabin Township (17˚1' 4" N - 95˚58' 54" E). Two hatcheries are located in Yangon Division (Plate. 1).

Study period

The prawn sample was collected from both hatcheries from November 2018 to October, 2019.

Specimen transportation and tissue sampling

Live Specimens were transported from each hatchery to the Aquatic Animal Diseases Laboratory, Fisheries and Aquaculture, University of Yangon. Samples were extracted RNA immediately after arriving at the Laboratory. From each hatchery, the pleopods of five broodstocks, eggs from the abdomen of broodstocks, thirty nine post-larvae and five juvenile stages were collected. The samples were collected in 1.5 ml tube separately.

For eggs, a total of twenty eggs were pooled into one sample tubes and altogether five sample tubes were prepared. Similarly, for post-larvae, three post-larvae were pooled into one single 1.5 ml tube and extracted for RNA. Therefore, there were a total of thirteen post-larvae tubes for RNA extraction for post-larvae. For juveniles, five juveniles were collected in the tube separately. The number of samples is described in Table 1.

Table 1 Number of samples collected from Twantay and Htantabin hatcheries

Tissue	Collected samples from each hatchery	Number of samples for RNA extraction
Pleopods of broodstocks	5	5
Eggs of broodstocs	100	5 (20 eggs in each sample tube)
Pleopods of juvenile	5	5
Post-larvae	39	13 (3 individuals in each sample tube)

RNA extraction

Total RNA was extracted from tissue using the PETNAD nucleic acid co-prep kit (GeneReach Biotechnology Corp) (Plate 2). Tissue (20-25 mg) was ground using disposable plastic grinder with 100 µl PB1 solution. Then PB1 solution (500 µl) was added and vortexed for one minute. A total of 600 µl PB2 solution, 600 µl of PB3 solution and 600 µl of PB4 solution were added step by step according to the manual instruction. The dry RNA pellet was eluted with 50 µl of PB 5 solution. Extracted RNA concentrations were measured using NanoDrop 2000 Spectrophotometer and stored at -20˚C.

RT-PCR and nRT-PCR analysis for *Mr*NV infection

Two set of primers were used to detect *Mr*NV using RT-PCR and nRT-PCR**.** First step RT-PCR was carried out for specific detection of *Mr*NV in serious infected prawns. Nested RT-PCR was detected low viral load of *Mr*NV in initial infection stage (Table 2).

Table 2 Primers used for RT-PCR and nRT-PCR analysis in *Mr*NV (Sahul Hameed et al., 2004 and Sudhakaran *et al*., 2007)

RT-PCR	Primers	Sequences 5' to 3'	Product Size
RT-PCR	Forward	GCG-TTA-TAG-ATG-GCA-CAA-GG	425bp
	Reversed	AGC-TGT-GAA-ACT-TCC-ACT-GG	
Nested PCR	Forward	GAT-GAC-CCC-AAC-GTT-ATC-CT	205bp
	Reversed	GTG-TAG-TCA-CTT-GCA-AGA-GG	

The volume of the reaction mixture for the first step RT-PCR was 20 µl containing 10.0 µl of RT-PCR MasterMix (WizPure™ RT-PCR 2X Master), 1.2 µl of MrNV RT-PCR Primer Mix, 6.8 µl of ddH2O, 2.0 µl of RNA. The total volume is 20.0 µl using the following cycles for RT-PCR: at 42°C for 30 minutes; initial denaturation at 95°C for 10 minutes, followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 20 seconds. The cycle was ended with an additional elongation step for 5 minutes at 72°C. The condition of PCR thermal cycler was described in Table 3.

In the nRT-PCR, the amplified products of the first step RT-PCR served as the template DNA for the nested PCR of amplification. After completion of the first step RT-PCR, nRT-PCR reaction was made with 10.0 µl of HS-PCR master Mix ((WizPure™ PCR 2X Master), 1.2 µl of *Mr*NV nRT-PCR Primer Mix, 7.8 µl of ddH2O, 1.0 µl of RT-PCR product. The total volume is 20.0 µl using the following cycles: 95°C for 5 minutes, followed by 25 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 10 seconds at 72°C with a final extension at 72°C for 5 minutes. The condition of PCR thermal cycler was described in Table 4. RT-PCR and nRT-PCR were conducted using miniPCR™ mini8 thermal cycler (minipcr^{TM} bio) (Plate 2).

Table 3 Thermal cycling condition for RT-PCR to detect *Mr*NV

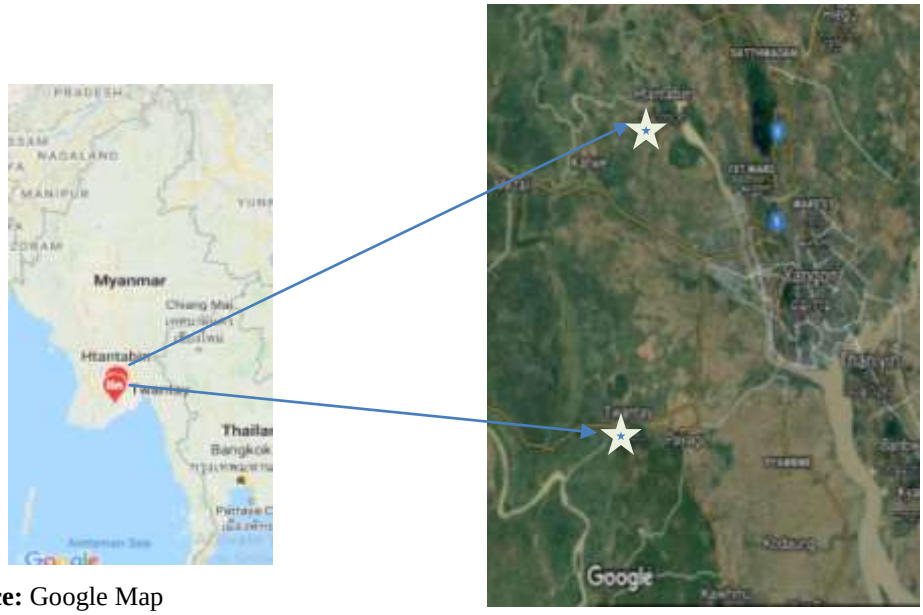
Temperature℃	Time	cycles	Step
42	30 minutes	1	Reverse transcription
95	10 minutes	1	Initial denaturing
95	30 seconds		denaturing
60	30 seconds	25	annealing
72	30 seconds		extension
72	5 minutes	1	Final extension

Table 4 Thermal cycling condition for nRT-PCR to detect *Mr*NV

Temperature℃	Time	cycles	Step
95	10 minutes	1	initial denaturing
95	30 seconds		denaturing
60	30 seconds	25	annealing
72	30 seconds		extension
72	5 minutes	1	final extension

Gel electrophoresis

Both RT-PCR and nRT-PCR products were separated and visualized by agarose gel electrophoresis using 1.5 % agarose gel containing 30ml of 1XTAE buffer. Gel were prepared with 0.15g of agarose powder diluted in 40ml of 1x TAE buffer together with 6 µl of Gel stain green (Wizbiosolutions Inc). A 100bp of DNA ladder marker (Wizbiosolutions Inc) was used as an indicator to compare the specific base pair of *Mr*NV. The gel was check under UV Blue light transilluminator (minipcrTM bio) for the result (Plate 4).



Source: Google Map

Plate 1 Location of Twantay and Htantabin Hatcheries

Plate 2 PETNAD nucleic acid co-prep kit (GeneReach Biotechnology Corp)

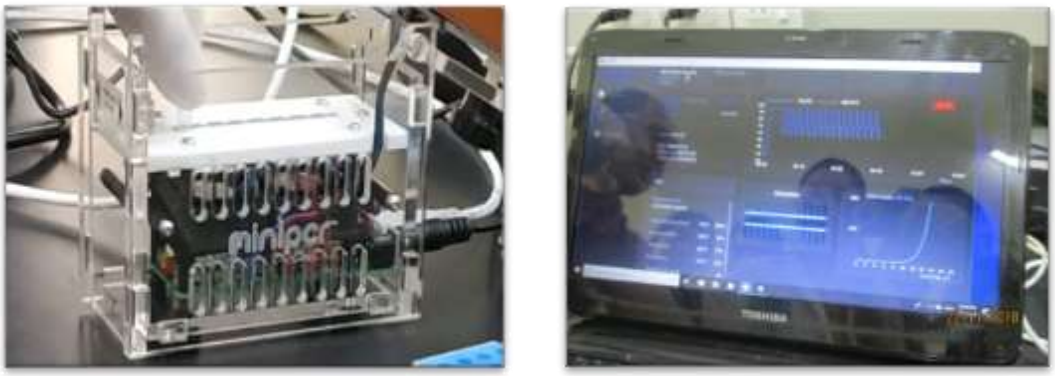


Plate 3 MiniPCR™ mini8 thermal cycler (minipcrTM bio)

Plate 4 Gel electrophoresis (minipcrTM bio)

Results

Concentration of extracted RNA

Concentration of RNA in all samples ranged 105 to 297 ng/µl and it has over 2.1 in 260/280 purity ratio.

Detection of *Macrobrachium rosenbergii* nodavirus (*Mr*NV)

Broodstocks, juveniles and post-larvae of *M. rosenbergii* were obtained from Twantay and Htantabin hatcheries to detect the *Mr*NV. In Twantay hatchery, all samples were showed negative in RT-PCR. However, three out of five broodstocks samples showed positive in nRT-PCR (Plate5, Table 4). However, eggs collected from the broodstocks were all negative (Plate 6 and Table 4).In juvaniles stage, two out of five samples were positive in nRT-PCR (Plate 6 and Table 4). Nine out of thirteen post-larvae were also showed positive in the samples collecd from Twantay hatchery (Plate 7 and Table 4). All positive samples indicated the presence of *Mr*NV at 205 bp.

All samples collected from Htantabin hatchery were negative in both RT-PCR and nRT-PCR (Plate 8,9,10 and Table 5).

Table 4 The RT-PCR and nRT-PCR results for *Mr*NV detection in prawn collected from Twantay Hatchery

Tissue	Examine samples	Positive in RT-PCR	Positive in nested PCR
Pleopods of broodstocks	5	0/5	3/5
Eggs	5 pooled sampes	0/5	0/5
Pleopods of juvenile	5	0/5	2/5
Post-larvae	13 pooled samples	0/13	9/13

Table 5 The RT-PCR and nRT-PCR results for *Mr*NV detection in prawn collected from Htantabin Hatchery

Tissue	Examine samples	Positive in RT-PCR	Positive in nested PCR
Pleopods of broodstocs	5	0/5	0/5
Eggs of broodstocs	5 pooled sampes	0/5	0/5
Pleopods of juvenile	5	0/5	0/5
Post-larvae	13 pooled samples	0/13	0/13

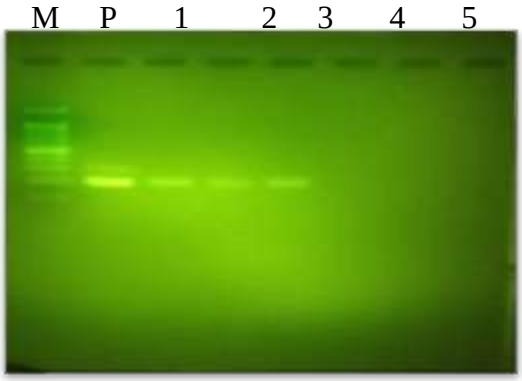


Plate 5 The result of nRT-PCR for the broodstocks collected from Twantay hatchery

M: 100bp DNA marker, P: positive control, N: negative control, Well 1 to 5: Broodstock samples

Note: Well 1 to 3 show positive for *Mr*NV.

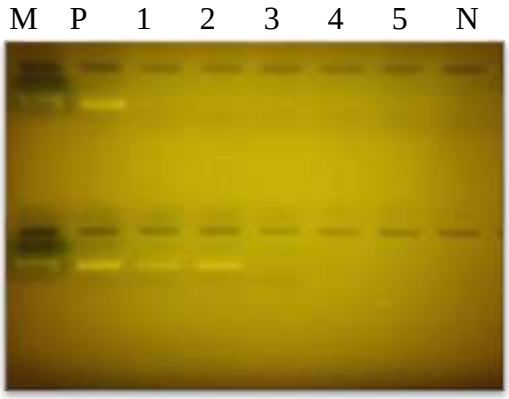


Plate 6 The result of nRT-PCR for the eggs (upper gel) and juveniles (lower gel) collected from Twantay Hatchery

M: 100bp DNA marker, P: positive control, N: negative control, Well 1 to 5 (upper gel): eggs pooled samples, Well 1 to 5 (Lower gel): Juveniles samples

Note: Well 1 and 2 (lower gel) show positive for *Mr*NV.

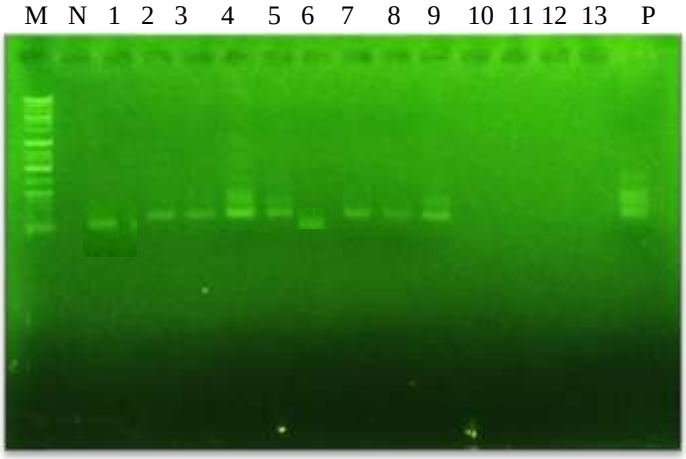


Plate 7 The result of nRT-PCR for post-larvae collected from Twantay Hatchery

M: 100bp DNA marker, P: positive control, N: negative control, Well 1 to 13 post-larvae pooled samples

Note: Well 1 and 9 show positive for *Mr*NV

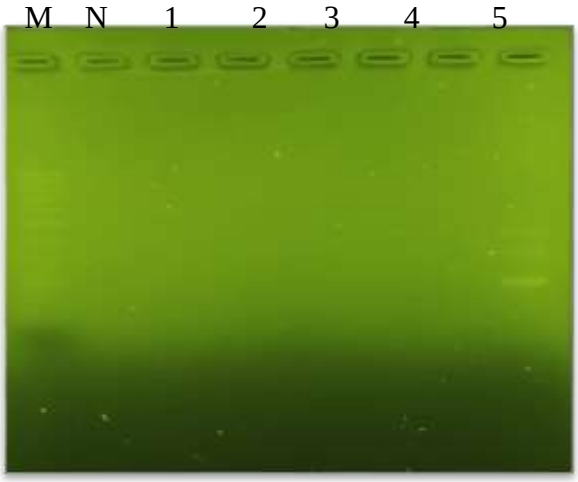
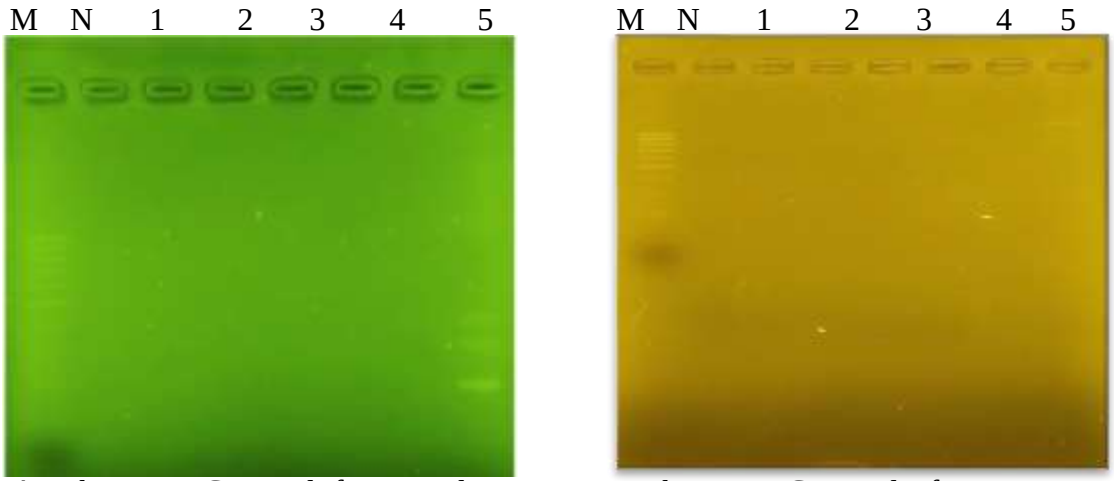


Plate 8 The result of nRT-PCR for the broodstocks collected from Htantabin Hatchery

M: 100bp DNA marker, P: positive control, N: negative control, Well 1 to 5: broodstock samples



A. The nRT-PCR result for juveniles B.The nRT-PCR results for eggs

Plate 9 The result of nRT-PCR for the juveniles and eggs collected from Htantabin Hatchery

M: 100bp DNA marker, P: positive control, N: negative control, Well 1 to 5: collected samples

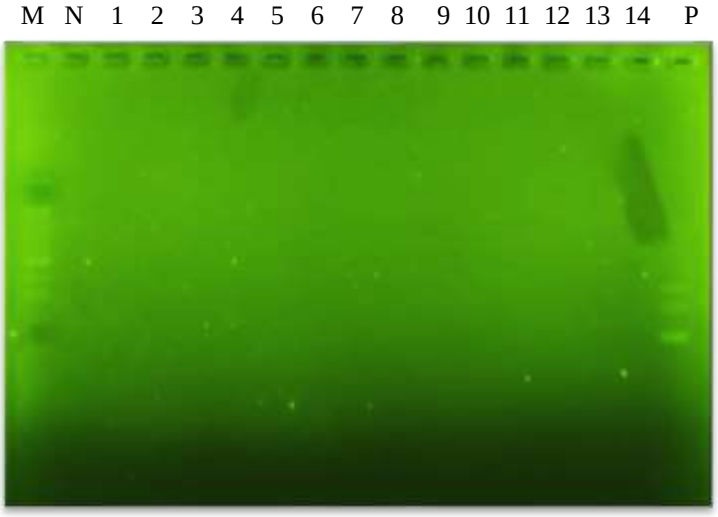


Plate 10 The result of nRT-PCR for the post larvae collected from Htantabin Hatchery

M: 100bp DNA marker, P: positive control, N: negative control, Well 1 to 13: collected samples

Discussion

M. rosenbergii nodavirus (*Mr*NV) has been identified the causative agent for white tail disease in the freshwater prawn and the diseases was reported in West Indies in 1994 (Arcier *et al.*, 1999), Taiwan and China in 1999 (Tung *et al*., 1999; Sri Widada *et al*., 2003), Thailand in 2007 (Sudthongkong *et. al.*, 2007) and Malaysia in 2012 (Tayebeh Azam Saedi *et al*. (2012). This report is first report of the scanning of *Macrobrachium rosenbergii* nodavirus *(Mr*NV) in giant freshwater prawns, *M. rosenbergii*, hatcheries in Myanmar. Different stages of freshwater prawns were obtained from Twantay and Htantabin hatcheries and diagnosed for *(Mr*NV) using RT-PCR and nRT-PCR methods.

We used both RT-PCR and nRT-PCR since nRT-PCR is more sensitive and useful for screening of *Mr*NV even in low viral load. Different diagnostic methods are available to detect MrNV/XSV in prawn and among these methods, RT-PCR is the most sensitive (Romestand and Bonami 2003; Sri Widada *et al*. 2003; Sahul Hameed et al. 2004; Yoganandhan *et. al*., 2005; Sudthongkong *et. al*., 2007). In the present study, *Mr*NV infection was negative in RT-PCR while it was positive result in nRT-PCR except from eggs at Twantay hatchery. The positive of 1st RT-PCR indicates a serious infection of *Mr*NV, when the nested PCR positive result shows low viral load. According to the results, it was identified that *Mr*NV infection has been introduced to Twantay hatchery through the broodstocks.

Yoganandhan *et. al.*, (2006) have been reported that *Mr*NV could be detected in broodstock of prawns but not showed gross sing of disease. Similarly the previous authors were reported that adults are tolerance to *Mr*NV infection, but act as carriers (Qian *et al*., 2003 and Sahul Hameed *et al*., 2004). In Twantay hatchery, although the broodstocks,post-larvae and juveniles were positive of *Mr*NV in the nested PCR, were not show any prominent signs of disease. The present study supports the collected prawns at Twantay hatchery are carrier state with light infection because positive results are only appeared in nRT-PCR. *Mr*NV was not detected in egg collect from Twantay hatchery. Egg has height concentration of lipid and it may take time for the virus to penetrate the eggs.

*Mr*NV have been reported to infect larvae and post-larval of *M. rosenbergii* but not adult prawn (Qian *et a*l., 2003, Sri Widada *et al.*, 2003).Larvae, post-larvae and early juveniles are susceptible, whereas adults are resistant (Qian *et al*., 2003; Sahul Hameed *et al*., 2004). The viral

transmission routes are vertical and horizontal (Sahul Hameed *et al*., 2004). In the present study, *Mr*NV was found in the broodstocks, post-larvae and juveniles by nRT-PCR methods in Twantay Hatchery.Therefore, it is assume that vertical transmission of *Mr*NV was occurred in the hatcheries. However, there was negative result in eggs samples. Further study are needed to confirm the reason of negative result in eggs.

All stage of prawns were negative in both 1stRT-PCR and nRT-PCR at Htantabin hatchery. It is assume that broodstocks introduced to Htantabin hatchery was free from the infection of *Mr*NV and as a consequent all life stages of prawns were free from the virus. Therefore, scanning on *Mr*NV infection before introducing to the hatchery is essential to increase the biosecurity in the area.The important role of biosecurity highted according to the results of the present study.

Conclusion

*Mr*Nv iinfection was scanning in two prawns hatcheries in Myanmar using RT-PCR and nRT-PCR methods. Broodstocks, post-larvae and juveniles were negative in 1st RT-PCR but they were positive in nested PCR except from eggs in Twantay hatchery. The present work revealed that collected prawns from Twantay hatchery were infection with light infection of *Mr*NVwhich are positive with nRT-PCR only. The virus can be transmitted horizontally from broodstocks to post-larve and juvinile. In the other hands, all life stages of prawn showed negative result in both first RT-PCR and nRT-PCR in Htantabin hatchery. Therefore, screen for *Mr*NV in broodstock should be undertaken before introducing to the hatcheries to control the *Mr*NV infection in farms.

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