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Mass larval mortality in a giant freshwater prawn *Macrobrachium rosenbergii* hatchery: an attempt to detect microbes in the berried and larvae

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Abstract. Early mass mortality of *Macrobrachium rosenbergii* larvae is a big problem of hatchery failure. *Vibrio* spp. and/or *M. rosenbergii* nodavirus (MrNV) have been reported to be the major causes of mass mortality of *M. rosenbergii* larvae. An attempt was made to detect the reported bacterial and viral pathogens in the berried and larvae of *M. rosenbergii*. The berried were collected from the Kocha river, Pirojpur and brought to the hatchery. For detection of bacterial pathogen, five berried were collected before the treatment with disinfectant and five berried were collected after the treatment and release of the hatchlings. Larvae samples were collected from the larvae rearing tanks after five days and 15 days post-hatching. The samples were processed, inoculated in TCBS agar media and PCR was done using primers designed from *groEL* and 16S rRNAgenes for *Vibrio* sp. MrNV detection kit was used to detect MrNV. We have identified two bacterial species namely *Morganella morganii* and *Citrobacter freundi* in the berried samples. However, no *Vibrio* spp. nor MrNV was detected in either of the samples of berried and larvae. However, mass mortality was observed in the hatchery resulting in a poor PL production. Therefore, other factors might be responsible for the mass mortality in the prawn hatchery that demands further in depth investigations.

Keywords: *Macrobrachium rosenbergii*, Bacteria, Virus, Larvae

Introduction

Bangladesh is considered to be one of the most suitable countries for *Macrobrachium rosenbergii* (Galda) farming in the region because of its favourable agro-climatic conditions. The prawn and shrimp industry of the country constitute the second largest industry in terms of export earnings. The increasing price of galda in local and international markets had promoted rapid expansion of galda farming in 1990s and early 2000s. The overall scenario on cultivation area, production and export of galda in Bangladesh is not satisfactory compared to its high potential as a freshwater aquaculture species. Unpredictability in post-larvae (PL) production in the hatcheries is considered as the major drawback of expanding galda farming in Bangladesh. Prawn hatcheries in Bangladesh have been encountering difficulties in PL production since 2012. Production level has drastically fallen and many hatcheries have already been closed. A total of 50 million PL was produced from 18 operating hatcheries in 2003-2004, which increased to 1.25 billion in 2011-2012 when 53 hatcheries were under operation (FRSS 2012). The PL production then drastically reduced to only 33.1 million in 2012-2013 and 43 million in 2014-2015 when only 18 out the 53 hatcheries were under operation.

The problems appear in larvae rearing period include reduced or complete cessation of feeding, high or complete mortality and delayed or no molting to PL. A number of factors including microbial infection, unsuitable water parameters, and temperature fluctuation were thought to be the reasons behind failure in prawn PL production. However, proper identification of the problems and their comprehensive solution have not been achieved yet. Infectious diseases in aquatic organisms are one of the main risks for economic losses in galda farming and many of these diseases are caused by bacteria. Bacteria of the genus *Vibrio* are important pathogens for farmed crustaceans and also have been reported as primary agents of bacterium-associated illness due to seafood consumption and handling, with emphasis on the species *Vibrio parahaemolyticus* (*V. parahaemolyticus*), *Vibrio vulnificus* (*V. vulnificus*) and *Vibrio harvayi* (*V. harveyi*). (Banerjee *et al*. 2012, Hossain *et al.* 2013, Robert-Pillot *et al*. 2014).

Mortality outbreaks with clinical signs of white tail disease (WTD were observed for the first time in hatchery-reared *M. rosenbergii* PL in Guadeloupe Island (French West Indies) in 1997 and the involvement of viral association was discovered (Arcier *et al.* 1999). Subsequently, this disease was reported in China (Qian *et al.* 2003), India (Hameed *et al.* 2004), Thailand (Yoganandhan *et al.* 2006), Taiwan (Wang *et al.* 2008) and Australia (Owens *et al*. 2009). The occurrence of WTD is still being reported from all prawn growing countries. The clinical signs of WTD -infected PL include lethargy and whitish appearance of the abdominal muscle extending to the tail region. The causative organisms for WTD are viruses which are named as *M. rosenbergii* nodavirus (MrNV) and extra small virus-like particle (XSV) (Bonami *et al.* 2005). MrNV is a small icosahedral non-enveloped particle of 26-27 nm in diameter. It contains two single-stranded RNAs: RNA1 (2.9 kb) and RNA2 (1.6 kb). It is closely related to the Nodaviridae family. The typical gross signs of WTD in infected PL are lethargy and opaque abdominal muscles. Mortality reached 100% within 2 to 3 days after the first appearance of prawns with whitish muscles. The causative agents of this disease are *M. rosenbergii* nodavirus (MrNV) and extra small virus (XSV). Research result shows that MrNV plays a key role in WTD and that XSV is a satellite virus dependent on MrNV. The objective of the study is to identify most common pathogenic bacteria and virus from *M. rosenbergii* berried and larvae.

Materials and Methods

***Collection of berried and larvae samples*:** A total of 350 berried were collected from the Kocha river, Pirojpur from artisanal fishermen, brought to the BRAC Prawn Hatchery, Barisal by truck in 200-L drums equipped with aerators for one cycle of hatchery operation. For the treated group, the berried were acclimatized to 6 ppt salinity for 2-3 hrs and then treated with formalin at a dose of 15 ml/50L for 30 min. The berried were then treated with povidone-iodine for 45 sec and transferred to the hatching tank. For this study, five treated and five untreated apparently healthy berried were randomly selected and preserved in freezer. The samples were aseptically transported to the Microbiology Laboratory of the Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh maintaining cool condition using flacked ice. The larvae samples were collected from the larvae rearing tank after 5 and 15 days post-hatching and preserved in 95% ethanol.

***Tissue preparation and culture of bacteria*:** The brain, hepatopancreas and intestine of each prawn was removed aseptically, placed in brain heart infusion (BHI) broth containing 2% NaCl and incubated at 37°C for 8 hrs with a view to enriching the growth of *Vibrio* spp. Each broth

culture was streaked on thiosulfate-citrate-bile salts-sucrose (TCBS) agar media and overnight incubated at 37°C. Subculture was done on same media to obtain pure culture.

***Extraction of chromosomal DNA and PCR amplification*:** One milliliter of overnight broth culture of each isolate was centrifuged at 12000 rpm for 3 min. Supernatant was discarded and total DNA from the cells was isolated following phenol: chloroform: isoamyl alcohol extraction method (Sambrook *et al*. 1989). One set of primer designed from *groEL* gene was used for the specific detection of *Vibrio* genus (Hossain *et al*. 2014) and for further confirmation the universal primer set (27f and 1492r) (Lane 1991) was used to amplify 16SrRNA gene fragment.

***Sequencing of DNA fragment generated by PCR*:** Sequencing of the 16SrRNA gene fragments of the isolates was done from Macrogen, South Korea. For bacterial identification, 16SrRNA gene sequences were submitted to the Gene Bank database of National Center for Biotechnology Information (NCBI), USA and homology with the closest known relatives were estimated using the BLAST program (Altschul *et al*. 1990). The 16SrRNA gene sequences of the isolates were used to construct a neighbor-joining tree following Saitou and Nei (1987) using the software MEGA7.0 (Kumar *et al*. 2016).

***Detection of MrNV infection*:** Samples were collected from one untreated and one spent berried; and larvae of first and second cycle and analyzed for MrNV infection using the MrNV detection kit IQ 2000 (GeneReach Biotechnology Corp, Taiwan).

***Water Quality Parameters Monitoring*:** Early life stages are the most sensitive phase in the complex life cycle of aquatic invertebrates and physicochemical parameters of water play important roles on embryonic and larval development and survival of *M. rosenbergii*. Among the physicochemical parameters temperature, pH and ammonia are very crucial and were monitored throughout the operation cycle. pH of the LRT waters was estimated using a benchtop pH Meter (Mi 150, Milwaukee). AQUA AM Ammonia Test (Made in Thailand) was used for estimation of Ammonia. A thermometer was used for reading the water temperature of the LRTs.

Results and Discussion

***Cultural characteristics of suspected Vibrio spp.*:** Both green and yellow colonies were observed on TCBS agar media after primary culture (Fig. 1a). Pure cultures of yellow and green colonies were used for PCR detection (Fig. 1b).

***Detection of Vibrio spp. by PCR*:** No amplification of target *groEL* gene fragment (1117 bp) from all the suspected isolates indicated that all collected samples were negative for the genus *Vibrio* (data not shown).

***Identification of bacterial species by sequencing of 16SrDNA*:** In order to identify the bacterial strains present in the treated and non treated berried, the 16SrDNA fragment was amplified using universal primers (Fig. 2). The 16SrDNA sequence information was used to identify most bacteria. We multiplied the bacteria by culturing in selective media. So, we hypothesized that sequencing of the PCR fragment would correctly identify the bacterial strain

present in the berried. This experiment would also give information about the difference in the infection in treated and non-treated berried.

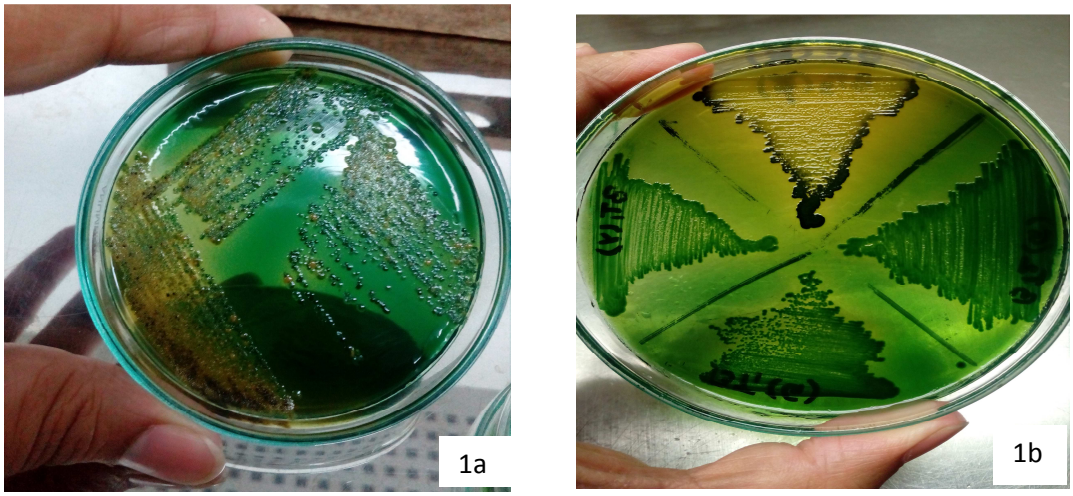

Fig. 1. Bacterial culture on selective TCBS agar media. Colony was observed both in un-treated and treated *M. rosenbergii* berried (a) Mixed and (b) back side of the petridish.

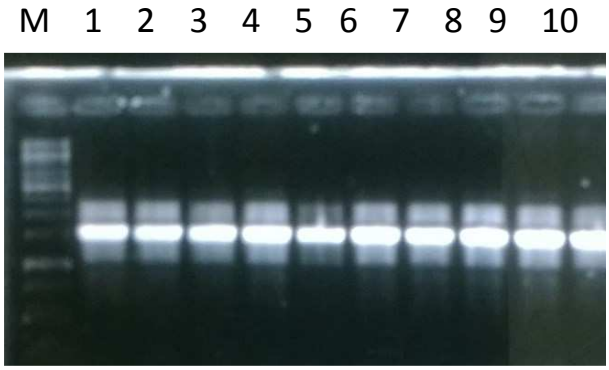

Fig. 2. Electropherogram of the PCR products amplified by universal primers 27f/1492r for 16SrDNA. M: Molecular weight marker; 1-4: Before treatment; 5-10: After treatment.

The results showed that there was no *Vibrio* spp. in both the non-treated and treated berried samples. Non-treated means the berried samples were collected before the antibiotic and other aseptic treatments in the hatching tank. Treated samples were collected after completion of hatching. We have, however, clearly identified two bacterial species from the samples showing 99-100% similarity with those of known sequence present in the genbank: (1) *Morganella morganii* and (2) *Citrobacter freundi*. *Morganella morganii* is a gram-negative rod shaped bacterium commonly found in the environment and in the intestinal tracts of humans, mammals,

and reptiles as normal flora. Al-Dulaimi *et al*. (2016) also detected *Morganella morganii* from very popular seafood named blood cockles (*Anadara granosa*) collected from wet and supermarket in Malaysia while isolating *Vibrio vulnificus*. *Citrobacter freundii* is a species of facultative anaerobic gram-negative bacteria of the Enterobacteriaceae family. *C. freundii* is a soil organism, but can also be found in water, sewage, food and in the intestinal tracts of animals and humans (Wang *et al*. 2000). The two bacteria, *M. morganii* and *C. freundii* identified in the samples have no known relation with diseases in prawn and shrimp. However, there is some evidence that they can cause human diseases along with other bacteria (Liu *et al*. 2016, Koshy *et al*. 2016).

In order to assess any phylogenetic relationship of the two bacterial species with the commonly reported pathogenic *Vibrio* spp., a Neighbor-Joining phylogeny tree was constructed based on the similarity of the sequence with those present in the genbank (Fig. 3). The phylogeny tree shows that all the seven *Vibrio* spp. *viz. Vibrio alginolyticus, V. parahemolyticus*, *V. harveyi*, *V. azureus*, *V. campbellii*, *V. vulnificus* and *V. navarrensis* have formed one cluster clearly separated from *Morganella morganii* and *Citrobacter freundii*. *M. morganii* and *C. freundii* belonged to two separate sub-clusters. We first tried to identify three *Vibrio* species namely *Vibrio alginolyticus, V. parahemolyticus*, *V. harveyi* by using specific primers but could not detect any of the three strains in none of the samples (results not shown).

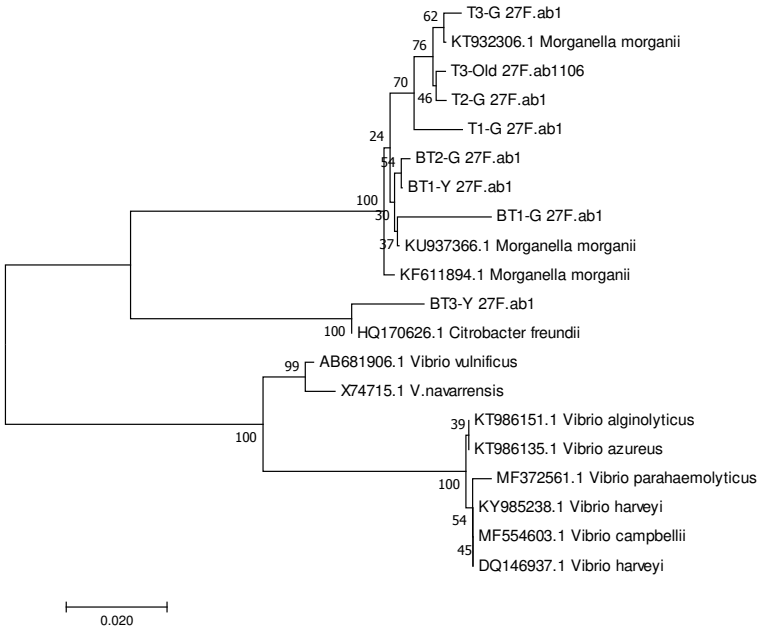

Fig. 3. Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. T1, T2, T3 indicates treated samples and BT1, BT2 and BT3 indicates untreated (before treatment) samples. G indicates green colony and Y indicates Yellow colony.

***Detection of MrNV infection*:** Viruses can be detected by almost all organs. They even presented in ovarian tissue which indicates the possibility of vertical transmission of WTD from brooders to larvae and PL. Since *M. rosenbergii* has a much lower fecundity of 20,000- 30,000, it is more prudent to have berried females tested to prevent the occurrence of WTD at the hatchery stage. We examined the occurrence of MrNV, one of the most devastating virus causing early mortality of *M. rosenbergii* larvae reported in different countries. We collected samples from one non-treated and one spent berried and larvae and analyzed for MrNV infection. MrNV was not detected in any of the four samples (Fig. 4).

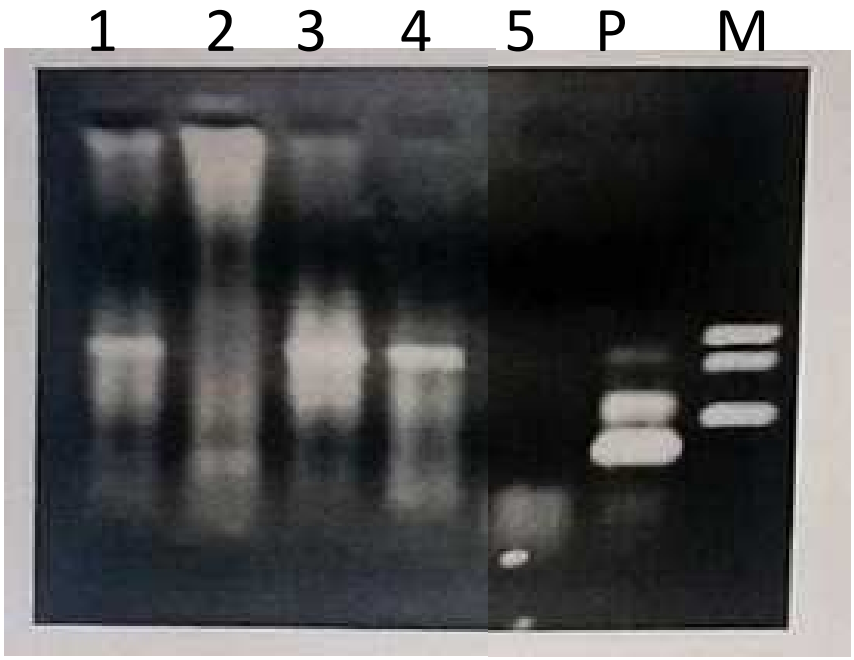

Fig. 4. Analysis of reverse transcriptase-polymerase chain reaction (RT-PCR) products with MrNV specific primers. Lane 1: Spent berried (i.e. treated); 2: Non-treated berried; 3: Larvae (Day 5); 4: larvae (day 15) Lane 5: Negative control; Lane P: Positive control; Lane M: Molecular weight marker.

***Water quality parameters*:** LRT temperature, pH and ammonia recorded during the operation cycle are presented in Table I. Temperature ranged from 26 to 31^{O}C, pH ranged from 7.4 to 8.1 and ammonia ranged from 0 to 5.0 ppm. The Prawn Hatchery under the study experienced mass mortality during the study cycle. In an effort to identify if any pathogen might have been the cause of the mass mortality, we looked for identifying the most commonly reported prawn pathogens such as MrNV-XSV virus and bacterial pathogen *Vibrio* spp. However, all the analysis for the presence of MrN virus and *Vibrio* pathogens indicated that the selected *Macrobrachium* hatchery was free from the common pathogens and the mortality is due to other cause. Dhar *et al*. (2019) inferred that the Early Mortality Syndrome (EMS) was caused by *Vibrio parahaemolyticus*, now known as AHPND which was not detected in this study. Therefore, it can be concluded that the mass mortality was not due to the commonly occurred viral and bacterial pathogen infection.

Table I. Water quality parameters recorded during the production cycle

Parameters	Tolerance limit range		Observed range		Mean±SD
	Lower limit	Upper limit	Lower limit	Upper limit	
Temperature (^{O}C)	28	31	26.0	31.0	28.87±0.696
pH	7.0	8.5	7.4	8.1	7.807±0.186
Ammonia (mg/l)	<0.3	0	5.0	1.056±1.069	

Temperature management is a critical and difficult job in a *Macrobrachium* hatchery. The optimum range of temperature for *Macrobrachium* larvae is from 29-31^{O}C. Temperature may go up beyond the upper limit of the optimal range in hot sunny days. On the other hand, on rainy cool days, it may go down the lower limit of the optimal range. Either situation can cause heavy mortality of the larvae. During the cycle, the LRT temperature went below 26^{O}C due to heavy rains that might be the crucial factor for the mass mortality and non-conversion of larvae into PL in the hatchery. We have observed that larvae survived more than 30 days but PL conversion was very low. So, not only the potential pathogens, we have to look for other reasons for mass mortality of galda larvae in the hatchery.

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Literature Cited

Al-Dulaimi, M.K., S.A. Mutalib, M.A. Ghani, Al-Hashimi, Hassan, A. Al-Kuwari and M.S. Alam, 2016. Antibiotic susceptibility and plasmid profile of *Vibrio vulnificus* isolated from cockles (*Anadara granosa*) in Selangor, Malaysia. *Asian J. Microbiol. Biotechnol. Environ. Sci.,* 18(2): 25-34.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990. BLAST: Basic Local Alignment Search Tool. *J. Molec. Biol.,* 215: 403-410.

Arcier, J.M., F. Herman, D.V. Lightner, R.M. Redman, J. Mari and J.R. Bonami,1999. A viral disease associated with mortalities in hatchery-reared post-larvae of the giant freshwater prawn *Macrobrachium rosenbergii*. *Dis. Aquat. Org.*, 38:177–181.

Banerjee, S., M.C. Ooi and H. Khatoon, 2012. Antibiotic resistant *Salmonella* and *Vibrio* associated with farmed *Litopenaeus vannamei*. *Sci. World J.*, ID 130136.

Bonami, J.R., Z. Shi and D. Qian, 2005. White tail disease of the giant freshwater prawn, *Macrobrachium rosenbergii*: separation of the associated virions and characterization of MrNV as a new type of nodavirus. *J. Fish Dis.*, 28: 23–31.

Dhar, A.K., P. Piamsomboon, L. Fernando, A. Caro, S. Kanrar, Jr.R. Adami and Y-S. Juan, 2019. First report of acute hepatopancreatic necrosis disease (AHPND) occurring in the USA. *Dis. Aquat. Org.,* 132: 241-247.

FRSS. 2012. Fisheries Statistical Yearbook of Bangladesh. Fisheries Resources Survey System (FRSS), Department of Fisheries, Bangladesh. Volume 28. 46 p.

Hossain, M.T., Y.O. Kim and I.S. Kong, 2013. Multiplex PCR for the detection and differentiation of *Vibrio parahaemolyticus* strains using the *groEL, tdh* and *trh* genes. *Mol. Cell Probes*, 27: 171-175.

Hossain, M.T., Y.R. Kim and I.S. Kong, 2014. PCR-restriction fragment length polymorphism analysis using *groEL* gene to differentiate pathogeniv *Vibrio* species. *Diagn. Microbiol. Infect. Dis.,* 78: 9-11.

Kimura, M.,1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Molec. Evol.*,16:111-120.

Koshy, M., R. Ralph, K.P. Abhilash and G.M. Varghese, 2016. *Citrobacter freundii* : A rare cause of native valve endocarditis. *J. Med. Soc.,* 30: 182-183.

Kumar, S., G. Stecher and K. Tamura, 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molec. Biol. Evol., 33:1870-1874.

Lane, D.J., 1991. 16S/23S rRNA sequencing, In E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. p. 115-147. John Wiley & Sons, New York, NY.

Liu, H., J. Zhu, Q. Hu and X. Rao, 2016. *Morganella morga*nii, a non-negligent opportunistic pathogen. I*nt. J. Infect. Dis.,* 50: 10-17.

Owens, L., K. La Fauce, K. Juntunen, O. Hayakijkosol, C. Zeng, 2009. *Macrobrachium rosenbergii* nodavirus disease (white tail disease) in Australia. *Dis. Aquat. Org.,* 85: 175–180.

Qian, D., Z.L. Shi, S.Y. Zhang, Z. Cao, W. Li. Liu, L.Z. Xie, Y.L. Cambournac and J.R. Bonami. 2003. Extra small virus-like particles (XSV) and nodavirus associated with whitish muscle disease in the giant freshwater prawn, *Macrobrachium rosenbergii*. *J. Fish Dis.,* 26: 521-527.

Robert-Pillot, A., S. Copin, C. Himber, M. Gay and M.L. Quilici, 2014. Occurrence of the three major *Vibrio* species pathogenic for human in seafood products consumed in France using real-time PCR**.** *Int. J Food Microbiol.*, 189: 75-81.

Hameed, A.S. Sahul, K. Yoganandhan, J.J. Sri Widada and R. Bonami, 2004. Studies on the occurrence of *Macrobrachium rosenbergii* nodavirus and extra small virus like particles associated with white tail disease of *M. rosenbergii* in India by RT-PCR detection. *Aquaculture,* 238: 127–133.

Saitou N. and M. Nei, 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molec. Biol. Evol.,* 4: 406-425.

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, New York Cold Spring Harbor Laboratory Press. 1546 p.

Sri Widada, J. and J.R. Bonami. 2004. Characteristics of the monocistronic genome of extra small virus, a virus-like particle associated with *Macrobrachium rosenbergii* nodavirus: possible candidate for a new species of satellite virus. *J. Gen. Virol.,* 85: 643–646.

Tran, L., L. Nunan, R.M. Redman, L.L. Mohney, C.R. Pantoja, K. Fitzsimmons and D.V. Lightner, 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Dis. Aquat. Organ.,* 105(1): 45–55.

Wang, J.T., S.C. Chang, Y.C. Chen, and K.T. Luh, 2000. Comparison of antimicrobial susceptibility of *Citrobacter freundii* isolates in two different time periods. *J. Microbiol. Immunol. Infect.,* 33 (4): 258–262.

Wang, C.S., J.S. Chang, C.M. Wen, H.H. Shih and S.N. Chen, 2008. *Macrobrachium rosenbergii* nodavirus infection in *M. rosenbergii* (de Man) with white tail disease cultured in Taiwan. *J. Fish Dis.,* 31: 415–422.

Yoganandhan, K., M. Leartvibhas, S. Sriwongpuk and C. Limsuwan, 2006. White tail disease of the giant freshwater prawn *Macrobrachium rosenbergii* in Thailand. *Dis. Aquat. Org.,* 69:255–258.

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