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Dual infections of IMNV and *Mr*NV in cultivated *Penaeus vannamei* from Indonesia

Saengchan Senapin [a,b,*], Kornsunee Phiwsaiya [a,b], Warachin Gangnonngiw [a,b], Matthew Briggs [c], Paisarn Sithigorngul [d], T.W. Flegel [a,b,e]

[a] National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani 12120, Thailand
[b] Center of Excellence for Shrimp Molecular Biology and Biotechnology, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand
[c] Vannamei 101 Co. Ltd., 178/5 Moo 1, Paklok, Thalang, Phuket 83110, Thailand
[d] Department of Biology, Faculty of Science, Srinakharinwirot University, Sukhumvit 23, Bangkok 10110, Thailand
[e] Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand

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ABSTRACT

Whitening of muscle tissue in farmed whiteleg shrimp *Penaeus* (*Litopenaeus*) *vannamei* can result from stress-induced muscle cramps and from viral infections caused by infectious myonecrosis virus (IMNV) or *Penaeus vannamei* nodavirus (*Pv*NV). A similar viral-induced whitening of muscles can be caused in the river prawn *Macrobrachium rosenbergii* and in larvae of the penaeid shrimp *Penaeus* (*Penaeus*) *monodon* and *Penaeus* (*Fenneropenaeus*) *indicus* by a mixed infection with *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV). Here we describe mixed infections of IMNV and *Mr*NV in juvenile cultivated *P. vannamei* from Indonesia, detected by nested RT-PCR and immunohistochemical analysis. Muscle lesions in the dually-infected shrimp gave positive immunohistochemical reactions for both IMNV and *Mr*NV, while connective tissue in the same samples gave positive reactions for *Mr*NV only, indicating some differences in tissue specificity between the two viruses. Although it is not known whether the dual infections are more lethal to shrimp than single IMNV infections, this is possible since earlier work has shown that *Mr*NV alone can increase mortality of *P. vannamei* under stress.

1. Introduction

Whitening of muscle tissue in the whiteleg shrimp *Penaeus* (*Litopenaeus*) *vannamei* can be caused by stress-induced cramps (Lightner, 1996) or by viral pathogens including infectious myonecrosis virus (IMNV) (Poulos et al., 2006) and *Penaeus vannamei* nodavirus (*Pv*NV) (Tang et al., 2007). A similar, viral-induced whitening of muscles can be caused in the river prawn *Macrobrachium rosenbergii* (i.e., white tail disease or WTD) by dual infections of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) (Qian et al., 2003), (Bonami et al., 2005). Recently, it has been reported that the mixed *Mr*NV/XSV infection can also cause WTD with high mortality in larvae of the brackishwater shrimp *Penaeus* (*Penaeus*) *monodon* and *Penaeus* (*Fenneropenaeus*) *indicus* (Ravi et al., 2009), although not in juveniles of the same species (Sudhakarana et al., 2006). Subsequent to the report of WTD mortality in penaeid shrimp, we began to screen specimens of farmed *P. vannamei* with whitened muscles for the presence of not only IMNV and *Pv*NV but also *Mr*NV. Accordingly, we reported the presence of *Mr*NV in cultivated *P. vannamei* in China and Vietnam (Senapin et al., 2012). Here we describe the discovery of specimens of farmed shrimp from Indonesia infected with both *Mr*NV and IMNV.

* Corresponding author at: National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani 12120, Thailand. Tel.: +66 2 201 5889; fax: +66 2 354 7344.
E-mail address: saengchan@biotec.or.th (S. Senapin).

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2. Materials and methods

2.1. Shrimp specimens

In 2007, specimens of farmed, juvenile whiteleg shrimp (*P. vannamei*) (n = 15) showing signs of whitened muscle were collected from shrimp ponds in Indonesia that were experiencing shrimp mortality accompanied by gross signs of whitened abdominal muscle tissue. Some specimens of living shrimp showing gross signs of whitened muscles were fixed with Davidson's fixative followed by normal tissue processing for histological analysis (Bell and Lightner, 1988). Some specimens were preserved in 95% ethanol for RNA extraction and assay (see below) within 7 days of collection.

2.2. Nested RT-PCR assays

Shrimp muscle tissues were ground in Trizol reagent (Invitrogen) and RNA was extracted following the manufacturer's instructions. RNA concentration and quality were measured by spectrophotometric analysis at 260 and 280 nm. In order to confirm that the shrimp tested were positive or negative for the presence of the two viruses, 3 sets of nested primers were used for IMNV detection and 2 for *Mr*NV detection (Table 1). The nested RT-PCR protocols for *Mr*NV detection were previously described (Senapin et al., 2012) while the RT-PCR protocols for IMNV were based on an established protocol (Senapin et al., 2007). The

Table 1
List of primers used in this study.

Virus	Primer set/genome position	Expected product size (bp)	Forward primer name/sequence	Reverse primer name/sequence	Reference
IMNV	Primer 1/481–1080	600	F2/AAT ACT ACA TCA TCC CCG GGT AGA C	R2/GAC TTT CTT CCC AAG ATG GAG TCT C	(Senapin et al., 2007)
		253	F2N/GTG GGA GTT TCA TTT GTG GG	R2N/GTG GGA GTT TCA TTT	This study
	Primer 2/3361–3960	600	F8/GTT GGT GTG GCC CTG CCA ACT GTA A	R8/ACT ACC TTG CAT TGA ACT CCA CGA A	(Senapin et al., 2007)
		264	F8N/GAA TGA AAA CAA CAC TGC CA	R8N/CGT ACC GAT TGT GGG CTG TG	This study
	Primer 3/5761–6360	600	F13/TTT ATA CAC CGC AAG AAT TGG CCA A	R13/AGA TTT GGG AGA TTG GGT CGT ATC C	(Senapin et al., 2007)
		282	F13N/TGT TTA TGC TTG GGA TGG AA	R13N/TCG AAA GTT GTT GGC TGA TG	
*Mr*NV	Primer 1/461–1141 of RNA-2 strand	681	MrNV-F/GAT ACA GAT CCA CTA GAT GAC C	MrNV-R/GAC GAT AGC TCT GAT AAT CC	(Yoganandhan et al., 2005)
		250	MrNV-FN/AGG CAG GET ACG TCA CAA GT	MrNV-RN/GCA TGG AAA ATC CAC AGA CC	(Senapin et al., 2012)
	Primer 2/1596–2324 of RNA-1 strand	729	Mr-RdRp-F/GCA TTT GTG AAG AAT GAA CCG	Mr-RdRp-R/CAT GTT CAA CTT TCT CCA CGT	
		234	Mr-RdRp-FN/TCG AGC TAA GCG TTT TGG TT	Mr-RdRp-RN/TGT TCG ACG ACG TAA TTC CA	

nested PCR reactions increased detection sensitivity. The nested PCR assay used 2 μl from the first RT-PCR amplification as template in 20 μl-reaction containing 0.25 μM of each nested forward and reverse primers (Table 1), 1 unit of Taq polymerase (Invitrogen), 0.2 mM dNTPs, and 1X reaction buffer. The reaction protocol comprised denaturation at 94°C for 5 min followed by 25 cycles of denaturation at 94°C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 30 s.

2.3. Immunohistochemistry

After deparaffinization and blocking steps, tissue sections were separately incubated with antibodies against viral capsid proteins at dilution 1:100 for *Mr*NV and 1:500 for IMNV at 37 °C for 1 h. Mouse polyclonal antibodies against capsid proteins of IMNV and *Mr*NV heterologously expressed in *Escherichia coli* were obtained from initial steps in generating monoclonal antibodies as previously published (Kunanopparat et al., 2011), (Wangman et al., 2012). Preparations were then washed and incubated with goat anti-mouse IgGs HRP conjugate for 1 h at 37 °C. Slides without first antibody served as negative controls. Signal detection was achieved using a Histostain-SP kit (Zymed) and sections were counterstained with tuloidine blue staining modified from a published method (Trump et al., 1961). Digital images were captured using an Olympus BX51 microscope.

3. Results

3.1. Nested RT-PCR assays for MrNV and IMNV

Shrimp specimens (15) collected from Indonesia in 2007 from ponds exhibiting white muscle disease and suspected of IMNV infection were obtained from 8 different farms from three provinces as listed in Table 2. Nested RT-PCR assays revealed that 10 were positive for IMNV with all three primer sets employed (Fig. 1a). Of these, 6 were also positive for *Mr*NV (Fig. 1b) with both primer sets employed. Only one specimen was positive for *Mr*NV but negative for IMNV. The remaining 4 specimens were negative for both IMNV and *Mr*NV using any primer set. It is known that some shrimp exhibiting white muscles do so because of the expression of non-infectious muscle cramp syndrome (Senapin et al., 2011). The results are summarized in Table 2.

3.2. Immunohistochemistry

Lack of cross reactions with the antibodies used against capsid proteins of the two viruses was revealed by immunodotblot assays (data not shown). Specimens positive by RT-PCR for both IMNV and *Mr*NV gave immunopositive results for capsid proteins of both viruses in muscle lesions characteristic of those caused by IMNV infection (Fig. 2). However, the *Mr*NV antibody also gave immunopositive results in the connective tissue, which gave negative reactions with the IMNV antibody (Fig. 3). These results also confirmed that the two antibodies did not cross-react.

4. Discussion

The purpose of this report is to inform shrimp farmers and shrimp pathologists that the whiteleg shrimp *P. vannamei* may sometimes be dually infected with IMNV and *Mr*NV. Similarly, natural co-infection of IMNV and IHHNV (infectious hypodermal and hematopoietic necrosis virus or recently called *Pst*DNV for *Penaeus stylirostris* densovirus) was previously reported in *P. vannamei* in Brazil (Teixeira-Lopes et al., 2011). Whether these co-infections result in more severe disease than

Table 2
Nested RT-PCR test results for juvenile *P. vannamei* shrimp collected from various locations in Indonesia in 2007. Sample 5 was also used for immunohistochemical analysis.

Sample no.	Age, weight	Source	IMNV	*Mr*NV	Dual infection
1	No record	Farm 1, Bali province	−	+	X
2	80 days old	Farm 2, Bali province	+	+	√
3	80 days old		+	+	√
4	40 days old	Farm 3, Situbondo, East Java province	+	+	√
5	80 days old	Farm 4, Situbondo, East Java province	+	+	√
6	105 days old	Farm 5, Lampung province	+	+	√
7	78 days old, 13 g	Farm 6, Lampung province	+	+	√
8	9 g	Farm 7, Jember, East Java province	+	−	X
9	9 g		−	−	X
10	9 g		+	−	X
11	40 days old, 5 g	Farm 8, Lumajang, East Java province	+	−	X
12	40 days old, 5 g		−	−	X
13	40 days old, 5 g		−	−	X
14	40 days old, 5 g		−	−	X
15	40 days old, 5 g		+	−	X