Aquaculture 499 (2019) 290–294

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Evidence that the whiteleg shrimp *Penaeus (Litopenaeus) vannamei* is refractory to infection by *Penaeus monodon* nudivirus (PmNV), also known as MBV

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ARTICLE INFO

Keywords:
Penaeus monodon nudivirus
PmNV
Monodon baculovirus
MBV
Penaeus vannamei
Unsusceptible
Histopathology
In situ hybridization

ABSTRACT

The name monodon baculovirus (MBV) was changed to *Penaeus monodon* nucleopolyhedrovirus (PemoNPV) and then, more recently to *Penaeus monodon* nudivirus (PmNV) based on genetic differences from baculoviruses. PmNV is endemic in Indo-West Pacific penaeid shrimp species, including *Penaeus (Penaeus) monodon*. Since *Penaeus (Litopenaeus) vannamei* was introduced to Asia around 2000 there have been no reports of PmNV infection despite an early report indicating its probable susceptibility. Thus, we hypothesized that *P. vannamei* was not susceptible to PmNV infection and tested the hypothesis using the susceptibility criteria of the World Organization for Animal Health (OIE) and employing a natural PmNV infection model used with *P. monodon*. By histological analysis, PCR detection and immunohistochemistry, we confirmed PmNV infections in positive-control *P. monodon* but failed to do so with *P. vannamei*. The results supported the OIE criteria for non-susceptibility and our hypothesis that *P. vannamei* is not susceptible to PmNV infection. The results allow us to dismiss PmNV as a threat to *P. vannamei* and to eliminate *P. vannamei* as a possible carrier for transmission of PmNV to other shrimp species.

1. Introduction

When exotic *P. vannamei* was translocated to Asia for aquaculture beginning in the late 1990's, local shrimp pathologists were on the lookout for the possibility of simultaneous import of exotic pathogens that might cause disease in endemic shrimp species (Flegel, 2006a), and for the possibility of imported stocks being infected by local pathogens. Indeed, import of exotic *P. vannamei* to Taiwan and Thailand led to disease outbreaks caused by exotic Taura syndrome virus (TSV) in the cultured, imported stocks (Flegel, 2006a, 2012; Nielsen et al., 2005; Thitamadee et al., 2016; Yu and Song, 2000). At the same time, lethal outbreaks of local white spot disease (WSD) caused by white spot syndrome virus (WSSV) and endemic yellow head disease (YHD) caused by yellow head virus (YHV) did occur and caused severe losses in farmed, exotic *P. vannamei* (Flegel, 2006a; Flegel, 2009; Senapin et al., 2010).

As with WSD and YHD outbreaks in *P. vannamei*, we were concerned that another, common, endemic virus originally called monodon baculovirus (MBV) in wild and cultivated *P. monodon* in Thailand might also present a threat to farmed, exotic *P. vannamei*. The whole genome of MBV was recently published (Yang et al., 2014) and found to differ significantly from viruses in the family *Baculoviridae* where it was formerly included as the tentative species *Penaeus monodon* nucleopolyhedrovirus (PemoNPV) (Fauquet et al., 2005). Because of this genetic difference, it has been put forward for inclusion in the newly proposed but yet unassigned family *Nudiviridae* (Jehle et al., 2013; Wang et al., 2007; Wang and Jehle, 2009) as *Penaeus monodon* nudivirus (PmNV) (Yang et al., 2014). Here, we will refer to it as PmNV.

PmNV was first reported as MBV from *P. monodon* in Thailand in 1991 (Fegan et al., 1991). The following summary of PmNV is derived from the publications of Fegan et al. (1991), Lightner (1996) and Flegel (2006a). The main route of PmNV infection in rearing ponds is contaminated postlarvae (PL) derived from grossly normal, captured, wild broodstock infected with PmNV. They produce microscopically visible crystalline polyhedrin protein bodies (occlusion bodies or OBs) with embedded bacilliform viral particles that are sloughed from the

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https://doi.org/10.1016/j.aquaculture.2018.09.029
Received 13 June 2018; Received in revised form 7 September 2018; Accepted 12 September 2018
Available online 21 September 2018
0044-8486/

hepatopancreas of infected-shrimp and shed in the feces. OBs are then ingested by larval offspring of the broodstock via filter-feeding, and the larvae produce more OBs for rapid exponential horizontal spread in a shrimp hatchery by filter-feeding and/or cannibalism. This is the normal natural transmission pathway for invertebrate bacilliform viruses embedded in polyhedrin protein. The only significant impact of PmNV in rearing ponds is associated with retarded growth in infected individuals during the later stages of pond cultivation (i.e., not associated with abnormal mortality) (Flegel et al., 2004).

Lightner (1996) wrote the following regarding the host range of PmNV or what he called MBV, "Observed hosts in apparent order of importance include: *P. monodon*, *P. merguiensis*, *P. semisulcatus*, *P. indicus*, *P. plebejus*, *P. penicillatus*, *P. esculentus*, *and P. kerathurus*, and possibly *P. vannamei*". We may now also add *Macrobrachium rosenbergii* to the list of potential hosts (Gangnonngiw et al., 2010). However, despite Lightner's speculation that *P. vannamei* is susceptible to PmNV, we have seen no histological evidence of PmNV to date in literally thousands of normal and diseased juvenile, farmed shrimp specimens examined since domesticated lines of *P. vannamei* have replaced *P. monodon* as the dominant species cultivated in Thailand. Nor have we found histological evidence of PmNV infection in the thousands of slides of cultivated *P. vannamei* that we have examined in the same time interval from Vietnam, China, Malaysia and Indonesia. Nor have we found any reports of PmNV infection in *P. vannamei* from anywhere else in Asia or the world.

On the other hand, it is not customary to include a PCR test for PmNV in the list of PCR tests used to screen for viruses in cultivated *P. vannamei* in Asia. As such, it is possible that *P. vannamei* infected with PmNV may have escaped notice, if they were infected but showed no gross or histological signs of disease. For example, grossly and histologically normal crayfish *Cherax quadricarinatus* infected with yellow head virus (YHV) can be RT-PCR positive for YHV and serve as a dangerous carrier capable of transmitting lethal YHV infections to the giant tiger shrimp *P. monodon* (Soowannayan et al., 2015). The ability to serve as grossly normal virus carriers is a common phenomenon for shrimp (Flegel, 2006b).

Another possible explanation for the absence of reports for PmNV in *P. vannamei* in Asia are the facts that most of the broodstock used by hatcheries are specific pathogen free (SPF) for PmNV (and other pathogens) and that the chance of horizontal transmission in rearing ponds may be extremely low. However, even with a low probability of horizontal transfer in ponds, one might expect to find at least a few histologically positive shrimp in surveys that include thousands of specimens.

These uncertainties regarding susceptibility of *P. vannamei* to PmNV led us to hypothesize that *P. vannamei* is not susceptible to PmNV infection. If this hypothesis proved to be correct, we would be able to dismiss PmNV as a threat to *P. vannamei* and also eliminate *P. vannamei* as a covert PmNV-carrier threat to other shrimp species. We tested this hypothesis using the very simple but dependable, natural PmNV infection model that we have used routinely in our annual international training course on shrimp pathogens since 2010 (unpublished). This consists of using stored (-80 °C), thawed and homogenized naturally-PmNV-infected PL of *P. monodon* for feeding to PmNV-negative PL of *P. monodon* and incubating them for 10 days in the laboratory. We show that following this protocol in parallel with *P. vannamei* does not result in its infection as determined by PCR detection, histological analysis and immunohistochemistry.

2. Materials and methods

2.1. Criteria for PmNV susceptibility

We used the criteria of the World Organization for Animal Health (OIE) in its Aquatic Animal Health Code Chapter 1.5 (Anonymous, 2017) to determine whether *P. vannamei* was susceptible to infection with PmNV. These criteria include evidence that transmission can be obtained by natural transmission routes AND that identity of the pathogenic agent can be confirmed AND that evidence of infection can be provided in accordance with 4 criteria (A to D in Article 1.5.6) Satisfaction of criterion A alone is sufficient to indicate susceptibility, and if not, satisfaction of any 2 of the remaining 3 criteria will do so. The 4 criteria are (A) evidence of multiplication or developing stages of the agent in the host; (B) isolation of viable agent from the proposed host followed by transmission to naive individuals; (C) demonstration in the proposed host of appropriate clinical or pathological changes associated with infection by the agent; (D) demonstration that the specific pathogen is present in the expected target tissues of the proposed host.

2.2. PmNV inoculum

As viral inoculum for challenge trials, we used a sub-lot from a large batch of approximately 200,000 post larvae (PL) of *P. monodon* infected with PmNV that were obtained from a local shrimp hatchery in 2010, subdivided into small packets and stored at −80 °C. A portion from one packet has been used each year since 2010 to successfully infect *P. monodon* per os for teaching purposes in our annual shrimp-disease training course (unpublished).

2.3. Experimental shrimp

For experimental shrimp, approximately 2000 PL of whiteleg shrimp *P. vannamei* (length approximately 12 mm) were obtained from a local hatchery in Chachoengsao province Similarly, approximately 2000 PL of the giant tiger shrimp *P. monodon* (length approximately 10 mm) were obtained from a local hatchery in Chonburi Province, Thailand. The PL were delivered to the laboratory within 3 h and transferred to 4 individual aquaria for acclimatization for 1 d (healthy shrimp) before challenge tests in covered tanks containing 12.5 L artificial seawater (Marinium, Mariscience International PC, Bangkok) at 15 ppt and at 28 °C. During acclimatization, 30 PL from each species were tested individually by PCR (see below) to ensure negative results for PmNV infection at approximately 10% or more prevalence (Cameron, 2002).

2.4. PCR assays

Whole PL were individually homogenized with a glass pestle homogenizer in PBS buffer (pH 7.4) at 4 °C. The homogenate was centrifuged at 1500 x*g* for 10 min at 4 °C. The pellet was resuspended in DNA extraction buffer for DNA extraction using a DNA extraction kit (Favorgen, Taiwan) according to the manufacturer's directions. Presence of PmNV was determined using MBV261 primers (Table 1) targeting a conserved region of the PmNV polyhedrin gene (amplicon 261 bp) and using a previously published protocol (Surachetpong et al., 2005). The quality of the DNA extracts was determined using an internal control reaction targeting the host shrimp gene for elongation factor 1-α (EF1-α) (Leelatanawit et al., 2008) (Table 1). The PCR reactions for the target (PmNV polyhedrin gene) and the control (*EF-1α*) were run separately with the same DNA template for each shrimp

Table 1
PCR primers used in this work to detect the PmNV (MBV) polyhedrin gene (Surachetpong et al., 2005) and the host shrimp gene for elongation factor 1-α (EF) (Leelatanawit et al., 2008). The PCR protocols are given in the respective publications.

Primer name	Sequence 5′ to 3′	Product size
MBV261F	AATCCTAGGCGATCTTACCA	261 bp
MBV261R	CGTTCGTTGATGAACATCTC	
EF-1 alpha-F	TTCCGACTCCAAGAACGACC	122 bp
EF-1 alpha-R	GAGCAGTGTGGCAATCAAGC	