Natural host-range and experimental transmission of Laem-Singh virus (LSNV)

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ABSTRACT: Slow growth caused by viral diseases has become a major constraint in shrimp aquaculture. Laem-Singh virus (LSNV), a positive-sense single-stranded RNA (ssRNA) virus, has been identified in *Penaeus monodon* showing slow growth syndrome. To examine the host-range and transmission modes of the virus, 6 species of penaeid shrimp of varying life stages, sourced from the wild and from farms, as well as juvenile mud crabs *Scylla serrata*, were screened using RT-nested PCR. LSNV was detected in *P. monodon, Fenneropenaeus merguiensis, Metapenaeus dobsoni*, and *Litopenaeus vannamei*, but not in *F. indicus, Marsupenaeus japonicus* or *S. serrata*. LSNV was most prevalent in *P. monodon* followed by *M. dobsoni, F. merguiensis*, and *L. vannamei*, and real-time quantitative RT-PCR (qRT-PCR) showed that LSNV infection loads were highest in *P. monodon*, followed by *L. vannamei, M. dobsoni*, and *F. merguiensis.* The nucleotide sequence of the LSNV RdRP gene fragment amplified by RT-nested PCR was highly conserved (99% identity) across these 4 penaeid species. LSNV was detected in both small and normal-sized *P. monodon* collected from the same pond. In experimental infections of both *P. monodon* and *S. serrata*, LSNV infection loads increased over time. The present study extends the known natural penaeid host-range and geographical distribution of LSNV and shows for the first time the potential susceptibility of *S. serrata*.

KEY WORDS: Laem-Singh virus (LSNV) · Penaeid shrimp · *Penaeus monodon* · PCR · Real-time PCR · Host-range · Experimental transmission

INTRODUCTION

Viral disease outbreaks have been increasing in penaeid shrimp aquaculture, and the number of viral pathogens has increased to more than 20 (Lightner 1996, Loh et al.1997, Lightner & Redman 1998, Umesha et al. 2008). Of several diseases of unknown or obscure aetiology that have emerged recently, monodon slow growth syndrome (MSGS) has been significant in hampering production yields of *Penaeus monodon*, particularly in Thailand where it was first reported (Chayaburakul et al. 2004). A similar condition has also been reported in *P. monodon* farmed in East Africa (Anantasomboon et al. 2006).

Investigations of MSGS-affected *Penaeus monodon* in Thailand identified a new virus that was named Laem-Singh virus (LSNV) after the name of the province where it was first identified (Sritunyalucksana et al. 2006). LSNV infection also occurs in *P. monodon* displaying no overt signs of MSGS, and experimental infections have identified no direct correlation between infection and slow growth (Anantasomboon et al. 2006, Sritunyalucksana et al. 2006). However, as LSNV is associated with retinopathy in slow growing *P. monodon*, it is believed to be a contributing factor to slow growth.

LSNV contains a positive-sense, single-stranded RNA genome, forms 25 to 30 nm diameter icosohedral particles, and is related genetically to insect-transmit-

ted plant viruses in the family *Luteoviridae* (Sritun-yalucksana et al. 2006, Sittidilokratna et al. 2009). The natural host-range of LSNV is presently unknown, but it occurs commonly in *Penaeus monodon*, and limited studies have suggested a geographical distribution restricted to south and southeast Asia (Sittidilokratna et al. 2009). It has also been detected in *Litopenaeus vannamei* in Thailand affected by abdominal segment deformity disease (Sakaew et al. 2008), suggesting that its natural host-range might include species other than *P. monodon*. There is limited evidence of LSNV occurring in *P. monodon* cultured in India (Prakasha et al. 2007, Rai et al. 2009), but it has not been associated with slow growth, and the virus has not been reported from any other crustaceans. Given this background, the natural host-range of LSNV was investigated for selected crustaceans present in India together with the potential for mud crabs to be a carrier of the virus.

MATERIALS AND METHODS

Samples. The commercially important penaeid shrimp species *Penaeus monodon*, *Fenneropenaeus merguiensis*, *F. indicus*, *Marsupenaeus japonicus*, *Litopenaeus vannamei*, *Metapenaeus dobsoni*, and the mud crab *Scylla serrata* were sampled. Tissue samples were collected from wild and cultured shrimp at postlarvae/juvenile/brooder stages or were supplied preserved in RNAlater (Ambion).

Overtly normal and slow-growing juvenile farmed *Penaeus monodon* originated from Pancham Aqua Farm, Safale, Maharashtra, India. *Fenneropenaeus merguiensis* and *F. indicus* postlarvae and juveniles originated from a hatchery and farm in Tamil Nadu, India. Juvenile *Marsupenaeus japonoicus* originated from a farm in Tamil Nadu, and juvenile wild *Metapenaeus dobsoni* were sampled from the Chennai coast of Tamil Nadu. *L. vannamei* postlarvae and juveniles originated from a hatchery and farm in Andhra Pradesh, and juvenile mud crabs were collected from Maharashtra. Pleopods collected from the 20 wild-caught *P. monodon* brooders from the Andaman Sea were preserved in RNAlater and transported to the laboratory. For LSNV transmission experiments, live shrimp were maintained in 500 l tanks containing seawater at 24‰ salinity. The ambient water temperature was 25 to 30°C. Mud crabs were maintained in rectangular plastic tanks with wet mud as a bottom substrate. Shrimp were fed commercial feed pellets twice daily, and crabs were fed chopped mussel meat.

Tissue collection. Crustaceans were anesthetised on ice before sampling gill, pleopod, hepatopancreas, lymphoid organ, stomach, heart, nerve cord, brain, and optical lobe tissues using sterile scissors. To avoid cross-contamination, dissecting instruments were flame-sterilized between individuals. RNA was extracted from tissues immediately after their collection. RNA was also extracted from tissues and whole postlarvae preserved in RNAlater soon after these were received at the laboratory.

Experimental infection. *Penaeus monodon* that tested RT-PCR-positive for LSNV were used to prepare a viral inoculum. Tissues such as gill, pleopod, and lymphoid organ were dissected aseptically, homogenized in sterile PBS using a mortar and pestle. The homogenate was centrifuged at 2432 × *g* for 20 min at 4°C to remove tissue debris, and the supernatant was filtered through a 0.22 µm membrane filter fitted to a syringe. Aliquots of the filtrate were stored at –80°C until used. RNA extracted from an inoculum aliquot was tested by RT-PCR to confirm the presence of LSNV RNA. Aliquots (100 µl) of inoculum were injected intramuscularly into the third abdominal segment of healthy shrimp, which had tested RT-PCR-negative for LSNV, or into the coxa of the swimming leg of mud crabs using a 1 ml syringe fitted with a 26-gauge needle. Injected animals were maintained as described earlier and were sacrificed at different times postinjection to collect tissue for RT-PCR analysis.

Total RNA extraction. Dissected tissues were placed into screw-cap tubes containing glass beads and 750 µl TRIzol (Invitrogen). The tissues were homogenized at 4000 rpm for 20 s in a Micro Smash MS-200 (TOMY), and total RNA was extracted according to the manufacturer's protocol. The RNA pellet was resuspended in 25 µl nuclease-free water, an aliquot quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and stored at –80°C.

cDNA synthesis. cDNA was synthesized using Superscript^{TM} II (Invitrogen) as per the manufacturer's protocol. Briefly, 1.0 µg total RNA was reverse-transcribed in a 10 µl reaction containing 100 µM random hexamers, 10 mM each dNTP (Fermentas), 200 U of Superscript^{TM} II reverse-transcriptase and reaction buffer containing 250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl$_2$, and 50 mM dithiothreitol (DTT). cDNA was stored at –20°C until use.

PCR. RT-nested PCR was performed with 2 sets of LSNV-specific PCR primers, using reaction components and cycling conditions as reported by Sittidilokratna et al. (2009). Briefly, 2.0 µl cDNA (200 ng RNA equivalents) was amplified in a 25 µl reaction volume in the first-step PCR, and 2.0 µl of this PCR was used as the template in a 25 µl nested PCR. After thermal cycling in an Applied Biosystems instrument, an aliquot of each PCR was resolved in 2% agarose-Tris-acetate-EDTA (TAE) gels containing 0.5 µg ml^{-1} ethidium bromide alongside a 100 bp DNA marker (Fermentas), and amplified DNA was visualized under UV

illumination using a gel documentation system (DNr Bio-imaging Systems).

Real-time RT-PCR. A 205 bp LSNV PCR product was cloned into pTZ57R/T vector (Fermentas) and plasmid DNA was purified and then quantified using a Nano-Drop 2000 spectrophotometer. The DNA copy number was calculated from the DNA concentration and the molecular mass of the plasmid DNA containing the 205 bp LSNV insert. Based on this calculation, plasmid DNA was serially diluted (1:10) from 1×10^9 DNA copies to 1 DNA copy. LSNV RNA copy numbers in clinical samples, after conversion to cDNA (assuming equivalence of RNA and cDNA) were quantified by extrapolating from the standard curve generated using the plasmid DNA dilution series. Real-time qRT-PCRs (25 µl) contained 12.5 µl Maxima™ SYBR Green qPCR Master Mix (Fermentas), 0.5 µl (0.9 µM) of each primer (LSNVQPF1: 5′-TAG CCT GCG GTG ATG ACA CTA-3′; LSNVQPR1: 5′-GCT TGC GAT CGA CAC TCT TAA C-3′), and 2 µl (equivalent to 200 ng) cDNA. Thermal cycling employed the ABI 7500 Real-time PCR system (Applied Biosystems). The thermal cycling profile used was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Melting curve analysis of amplified DNA products was performed at the end of each PCR to confirm amplicon specificity. For each cDNA, 3 well replicates were used.

Statistical analysis. Analysis of variance to determine the significance of variation between uninfected and LSNV-infected shrimp and production traits such as body length and weight used the PROC GLM program (SAS 2010).

RESULTS

RT-PCR detection of LSNV in *Penaeus monodon*

A 2-step LSNV RT-PCR amplified 357 bp primary and 205 bp nested PCR products from cDNA prepared to gill RNA from *Penaeus monodon* infected with LSNV. Sequencing of the 357 bp product showed 99.0% identity with LSNV detected in Thai *P. monodon* (Sritunyalucksana et al. 2006). To select the best tissue for screening different shrimp species for LSNV, various tissues from *P. monodon* and *F. merguiensis* including gill, pleopod, lymphoid organ, stomach, hepatopancreas, heart, nerve cord, brain, and optical lobe were tested. LSNV RNA amounts vary from tissue to tissue in both species, and whilst gill and pleopod showed relatively consistent PCR product yields, products were detected in nested PCR analyses of most other tissues (Fig. 1). Based on these data, gill and pleopod were employed as reference tissues to screen shrimp, whilst gill and stomach were used to screen crabs.
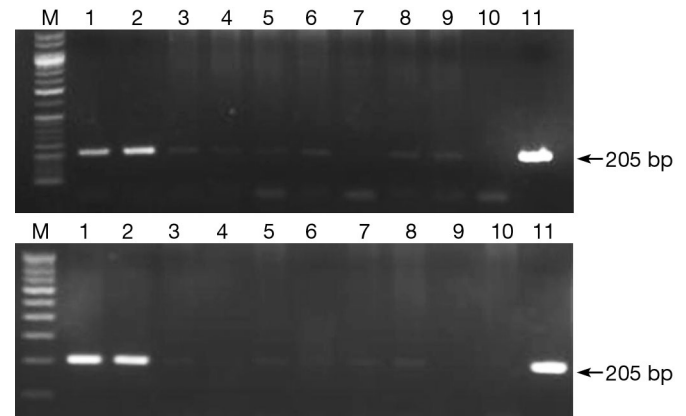

Fig. 1. RT-nested PCR amplification of Laem-Singh virus (LSNV) RNA from different tissues of *Penaeus monodon* (upper panel) and *Fenneropenaeus merguiensis* (lower panel). Lanes 1 to 9, respectively: gill, pleopod, lymphoid organ, hepatopancreas, stomach, heart, nerve cord, brain, optical nerve; Lane 10: negative control; Lane 11: positive control. Lane M: 100 bp DNA ladder

Prevalence and host-range

To examine the host-range and prevalence of LSNV in India, samples from various life stages of 6 penaeid shrimp species and of mud crab *Scylla serrata* were screened by RT-nested PCR. As shown in Table 1, LSNV was detected in *Penaeus monodon, Fenneropenaeus merguiensis, Metapenaeus dobsoni,* and *Litopenaeus vannamei* but not in *F. indicus, Marsupenaeus japonicus* or *S. serrata.* Among the LSNV-positive shrimp, the highest prevalence (48.6%) occurred in farmed juvenile *P. monodon,* followed by *P. monodon* brooders (20.0%) collected from the Andaman Sea. Among the 16 pools of 4 *P. monodon* postlarvae tested, 5 tested LSNV-positive by first-step PCR, and another 2 tested positive by nested PCR. LSNV RNA was detected by RT-nested PCR in 20.0% of juvenile *M. dobsoni*, 13.9% of juveniles, and 1 of 12 pools of *L. vannamei* postlarvae, and in 7.7% of juveniles and 2 of 10 pools of *F. merguiensis* postlarvae. Except for the 5 pools of *P. monodon* postlarvae that tested positive by PCR, LSNV RNA levels in all other positive samples required nested PCR for detection of an amplicon.

Quantification of LSNV RNA in clinical samples

Real-time qRT-PCR was used to quantify the LSNV RNA loads in samples of different life stages of penaeid shrimp species that tested positive by conventional RT-nested PCR (Fig. 2). Amongst samples of juvenile shrimp, the highest RNA loads were evident in *Penaeus monodon* (1.2×10^6 copies µg⁻¹) followed by *Litopenaeus vannamei* (2.9×10^5 copies µg⁻¹), *Meta-*

Table 1. RT-PCR screening of penaeid shrimps and mud crabs for Laem-Singh virus (LSNV) in India. (–): not available

| Stage	Mean length (cm)	Mean weight (g)	Collection location	Tissue screened	No. of animals tested	LSNV-RT-PCR 1st-step	2nd-step
Penaeus monodon							
Juveniles	13.0	20.0	Maharashtra	Gill and pleopod	35	0	17
Brood stock	23	110.0	Andaman	Pleopod	20	0	4
Postlarvae	–	–	Andhra Pradesh	Whole	64 (16 pools)	5	7
Fenneropenaeus merguiensis							
Juveniles	13.0	18.0	Tamil Nadu	Whole	13	0	1
Postlarvae	–	–	Tamil Nadu	Gill and pleopod	40 (10 pools)	0	2
Fenneropenaeus indicus							
Juveniles	9.0	9.5	Tamil Nadu	Gill and pleopod	22	0	0
Postlarvae	–	–	Tamil Nadu	Whole	24 (6 pools)	0	0
Litopenaeus vannamei							
Juveniles	14.0	17.0	Andhra Pradesh	Gill and pleopod	36	0	5
Postlarvae	–	–	Andhra Pradesh	Whole	48 (12 pools)	0	1
Marsupenaeus japonicus							
Juveniles	12.0	15.0	Tamil Nadu	Gill and pleopod	15	0	0
Metapenaeus dobsoni							
Juveniles	11.0	12.0	Tamil Nadu	Gill and pleopod	15	0	3
Scylla serrata							
Juveniles	–	32.0	Maharashtra	Gill and stomach	10	0	0
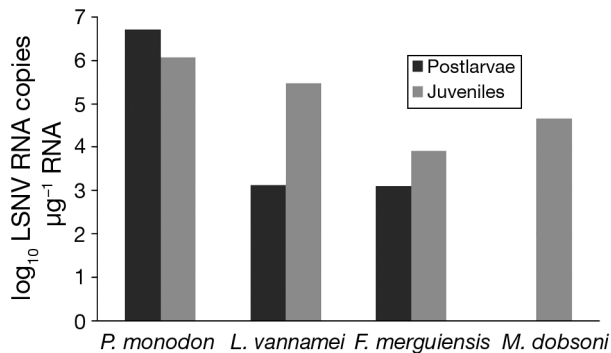
Fig. 2. Laem-Singh virus (LSNV) RNA copy number estimated in postlarvae and juveniles of different penaeid shrimp by real-time quantitative RT-PCR. See Table 1 for full species names

penaeus dobsoni (4.7×10^4 copies µg^{-1}), and *Fennero-penaeus merguiensis* (8.2×10^3 copies µg^{-1}). Among samples of postlarvae, the highest LSNV RNA loads were evident in *P. monodon* (5.1×10^6 copies µg^{-1}), followed by *L. vannamei* (1.3×10^3 copies µg^{-1}) and *F. merguiensis* (1.2×10^3 copies µg^{-1}).

Non-association of LSNV with slow growth in *Penaeus monodon*

To investigate whether LSNV was associated with slow growth in *Penaeus monodon*, shrimp collected from a farm which held both slow-growing and normal shrimp in the same pond were screened by real-time qRT-PCR after 120 d of culture. Of 35 shrimp collected, 16 showed signs of slow growth (8.7 cm mean length, 8.2 g mean weight) and 19 did not (16.5 cm mean length, 30.8 g mean weight). Of these, 10 (62.5%) slow-growing shrimp and 7 (36.8%) normal shrimp were positive by real-time qRT-PCR. No significant differences in LSNV RNA copy number were evident either between slow-growing and normal shrimp, or between infected and uninfected shrimp in their body length or weight (Table 2). The model R^2 value was found to be 7.5 and 9.4% for weight and length, respectively.

Experimental infection of *Penaeus monodon* and *Scylla serrata* with LSNV

A virus inoculum was prepared from *Penaeus monodon* that were RT-PCR-positive for LSNV and PCR-negative for WSSV. RT-nested PCR analysis of RNA isolated from the inoculum generated no amplicon by 1-step PCR but a 205 bp amplicon by nested PCR.

In healthy LSNV-free *Penaeus monodon* (21 ± 3 g) injected with inoculum and sacrificed at 48 h post-injection, LSNV was detected by RT-nested PCR in RNA extracted from gill, pleopod, lymphoid organ, nerve cord, and brain tissue but not optical lobe tissue. At Day 8 post-injection, LSNV infection loads were

Table 2. Relation between body weight and length and Laem-Singh virus (LSNV) infection (means ± SE). Superscript letter indicates that means within a group show no significant difference (p < 0.05) from one another

Status	No. of observations	Weight (g)	Length (cm)
Overall	35	15.03 ± 1.14	13.12 ± 0.73
Infection status			
Negative	17	18.73[a] ± 1.21	14.47[a] ± 1.02
Positive	18	12.06[a] ± 1.21	11.76[a] ± 1.05
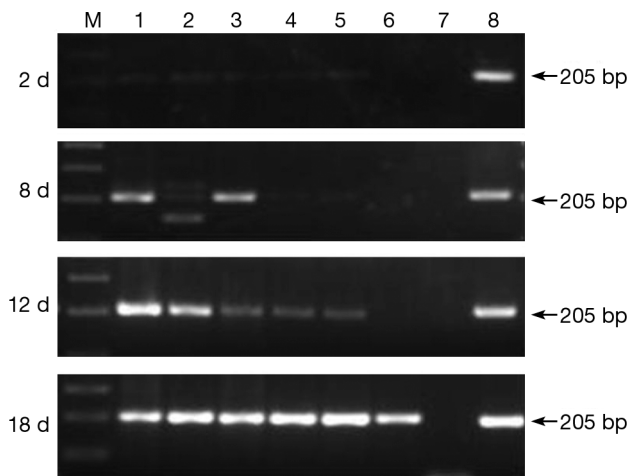
Fig. 3. RT-nested PCR of different tissues of *Penaeus monodon* infected experimentally with Laem-Singh virus (LSNV) collected at 4 time-points post-injection. Lanes 1 to 6, respectively: gill, pleopod, lymphoid organ, nerve cord, brain, optical lobe; Lane 7: negative control; Lane 8: positive control. Lane M: 100 bp DNA ladder
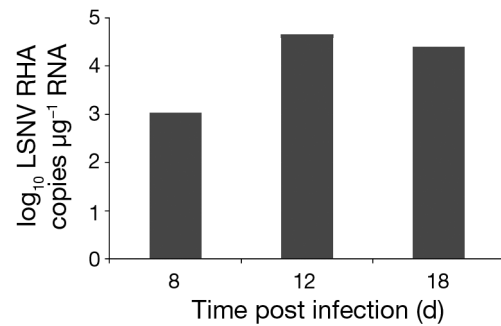

Fig. 4. Laem-Singh virus (LSNV) RNA copy number in gill tissue of experimentally-infected *Penaeus monodon* determined by real-time qRT-PCR
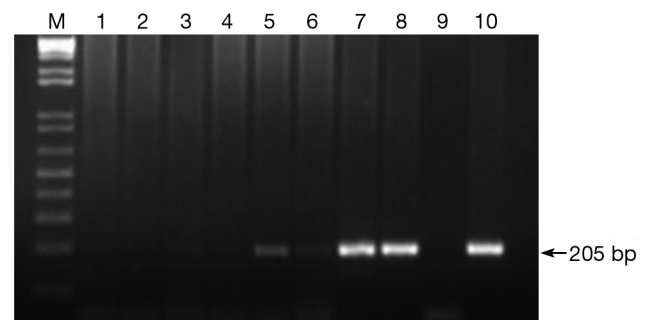

Fig. 5. RT-nested PCR amplification of Laem-Singh virus (LSNV) RNA from gill and stomach tissues *Scylla serrata* infected experimentally with LSNV collected at 4 time-points post-injection. Lanes 1 & 2: 12 h; Lanes 3 & 4: 24 h; Lanes 5 & 6: 8 d; Lanes 7 & 8: 20 d; Lane 9: negative control; Lane 10: positive control. Lane M: 100 bp DNA ladder

increased noticeably in gill, pleopod, and lymphoid organ tissue, and increased progressively until Day 18 post-injection, except in optic lobe tissue (Fig. 3). Real-time qRT-PCR analysis of RNA extracted from gill was used to quantify the increases in LSNV infection loads over time post-injection (Fig. 4).

To examine the susceptibility of *Scylla serrata* to LSNV infection, the inoculum was injected into the coxa of the swimming leg, and crabs were sacrificed at 12 and 24 h, and 8 and 20 d post-injection to collect gill and stomach tissue. RT-nested PCR only generated amplicons at the Day 8 and Day 20 post-injection sampling points (Fig. 5).

LSNV sequence analysis

Cloning and sequence analysis of 205 bp RT-nested PCR amplicon from LSNV-infected *Penaeus monodon*, *Fenneropenaeus merguiensis*, *Litopenaeus vannamei*, and *Metapenaeus dobsoni* showed them to be identical (GenBank Accession no. HQ825248), except for *F. merguiensis*, which possessed a single T → C transition (alanine to valine change) and was identical to a LSNV sequence obtained from Thai *P. monodon* (GenBank Accession no. DQ127905). The 357 bp first-step RT-PCR product amplified from *P. monodon* postlarvae (GenBank Accession no. HQ728430) possessed 99.0% identity to the Thai LSNV sequence.

DISCUSSION

Whilst mainly detected in *Penaeus monodon* (Sritunyalucksana et al. 2006, Pratoomthai et al. 2008, Sittidilokratna et al. 2009), LSNV has also been detected in Thai *Litopenaeus vannamei* investigated for abdominal segment deformity disease (Sakaew et al. 2008). In India thus far, LSNV has been reported only in *P. monodon* (Prakasha et al. 2007, Rai et al.

2009). Of the 6 shrimp species and mud crab species from India examined here, RT-nested PCR evidence of LSNV infection was found in *P. monodon, Fenneropenaeus merguiensis, L. vannamei,* and *Metapenaeus dobsoni* at all life stages tested but not in *F. indicus, Marsupenaeus japonicus*, or *Scylla serrata*. The data thus confirm the existence of LSNV infection in *L. vannamei* farmed in India and suggest for the first time that *F. merguiensis* and *M. dobsoni* are similarly susceptible to natural infection. The study also shows infection to occur in *P. monodon* collected 2 km off the coast of Betapur, Andaman and Nicobar Islands, a popular ground for broodstock collection. The high prevalence of LSNV detected amongst *P. monodon* and the comparatively higher infection loads detected indicate that this species is a primary host of LSNV. Whilst the present study helps define that other crustacean species of commercial relevance in India might be sources of LSNV infection, far more extensive surveys are needed to elucidate its range of natural hosts more thoroughly. With the exception of *P. monodon* postlarvae, no samples of any tissue type from *P. monodon* or any other shrimp or crab species examined possessed infection loads sufficiently high to be detected by RT-PCR without the use of nested PCR. This is suggestive of a high infection prevalence amongst *P. monodon* postlarvae, this life stage being highly susceptible to infection, and of the likelihood of their infections being transmitted vertically from brooders. The finding also suggests that it might be worthwhile screening batches of *P. monodon* postlarvae for LSNV before stocking of aquaculture ponds. As LSNV detected in *L. vannamei* postlarvae and cultured juveniles was identical in sequence across the 205 nt region amplified by RT-nested PCR to that amplified from *P. monodon*, it seems likely that *P. monodon* used for aquaculture purposes might be the source of these infections, although additional studies are needed to confirm this.

The 357 nt region of the LSNV RNA genome amplified by RT-PCR from *Penaeus monodon* postlarvae displayed 99.0% nucleotide sequence identity to isolates detected in Thai *P. monodon.* These data support the findings of Sittidilokratna et al. (2009) that the extent of genetic variation in LSNV across India and southeast Asia is relatively low (>98%).

Gill, pleopod, and lymphoid organ have been commonly used for RT-PCR screening for LSNV (Sritunyalucksana et al. 2006, Prakasha et al. 2007, Sittidilokratna et al. 2009). Study of the tissue tropism of LSNV has also shown infection to occur throughout neural tissues such as the thoracic ganglion, abdominal ganglion, brain, and optical lobe (Pratoomthai et al. 2008). Of several tissue types of the various shrimp species examined here, gill and pleopod generated the most consistent RT-nested PCR products amongst LSNV-infected *Penaeus monodon* and *Fenneropenaeus merguiensis*.

MSGS in farmed *Penaeus monodon* is a serious problem and has reduced the farm profitability and damaged export industries based on this species (Chayaburakul et al. 2004, Sritunyalucksana et al. 2006, Pratoomthai et al. 2008). In severe occurrences in Thailand, MSGS could result in up to 30% of pond stocks of *P. monodon* averaging only 6 g in weight after 4 months of culture, with unaffected shrimp averaging 30 g in weight (Withyachumnarnkul 2005). *P. monodon* farmed in India often also display lengths and body weights 2- and 4-fold lower, respectively, and appear more darkly colored than normal shrimp in the same pond 120 d after stocking. Unlike occurrences of MSGS in Thailand, however, unusually bright yellow markings, 'bamboo-shaped' abdominal segments, or brittle antennae have not been evident in smaller shrimp at farms in India. Real-time qRT-PCR data showing no significant differences in LSNV infection loads in small and normal-sized *P. monodon* are consistent with it occurring in both MSGS affected and unaffected *P. monodon* in Thailand (Sritunyalucksana et al. 2006, Pratoomthai et al. 2008), indicating that other factors are needed to induce MSGS.

It has been reported that slow growth syndrome in *Penaeus monodon* can be induced when lymphoid organ extracts of MSGS-affected shrimp are injected into healthy *P. monodon* (Withyachumnarnkul 2005). However, no experimental LSNV infections have thus far been reported in any crustacean species in which increases in LSNV infection loads have been tracked. Such transmissions were undertaken here with LSNV RNA levels tracked by RT-nested PCR and real-time qRT-PCR. In *P. monodon,* although LSNV infection loads increased between 2 and 18 d post-injection, they did not reach levels sufficient for detection by RT-PCR without the use of nested PCR. Experimental LSNV infection of the mud crab *Scylla serrata* also induced gradual increases in viral load between 12 h and 8 d post-injection, after which time loads appeared to stabilize until 20 d post-infection. Whilst infection loads remained below the threshold needed for detection by RT-PCR without the use of nested PCR, and LSNV could not be detected in wild *S. serrata* collected from Maharashtra, the ability of LSNV to cause infection when injected into a crustacean species other than shrimp suggests that this and other species might be potential natural carriers of LSNV infection. As the data presented here indicate that the host-range of LSNV could involve crustacean species other than shrimp, this hypothesis warrants more thorough examination.

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