Indian J. Virol. (July–September 2012) 23(2):215–225
DOI 10.1007/s13337-012-0099-7

REVIEW ARTICLE

Laem-Singh Virus: A Probable Etiological Agent Associated with Monodon Slow Growth Syndrome in Farmed Black Tiger Shrimp *(Penaeus monodon)*

M. Poornima · Y. Seetang-Nun · S. V. Alavandi · J. Syama Dayal

Received: 11 December 2011 / Accepted: 25 July 2012 / Published online: 18 September 2012

Abstract Among the emerging diseases in shrimp aquaculture, monodon slow growth syndrome (MSGS) is a major concern in South and Southeast Asia. Shrimp farming in Thailand was severely affected during 2000–2002 due to MSGS, which caused an economic loss, of about US$ 300 million. MSGS is characterized by abnormally slow growth with coefficients of size variation of >35 %, that has impacted *P. monodon* production in Thailand. A new shrimp virus, Laem-Singh virus (LSNV) was identified to be associated in MSGS affected shrimp. LSNV a RNA virus of about 25 nm diameter is phylogenetically related to the insect-borne viruses in the families *Barnaviridae*, *Tymoviridae* and *Sobemoviridae* an important histopathological observation is exclusively noticed in growth-retarded shrimp. The LSNV infections have been confirmed in various organs of infected shrimp such as lymphoid organ, gills and nervous tissues by various diagnostic techniques such as reverse transcription polymerase chain reaction (RT-PCR), in situ hybridization, quantitative real-time RT-PCR and reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick (RT-LAMP-LFD) and these tools are available for the diagnosis of LSNV. Recently, an integrase containing element has been identified in absolute association with LSNV in stunted growth shrimp. The transmission of LSNV through horizontal and vertical routes has been experimentally demonstrated. The known natural host-range of LSNV includes *P. monodon* and other penaeid shrimp. The putative *RdRp* gene involved in replication of LSNV was targeted for dsRNA-mediated gene silencing and appeared to be effective in a dose-dependent manner. Since the discovery of LSNV in 2006 in Thailand, it has been added to the list of viruses to be excluded from domesticated specific pathogen-free stocks of *P. monodon* and it has been recommended that shrimp farmers avoid stocking post larvae positive for LSNV to prevent MSGS in their farms.

Keywords LSNV · MSGS · Retinopathy · ICE · *P. monodon*

M. Poornima (✉) · S. V. Alavandi · J. S. Dayal
Central Institute of Brackishwater Aquaculture, Chennai 600028, India
e-mail: poornima_vet@yahoo.com

Y. Seetang-Nun
Centex Shrimp, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand

Y. Seetang-Nun
National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Pathum Thani 12120, Thailand

Introduction

Shrimp is the largest single seafood commodity accounting for 17 % by value, of internationally traded fishery products. Approximately 75 % of the production is from aquaculture, which is almost entirely dominated the black tiger shrimp (*Penaeus monodon*) and the Pacific white shrimp (*Litopenaeus vannamei*) [13, 57]. Diseases are major constraints in the sustainability of aquaculture worldwide. Intensification of aquaculture production systems continues to create new archetypes for disease expression. Among diseases with infectious causes, viruses are the most devastating agents. Approximately 20 viruses are currently known to infect penaeid shrimp worldwide [14, 23, 24, 27]. In stressful environments such as culture systems, some of these viruses can become more virulent and cause significant economic loss by causing mortality or

retarded growth [55]. Besides the major viral diseases in shrimps, an emerging disease characterized by stunted growth and size variation in farmed black tiger shrimp called Monodon Slow Growth Syndrome (MSGS) is on the rise in recent past in South and Southeast Asian countries. The impact of MSGS in tiger shrimp has been substantial on national economies due to significant production losses and poor productivity.

Monodon Slow Growth Syndrome (MSGS)

The MSGS condition was first noticed in Thailand in 2001 when farmers found an unusual abnormally slow growth and a large size variation of their shrimp. In culture affected by MSGS, shrimp reached an average size of 5–10 g instead of the regular size of 24–40 g after 4 months of culture and very high (30–80 %) coefficient of variation (CV) in weight [58]. The term MSGS was coined by Thai shrimp farmers to refer this unusual retarded growth that has occurred in cultivated *P. monodon* [10, 16, 55] (http://www.biotec.or.th/rdereport/prjbioteceng.asp?Id=882). MSGS is considered as the most serious problem, probably ranks third next to WSSV and YHV in *P. monodon* [19, 42]. The cause of this slow growth is not determined but considered to be implication of an unknown infectious agent [10]. This contention was supported by laboratory trails suggesting involvement of a filterable infectious agent as injection of 0.45 µm filtered lymphoid extracts from MSGS shrimp caused similar slow growth symptoms in normal growing shrimp [58, 59]. It was reported based on a survey that there was no association of known shrimp viruses with MSGS [10]. MSGS in farmed *P. monodon* has reduced the farm profitability, caused serious economic loss, damaged export industries based on this species in Thailand [10, 55] and has been responsible for the major switch to *L. vannamei* farming. [10]. Subsequently, a similar condition was also reported in *P. monodon* from East Africa also [2]. Emergence of MSGS started following large-scale importations of Pacific white shrimp (*L. vannamei*). Hence it was thought that this syndrome would have arisen due to introduction of an exotic virus through such importation (http://www.biotec.or.th/rdereport/prjbioteceng.asp?Id=882).

Working Case Definition of MSGS

Thai researchers adopted a case definition to distinguish ponds exhibiting MSGS from slow growth caused by other problems, for surveillance and epidemiological purposes [19, 55] (http://www.biotec.or.th/rdereport/prjbioteceng.asp?Id=882) and suggested following working case definition: The suspected population should be RT-PCR positive for LSNV and must have a CV of more than 35 % by weight and absence of hepatopancreatic parvovirus (HPV) or of other severe hepatopancreatic infections by known agents while also complying with any three of the following gross signs: (i) unusually dark colour, (ii) average daily weight gain of less than 0.1 g day–1 g at 4 months, (iii) unusually bright yellow markings, (iv) "bamboo-shaped" abdominal segments, and (v) brittle antennae.

Nomenclature and Taxonomy

Laem-Singh virus (LSNV) was identified in 2006 while investigating the cause of MSGS in black tiger shrimp reared at Laem-Singh district, Chanthaburi Province, Thailand [55]. An initial study using random shotgun cloning technique yielded a partial deduced amino acid sequence homologous to viral RNA-dependent RNA polymerase (RdRp) sequences from tissues of MSGS shrimp. This fragment gave relatively low but significant homology to mushroom bacilliform virus (MuBV), type species and only member of genus *Barnavirus* of family *Barnaviridae* and to insect-plant viruses in the family *Luteoviridae*. However, phylogenetic analysis showed that the virus sequence did not cluster with the *Luteoviridae* or other known RNA virus sequences, and the MuBV and LSNV fell between clades of representatives from the families *Togaviridae*, *Flaviviridae* and the dsRNA-viruses, infectious bursal disease virus (IBDV) and bluetongue virus (BTV), with low bootstrap values. Thus, in accordance with frequent practice, it was named according to the area where it was first collected as LSNV [55]. In an attempt to identify the nature of viral genome, when the total nucleic acid extracted from the purified virions digested with RNase enzyme resulted in no RT-PCR product of LSNV. The presence of *RdRp* gene and the results from RNase digestion confirmed that LSNV is an RNA virus [54, 55]. The subsequent work on extending the fragment to a full-length *RdRp* gene using the rapid amplification of cDNA ends (RACE) followed by further phylogenetic analysis revealed possible relationships to insect-borne viruses in the families *Barnaviridae*, *Tymoviridae* and *Sobemoviridae*. Additional sequence information revealed the presence of a protease gene and other structural motifs suggestive of the family *Sobemoviridae* [49, 50, 54]. However, more genome information is required for proper classification of this virus (Table 1).

LSNV: Transmission Electron Microscopy (TEM)

Transmission electron microscopic description of the putative etiological agent of MSGS has been reported by

several researchers [32, 36, 38, 53–55]. Detection of abundant, naked, icosahedral viral-like particles of approximately ~25–27 nm by TEM in the lymphoid organ (LO), gills, neural tissues including optic lobe, supra-esophageal ganglion or brain, thoracic ganglion, abdominal ganglion and ventral nerve cord of both small and normal shrimp from the MSGS pond has been reported by many investigators [1, 38, 49, 50]. Membrane-bound, vacuolated, intra-cytoplasmic inclusions that contained small granular bodies associated with naked, viral-like particles of 25–30 nm diameter located in LO tubule matrix cells at the outer tubule rim has been also observed [55]. It was reported that LSNV was present in the fasciculated zone and onion bodies of organ of Bellonci in the small shrimp, but not in the normal shrimp of both the MSGS and normal ponds. In this study the viral-like particles were observed in unidentified cells and in intercellular spaces in the fasciculated zone. In the neural tissues of the small shrimp, the virus particles were mostly in the connective tissue in intercellular spaces or in the cell cytoplasm. Few particles were seen in the cytoplasm of glial cells and neurons [36]. Icosahedral particles of about 25–33 nm diameter was reported by TEM in fractions between 45 and 60 % (w/w) sucrose gradients from ultra-purified gill tissue of affected shrimp by negative staining technique [32, 54]. Further, a second step purification yielded two bands at approximately 54 and 58 % (w/w) sucrose gradient but the particle sizes and substructure of virions were unclear though confirmed positive by RT-PCR [54]. Presumptive viral-like particles were observed from band obtained at 20–25 % (w/w) CsCl by second step ultracentrifugation. Further, a third round of gradient separation yielded a distinct band at 21 % CsCl (buoyant density 1.1843 g cm^{-3}) which revealed the presence of non-enveloped, icosahedral viral-like particles of approximately 25 and 15 nm diameter using negative staining by TEM, similar to those of previously shown by TEM in tissue sections of stunted shrimp from MSGS ponds [32].

LSNV Genome

Initial work on LSNV showed significant deduced amino acid sequence similarity to *RdRp* of the RNA viruses and homology with viruses in the family *Luteoviridae*. The sequence alignment revealed 3 out of 8 conserved motifs of RdRp gene sequences of viruses in the family *Luteoviridae*. These were acidic SGT motif 2, a GDD (Gly-Asp-Asp) motif 3 and a modified FCG motif 5. The FCG consensus motif 5 was present as FMG in this sequence and as FCS in the others. As with the *Luteoviridae*, there was no basic motif 4 (conserved K between motifs 3 and 5) and motifs 6, 7 and 8 were not found (not ubiquitous in the *Luteoviridae*) and motif 1 fell outside the region of cloned fragment [55]. The GDD motif is found in many RdRp of plant, animal and bacterial viruses [8]. The GDD sequence in motif 3 is conserved in almost all polymerases and is postulated to be located at the enzyme active site [31]. Subsequent work and additional sequence information using RACE revealed the presence of a protease gene and other structural motifs suggestive of the family *Sobemoviridae*. A total sequence of 2,206 bp (LSNV2206) was assembled in a recent study. LSNV contains at least two overlapping open reading frames comprising a peptidase enzyme and RdRp. Although RdRp of LSNV showed high similarity to *Luteoviridae* and *Sobemovirus*, LSNV did not fit with both groups on phylogenetic analysis [54]. The large genome part (2,206 bp) of LSNV approximately constitutes 71 % of LSNV genome encodes a large polyprotein. Sequencing of LSNV2206 revealed part of consensus amino acid sequence (TXXGXSG) of the catalytic triad which is H(X-35)(D/E)(X61-62)TXXGXSG. The C-terminal of LSNV polyprotein predicted to encode RdRp. The putative LSNV-RdRp contains 1,149 bp encoding 382 deduced amino acids, with a predicted molecular mass of about 44 kDa. The conserved motifs reported in other viral RdRps including consensus amino acid sequence (GX3TX3NXnGDD) was found in LSNV-RdRp [6, 54]. The enzyme contains 8 conserved motifs of which I-VI were found to be conserved in LSNV and four of these motifs are in catalytic palm domain [54]. It was also reported that the fragments of the LSNV genome could be detected in the shrimp host genome by Southern blot analysis and RT-PCR, indicating that one or more inserts can occur in shrimp host genome at unknown locations. However, it is yet to be understood whether shrimp and other arthropods have mechanisms of viral integration into their host genomes that could lead to persistent infections without signs of disease [54]. It was

Table 1 Details of LSNV—an emerging pathogen of shrimp

Virus	Abbreviation	Genome	Taxonomic classification[a]	Year emerged	Known geographic distribution	OIE listed disease[b]	Reference
Laem-Singh virus	LSNV	[+] RNA	Luteovirus-like (unclassified)	2003	South and Southeast Asia	No	[52]

[a] ICTV, 2009

[b] OIE, 2009

reported that northern blot analysis indicated LSNV genome from infected shrimp samples was larger (3.4 kb) than those from the purified virus (2.8 kb). Sequence comparison with the *Sobemo* and *Luteo* viruses suggests that 3.4 kb is likely to be the total genome size for LSNV [49, 50, 54]. Further work is required to determine whether single genome fragment breaks during viral extraction or virus contain two RNA fragments [54].

Recently, a unique clone containing an integrase domain that appeared to arise from RNA of viral-like particles from ultra-purified tissue homogenates of MSGS shrimp was identified by shotgun cloning and sequencing. In this study, it was reported that a frame +1 translation of the 2,233 bp sequence yielded a single uninterrupted sequence of 744 deduced amino acids that had no known homology to any protein or translated protein sequence at GenBank except for an integrase protein of bacteriophage SH046 of *Acinetobacter johnsonii* near the 3′ end, with low identity (29 %). Further, it was observed that the deduced amino acids at positions 1,894-2,025 (44 amino acid residues) in the C-terminal portion of the sequence had homology to a conserved domain for phage integrases and DNA breaking enzymes in the DNA_BRE_C family. The sequence was called an integrase containing element (ICE) [32]. Northern blot of RNA from the 21 % (w/w) CsCl gradient band when subjected to northern blot analysis using ICE probe gave a positive hybridization signal at approximately 3.0 kb for ICE, while no signal was detected in the RNA extracted from specific pathogen-free (SPF) shrimp [32].

The consensus sequence of 2,233 bp of ICE identified together with LSNV exclusively in stunted shrimp revealed no significant identity to known nucleic acid sequences except for 100 % identity in 46 bases near the 3′ end of the sequence with thrombospondin of genus *Penaeus* (*Marsupenaeus*). Also, the ICE sequence shared no significant identity to the XSV genome sequence or any other known satellite virus sequences. Neither the XSV genome nor other known satellite genomes contain integrase genes [28]. Similarly, the ICE sequence showed no significant homology to the genomes of naked RNA viruses with integrase genes that can be found in the families *Pseudoviridae* [7] and *Metaviridae* [12] that are related to enveloped viruses in the family *Retroviridae* [26]. ICE contained an integrase domain with a conserved catalytic triad H-R-H and a conserved tyrosine (Y) in a binding region that may be involved in host genome insertion [22, 30]. The 44 amino acids of integrase domain found in LSNV affected shrimp showed homology only to integrases or recombinases of bacteria and bacteriophages and not viral-like members of the families *Pseudoviridae* and

Table 2 Sequencing of LSNV PCR products and genome fragments

Species	Country	Milestone year		Genome		References
		Disease observed	Virus observation	Sequence Accession	Product size (bp)	
P. monodon	Thailand	2001	2006	DQ127905	615	[55]
P. monodon	Vietnam	2002	2009	FJ811836	199	[54]
P. monodon	Vietnam	2002	2009	FJ811835	199	[54]
P. monodon	Malaysia	2003	2009	FJ811834	199	[54]
P. monodon	India	2006	2007	EF593037	194	[35]
P. monodon	Thailand	2008	2008	FJ356710	602	
P. monodon	Thailand	2008	2008	FJ356709	604	
P. monodon	India	2007	2009	FJ811832	199	[54]
P. monodon	India	2007	2009	FJ811833	199	[54]
P. monodon	India	2007	2009	FJ811829	199	[54]
P. monodon	India	2007	2009	FJ811831	199	[54]
P. monodon	India	2007	2009	FJ811830	199	[54]
P. monodon	India	2007	2009	FJ811837	199	[54]
P. monodon	India	2007	2009	EF593037	199	[54]
P. monodon	India	2007	2009	FJ811838	199	[54]
L. vannamei	India	2010	2010	HQ825248	205	[51]
P. monodon	India	2010	2010	HQ728430	357	[51]
P. monodon	Thailand	2008	2011	FJ498866	2214	[32]
P. monodon and *L. vannamei*	India	2008–2011	2008	JQ219262–JQ219307	340–597	[33]

Metaviridae. The nature of ICE and its phylogenetic relationship to other viral-like entities known to occur in invertebrates still remains an enigma [32].

Genetic Lineage of LSNV

Many studies [47, 51, 52] (http://www.vienthuysan2.com/index.php?do=news&act=detail&id=32&lang=en) have found that the sequences obtained from different geographical locations had high homology (97–98 %) with the reference Thai isolates (Table 2). It was observed that sequences of eight viruses from Thailand, Malaysia and India, were identical to the Thai reference strain and there was no clustering according to geographic location or from the healthy or MSGS affected shrimp. Phylogenetic analysis based on nucleotide sequence of *RdRp* gene of ten Indian isolates and their comparison with published nucleotide sequences of LSNV suggested that LSNV exists as a single genetic lineage (Fig. 1) [33, 34] and occurs commonly in healthy *P. monodon* in parts of Asia [52]. The nucleotide sequence of *RdRP* gene fragment of LSNV was found to be highly conserved (99 % identity) across the four penaeid species [51]. Further phylogenetic studies reported LSNV peptidase had high sequence similarity to those of *Sobemovirus* whereas the LSNV-RdRp had high sequence similarity to RdRps of MuBV in the family *Barnaviridae* and viruses in the genus *Polerovirus* of the family *Luteoviridae* [54].

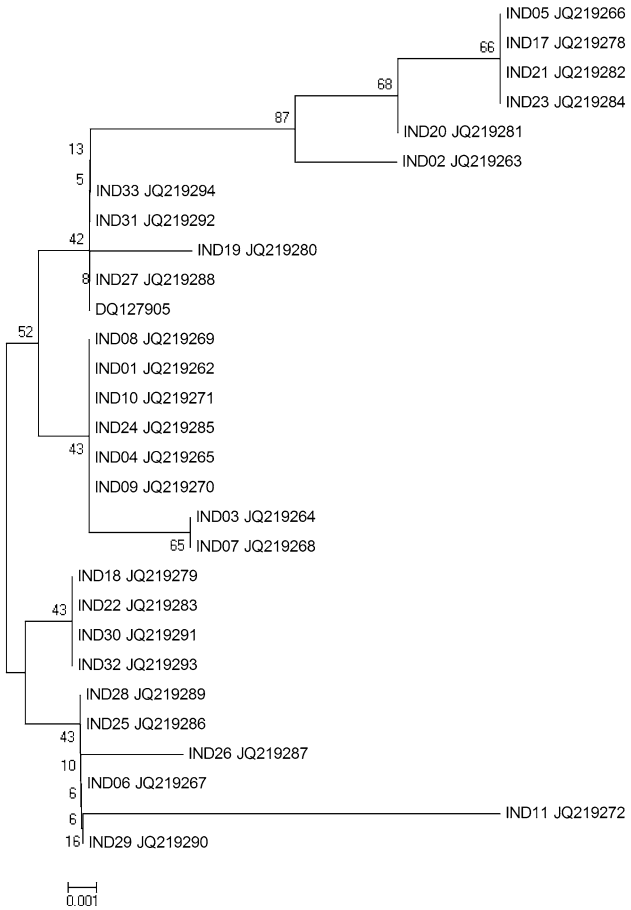

Fig. 1 Phylogenetic tree of LSNV isolates based on the partial sequencing of RdRp coding region. The numbers at each node represent the percentage bootstrap scores (10,000 replicates) [33]

Geographical Distribution of MSGS and Prevalence of LSNV

LSNV was reported in association with MSGS in both healthy and infected *P. monodon* first from Thailand and later in Malaysia, Indonesia, India and Vietnam suggested a geographical distribution restricted to South and Southeast Asia [52]. Initial studies reported low incidence of LSNV from India [35, 40] probably the disease was newly reported from India. A later study reported 56.8 % prevalence of LSNV infection from 15 to 135 days culture period in Andhra Pradesh during 2007 and was no obvious clustering of positive samples according to the site of collection. Recent reports indicated much higher prevalence from the farms from both east and west coast of India with the history of extended culture period and growth retardation during past few years [10, 33]. Recently relatively high prevalence of LSNV was observed in brooders from different locations like Tamilnadu, Orissa and Maharashtra of India [33] including Andaman and Nicobar [33, 51]. LSNV was reported in brooders and post larvae for the first time in Sri Lanka in 2010 [39]. Case of growth retardation also found in *P. monodon* in East Africa in 2004. It was reported that the mean body weight of the shrimp at the sixth month in affected ponds was 19 ± 4 g, which was 30 % less than the expected size of normal shrimp grown within the same period. However, the phenomenon of MSGS occurring in this region and the growth retardation reported in farmed shrimp from East Africa is the same, remains unknown [2].

Economic Impact

Since 2000, economic losses due to MSGS became apparent. The most drastic consequence to the farmer is the uncertainty of final harvest yield and value. In a typical case, after 3-4 months of culture, the size variations ranges from 80 to 300 pieces kg^{-1} in a single pond [25] and at similar stocking density, the average growth rate in MSGS ponds is approximately half that of normally expected by shrimp farmers [36]. Shrimp farming in Thailand was reported to have suffered great economic damage as the

production value of farmed *P. monodon* dramatically dropped to 40-68 % over a 2 year period. The accumulated damage caused by this phenomenon was estimated at 13 billion Baht (US$ 300 million) [10, 58]. In India, white spot disease, LSS and slow growth have been primarily responsible for economic losses to the shrimp farming sector. The production loss due to slow growth and white gut disease was estimated to be 5726 MT amounting to Rs.120 crores per year (about US$21.64 million annually) [5, 21].

Host-Range and Potential Vectors

The natural host-range of LSNV is presently unknown, but it occurs commonly in *P. monodon* [33, 35, 36, 40, 51, 52, 55]. LSNV was detected in *P. monodon*, *Fenneropenaeus merguiensis*, *Metapenaeus dobsoni* and *L. vannamei*, but not in *F. indicus*, *Marsupenaeus japonicus* and *Scylla serrata*. LSNV was most prevalent in *P. monodon* followed by *M. dobsoni*, *F. merguiensis* and *L. vannamei* in India [51]. *L. vannamei* may act as carrier as evidenced by experimental transmission study [1]. It was also detected in *L. vannamei* in Thailand affected by abdominal segment deformity disease [46]. An earlier study reported no evidence of LSNV infection in healthy juvenile or adult *P. monodon*, *F. merguiensis* or *P. japonicus* from northern and eastern Australia between 1998 and 2006. This is probably due to the fact that the numbers of Australian *P. japonicus* and *P. mergiuensis* tested were small and *P. vannamei* sampled from Thailand had origins as SPF stock imported from Hawaii [52].

Modes of Transmission

LSNV has been reported to be transmitted vertically as well as horizontally, as indicated by its widespread detection in wild and domesticated brood stock and post larval shrimp in Thailand and India [33, 41, 48, 51, 52]. High prevalence of LSNV was reported in female shrimp broodstock and various developmental stages of shrimp viz., nauplius, zoea, mysis, post larvae 5 (PL5) and PL15 [47]. It has been also reported that the slow growth syndrome in *P. monodon* could be induced by injection of LO extracts of MSGS affected shrimp into healthy *P. monodon,* that result in severe growth retardation and extreme size variation of 20–45 % in experimentally induced and 17–24 % in control groups [58, 59]. In another study, Pacific white shrimp, *L. vannamei* that were co-cultured with MSGS affected *P. monodon* were reported to grow normally. However, membrane filtered LO extracts from these co-cultured *L. vannamei* caused MSGS when injected into healthy *P. monodon.* These experimental transmission studies suggest *L. vannamei* might act as asymptomatic carrier [1]. Bioassay experiments by oral feeding of MSGS affected shrimp homogenates in healthy tiger shrimp showed 100 % LSNV-positives by 10 weeks along with signs of size variation, dark discoloration, bright yellow markings (Fig. 2) and 90 % mortalities by end of four and half months of experimental period in infected group [33]. Further, the cohabitation experiments of tiger shrimp showed 100 % positives by 5 weeks along with size variation and 65 % mortalities by end of five and half months of experimental period [33, 47]. In another study on experimental transmission in mud crab *S. serrata* a gradual increase in viral load 8 days post-injection was reported, which appeared to stabilize until 20 days post-infection. The ability of LSNV to cause infection when injected into a crustacean species other than shrimp suggests that other species might be potential natural carriers of LSNV infection [51].

Predisposing Factors

It was concluded that LSNV is a necessary but insufficient cause of MSGS. The other component cause (s) that leads to LSNV-associated stunting of shrimp in MSGS ponds is still unknown but may involve other pathogens and/or environmental factors [16, 17]. The prevalence of LSNV infection can vary significantly in different populations of *P. monodon* and, as stress is commonly a stimulus for the onset of disease. More work is required to elucidate possible associations with loose shell, slow growth or other pathologies. The best way to prevent MSGS is through adapting better management practices during culture [52].

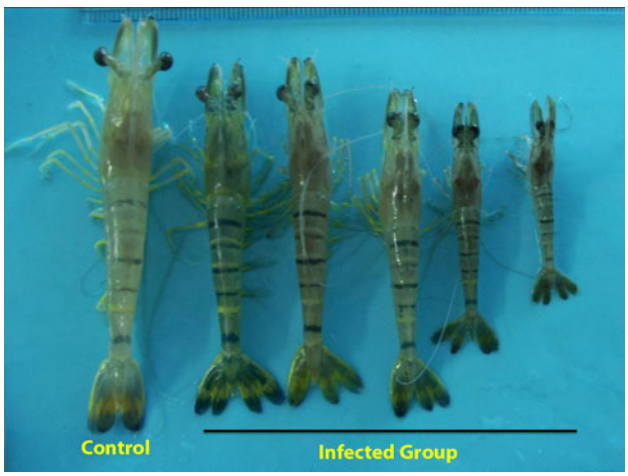

Fig. 2 Shrimp exhibiting size variation with dark discoloration and stunted growth by experimental challenge study [34]

Pathogenesis

The LO is suggested to be a major site for viral predilection as indicated by formation of LO spheroids as reported in a number of viral diseases of penaeid species [2, 3, 44, 45]. In LSNV-infected *P. monodon,* viral-like particles could be observed by TEM only in the LO tubules which were ISH positive [55]. Further work suggested that slow growth in the small shrimp from the MSGS ponds may be due to a specific infection of LSNV in the fasciculated zone and onion bodies of organ of Bellonci of the eyes. It is thought that progression of infection in the optic nerve may be the cause of slow growth. It is yet unclear whether damage to the fasciculated zone and onion body of the organ of bellonci is due to LSNV infection or other pathogens that finally lead to slow growth in *P. monodon*. Also, it could be possible that shrimp may suffer from growth retardation caused by retinopathy resulting from interaction between LSNV and some other agent or factor(s) like ICE or other mechanisms which may predispose their fasciculated zones and organs of Bellonci to infection and damage [38]. A comparative study on moulting frequency and the transcription level of moult-inhibiting hormone (MIH) in small and large shrimp from MSGS pond with normal shrimp showed that the moult frequency of small shrimps was higher than other groups. The transcription of MIH in small shrimps was lower than other groups in all stages of moulting suggesting that retinopathy may be involved in the reduction of MIH levels [37].

Histopathology

Histopathological studies of growth-retarded shrimp from MSGS ponds have revealed pathognomonic lesions in eyestalk [5, 33, 34, 36, 38, 41]. The striking difference between the small and large LSNV-positive shrimp was that retinopathy was observed exclusively in the small shrimp from the MSGS ponds. Retinopathy comprised of abnormally enlarged haemolymphatic vessels, haemocytic infiltration in the fasciculated zone of the small shrimp [33, 36, 38, 41] and rupture of the membrane that separated the fasciculated zone from the overlying row of retinular cells [36, 38]. There was no degeneration of nerve fibers or neurons, abnormal inclusions except haemocytic infiltration in the crystalline tracts in a few small shrimp. Lymphoid organs spheroids (LOS) of affected shrimp show unusually large, magenta cytoplasmic inclusions [55, 58]. It was suggested that slow growth in the small shrimp from the MSGS ponds may be due to specific infection of LSNV in the fasciculated zone and onion bodies of organ of Bellonci of the eyes [36]. A fatal disease, 'peripheral neuropathy and retinopathy' (PNR), associated with minor to heavy mortalities was reported from eastern Australia in shrimp infected with yellow head-like virus. However, the histopathological symptoms differed, in that, these shrimp showed mild to severe, focal to diffuse degeneration and necrosis of axons and their sheaths, together with associated glial cell apoptosis, in peripheral nerve fibres [9].

LSNV Diagnostics

Nucleic acid based diagnostic methods like RT-PCR, nested RT-PCR, loop-mediated isothermal amplification combined with a lateral flow dipstick (RT-LAMP-LFD) have been used for diagnosis of LSNV with improved sensitivity and specificity [4, 29, 32–36, 40, 51, 52, 55] using various primers (Tables 3, 4, 5, and 6). Methods of quantification of LSNV by real-time quantitative RT-PCR (qRT-PCR) has been also developed and the authors also reported variation in LSNV loads in different shrimp species tested [51].

In Situ Hybridisation (ISH)

A number of studies have demonstrated LSNV in various tissues of MSGS affected shrimp using ISH [15, 36, 38, 49, 50, 55]. In situ hybridization tests revealed the presence of LSNV in the cytoplasm of cells in the LO, heart, hepatopancreatic, interstitial cells [20, 55], neural tissues including optic lobe, supra-esophageal ganglion, thoracic ganglion, abdominal ganglion, ventral nerve cord and connective tissue of the brain. LSNV was detected in the fasciculated zone and onion bodies of organ of Bellonci in the small shrimp, but not in the normal shrimp of both the MSGS and normal ponds [36, 38]. Also in situ hybridization tests with experimentally challenged shrimp showed positive reactions in LO, eyes and gills of the test shrimp only for both ICE and LSNV [32] supporting the link between retinopathy and LSNV [36].

Therapeutics

RNA interference (RNAi) based technology targeting *RdRp* gene involved in the replication of LSNV was exploited to inhibit replication of LSNV [29, 48]. It was observed that interference mediated by viral sequence-specific dsRNA appeared to be effective in a dose-dependent [48]. In another study, dsRNA targeting *PmRab7* gene (dsRNA-PmRab7) was employed to investigate the inhibitory effect on LSNV replication in *P. monodon*. It was reported that dsRNA-PmRab7, could significantly knockdown *PmRab7* gene resulting in the prevention of LSNV

Table 3 Details of RT-PCR primers used in different studies for detection of LSNV

Primer	Sequence 5′–3′	Expected product (bp)	Reference
LLV-F	ttgccttctcccgagtggtc	200	[33, 35, 36, 40, 55]
LLV-R	ccggctgaggtagctgcttg		
BLF	cgttgccttctcccgagtggt	357	[51, 52]
LR1	aatctcaccatgaagctcctcac		
LSNVF	gctctttgcgcctatgaatg	850	[29]
LSNVR	gccccagaaacgtattggcac		
LSNVF	gttgccttctcccgagtg	597	[33]
LSNVR	gccccagaaacgtattgg		
LSNVF1	ttctcccgagtggtcaggttta	588	[4]
LSNVR1	ccagaaacgtattggcacacg		
LSNV-Fr	cgttcgcttctcccgagtggt	600	[32]
LSNV-Rv	ttgccccagaaacgtattggca		
Nested primers			
LSNVnF	gcgcaagagttctcaggctt	140	[33, 35, 40]
LSNVnR	atcaccgcaggctaatatag		
LF2	agatcatgctgcatatgcttgc	205	[51, 52]
LR2	gtgtagattggttgcatggcg		
LSNVFPn	acgcgcaagagttctcag	340	[33]
LSNVRPn	aggggtggtgagcgtac		
LSNV-NFr	ttgccttctcccgagtggtc	195	[32]
LSNV-NRv	ccggctgaggtagctgcttg		

Table 4 Details of probes used for in situ hybridisation [36, 55]

Probe	Labelling of probe	Oligonucleotide probe sequences (5′–3′)
20AF	DIG-labelled probe	ttgccttctcccgagtggtc
20AR		ccggctgaggtagctgcttg

Table 5 Details of oligonucleotide sets used for SYBR Green qRT-PCR assay [51]

Primer	Gene	Sequence [5′–3′]
LSNVQPR1	RdRp	gcttgcgatcgacactcttaac
LSNVQPF1		tagcctgcggtgatgacacta

replication. Injection of dsRNA-PmRab7 24 h before challenge with the virus resulted in a drastic decrease of *PmRab7* mRNA and complete inhibition of LSNV replication by 3 days post-challenge. However in a therapeutic mode, shrimp injected with dsRNAPmRab7 only one day but not at 3 or 5 days post-LSNV challenge resulted in

Table 6 Primers and probe used for LSNV RT-LAMP detection [4]

Primer and probe	Genome position	Sequences (5′–3′)
LSNV-F3	101–120	tcatgctgcatatgcttgct
LSNV-B3	318–299	tgcgatgtgtttcatggtgt
LSNV-FIP-biotin	196–176/tttt/134–151	biotin-cggctgaggtagctgctt gaattttgtgagcccgtgactccta
LSNV-BIP	214–233/tttt/284–265	gcgaaggcagggtgcattgttttt gcgccctcaaagttaaaacc
LSNV-LF	155–172	tgtcatcaccgcaggcta
LSNV-LB	238–255	agtgtcgatcgcaagcta
FITC-probe	197–211	fitc-gttatattgaagagc

inhibition of LSNV replication [29]. The reason for this was attributed to the possibility of prior infection of target cells by LSNV by day 3 or 5 or an increase in viral load before dsRNA injection, minimizing the protective effect of dsRNA. Also there may be loss of RNAi potency due to the virus produced RNAi suppressor [11]. However, whether some LSNV proteins act as suppressor needs to be investigated. These two studies suggested a promising use of RNAi in therapeutic intervention. Hence, RNAi may be developed as a tool for cleaning up domesticated or wild shrimp stocks found to be infected with LSNV [29, 48].

Strategies for the Control of LSNV

Shrimp farmers have been reporting slow growth of black tiger shrimp since 2002. The fifth meeting of the Asia Regional Advisory Group (AG) on Aquatic Animal Health (29) suggested that MSGS seriously affects cultured *P. monodon*. The specific cause of MSGS has yet to be determined unequivocally, and whilst its occurrence is strongly associated with infection by LSNV [17, 55], there is no evidence that LSNV infection alone can cause the syndrome [36]. Since the discovery of LSNV in 2006, it has been added to the list of viruses to be excluded from domesticated SPF stocks of *P. monodon* in Thailand and it has been recommended that shrimp farmers avoid stocking LSNV positive post larvae to avoid MSGS [4, 25, 32]. It is recommended that in countries where *L. vannamei* has already been introduced, *L. vannamei* and *P. monodon* should be reared separately, particularly at the maturation and hatchery phases [16, 42, 43] (http://www.biotec.or.th/rdereport/prjbioteceng.asp?Id=882). The reason is not only to prevent disease spreading but the cultivation method of the two species is quite different [58]. Incidence of MSGS has been reported to be low in farms adopting GMP, GAqP or using SPF PL [56]. It would be prudent to limit the potential spread of MSGS by initiating quarantine

safeguards in the movement of living shrimp stocks for aquaculture and to exercise due caution to prevent its spread by live broodstock or PL for aquaculture [2, 15]. Further, the National authorities should increase surveillance for slow growth syndrome in *P. monodon*. These measures will help reduce the risk of importing exotic viral pathogens that may damage local aquaculture [20].

Concluding Remarks

MSGS an emerging new syndrome in farmed *P. monodon* is a serious problem that reduced the farm profitability and made farmers to switch over to cultivation of American white shrimp. The anecdotal reports of unusual slow growth in cultivated *P. monodon* revealed common occurrence of LSNV in South and South East Asian countries and probably *P. monodon* a primary host for LSNV. Extensive surveys are needed to explore the range of natural hosts of LSNV as other crustacean species might be sources of infection. In view of the severity of the loss in production, the specific cause of MSGS yet to be determined unequivocally. Although MSGS occurrence is strongly associated with infection by LSNV, it is essential to understand pathogenesis causing retinopathy and effect on moulting. The other cause(s) that lead to LSNV-associated stunting of shrimp in MSGS ponds is still unknown but may involve other pathogens and/or environmental factors. More genome information is needed for proper classification of LSNV. The sources of LSNV and the cause of MSGS agent are unknown, but it is possible that they might have been originated from exotic crustaceans that have been imported for aquaculture and for the ornamental aquarium trade [18]. The problem is further complicated by the fact that it is transmitted by both vertical and horizontal routes. Also detailed studies that address the epizootiology of MSGS infection among cultured species to assess the risk of potential danger of transfer from the wild or asymptomatic carriers to the cultured species needs to be taken up. To date, no effective therapeutic measures are available to cure LSNV infection. Despite the fact that the actual etiology of MSGS is unknown, it is recommended that farmers avoid stocking ponds with LSNV-infected post larvae. Further, *L. vannamei* and *P. monodon* should be reared separately, particularly at the maturation and hatchery phases, and LSNV should be added to the list of pathogens for exclusion. The RT-PCR methods will be useful tools to select and maintain virus free penaeid shrimp stocks. Further, information on survival and stability of the agent outside the host and possible role of vectors needs to be elucidated which would help in formulating control strategies of MSGS. Also, large-scale outbreaks of emerging viral diseases among shrimp illustrate the need for new approaches towards disease surveillance and control.

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