Vol. 77: 83–86, 2007
doi: 10.3354/dao01835

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published August 13

NOTE

Detection of Laem-Singh virus (LSNV) in cultured *Penaeus monodon* from India

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ABSTRACT: Viral diseases have become a major constraint on the growth of shrimp aquaculture in India. During a study of new diseases in cultured shrimp *Penaeus monodon*, diseased samples randomly collected from the southwestern and southeastern coasts of India were analyzed for white spot syndrome virus (WSSV), monodon baculovirus (MBV), and hepatopancreatic parvovirus (HPV) by nested PCR, and for Laem-Singh virus (LSNV) by reverse transcription PCR (RT-PCR). Of the 56 samples analyzed, 3 were positive for LSNV. These samples had signs of loose shell syndrome (LSS). Of the 3 samples that tested positive for LSNV, 2 were also positive for WSSV and MBV, and of these 2 samples, 1 was also positive for HPV. This is the first reported presence of LSNV in cultured shrimp in India.

KEY WORDS: Laem-Singh virus · Loose shell syndrome · *Penaeus monodon* · Reverse transcription PCR (RT-PCR)

INTRODUCTION

The sustainability and economic success of the shrimp culture industry have been adversely affected by viral diseases. In the presence of predisposing environmental conditions, viruses cause disease outbreaks resulting in massive losses to the industry. Intensive shrimp cultivation, inadequate sanitation, poor pond management and irresponsible movement of broodstock and larvae have exacerbated the disease incidence and enhanced its spread. With expansion of the industry, the number of viral pathogens of penaeid shrimp has also increased, reaching a new high of 20, as compared to only 6 in 1988 (Lightner 1988, 1993, 1996).

Among all known viruses of shrimp, white spot syndrome virus (WSSV) is the most prevalent and is considered to be pandemic, affecting most countries that culture shrimp. In addition to single viral pathogens, multiple viral infections of cultured shrimps have also been reported. These include triple infections with yellow head virus (YHV), hepatopancreatic parvovirus (HPV) and monodon baculovirus (MBV) in *Penaeus monodon* (Chantanachookin et al. 1993), HPV, MBV and WSSV in post larvae (Manivannan et al. 2002) and, recently, MBV, HPV and WSSV in adult prawns (Umesha et al. 2006).

Recently, several new diseases of unknown or obscure etiology have been reported in the shrimp culture industry. These include swollen hindgut syndrome (SHG) (Lavilla-Pitogo et al. 2002), monodon slow growth syndrome (MSGS) (Chayaburakul et al. 2004) and loose shell syndrome (Mayavu et al. 2003). MSGS was first observed in cultured *Penaeus monodon* in Thailand, and in the absence of known viral pathogens, the causative agent was designated as monodon slow growth agent (MSGA) (Chayaburakul et al. 2004). Later investigations revealed the presence of a virus called Laem-Singh virus (LSNV) (Sritunyalucksana et al. 2006). The unusually retarded growth and wide variation in size, without abnormal

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mortality (which is similar to MSGS in Thailand), have also been reported from East Africa (Anantasomboon et al. 2006). Though such disease problems have been frequently observed in hatcheries and growout systems in India, no studies have been conducted to determine the possible association of viral/bacterial pathogens. Here, we present a first report of the occurrence of LSNV in cultured shrimp (*P. monodon*) from the Indian coast.

MATERIALS AND METHODS

Diseased samples of tiger shrimp *Penaeus monodon* were collected randomly from shrimp farms in Kundapura (Karnataka) along the southern west coast and in Kakinada and Amalapuram (Andhra Pradesh) along the southern east coast of India. Post larvae (PL) were collected from different hatcheries along the southeastern and southwestern coasts of India. The PL were preserved in RNAlater (Ambion) and brought to the laboratory. Juvenile and adult shrimp samples were processed immediately upon collection for extraction of RNA.

Extraction of viral DNA. Approximately 100 mg of hepatopancreas, gills and pleopods were taken separately from each sample, and DNA was extracted using the method described by Otta et al. (1999). DNA extracted from the hepatopancreas was used for the detection of both HPV and MBV by PCR, while the DNA from gills and pleopods was used for detection of WSSV. In the case of PL, more than 30 individuals were taken for DNA extraction.

Total RNA was extracted from samples using TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 750 µl of TRIzol reagent was added to the tissue (hepatopancreas/PL) and homogenized. After incubating for 5 min, it was centrifuged at 12 000 × *g* for 15 min at 2°C. RNA was precipitated from the aqueous phase with isopropanol, washed with 75% ethanol and dissolved in 50 µl of diethylpyrocarbonate treated water. This was stored at –80°C.

Polymerase chain reaction. Nested PCR was performed for detection of WSSV, MBV and HPV using primers and thermocycling conditions previously described for WSSV (Hossain et al. 2001), MBV (Belcher & Young 1998, Otta et al. 2003) and HPV (Phromjai et al. 2002, Umesha et al. 2006). All the amplification reactions were carried out in a 30 µl reaction mixture consisting of a 1X assay buffer (Bangalore Genei), 10 pmol of each forward and reverse primer, 200 µM of each of 4 deoxyribonucleotide triphosphates (dNTPs), 1 U of Taq DNA polymerase (Bangalore Genei), and 2 µl of DNA extract as template. All PCR reactions were performed in a PTC 100 thermocycler (MJ Research). The amplified PCR products were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide (0.5 µg ml^{-1}) and photographed using a gel documentation system (HeroLab).

Reverse transciption-PCR (RT-PCR). One pair of primers specific to LSNV (described by Sritunyalucksana et al. 2006) was used for amplification of LSNV RNA, yielding a product of 200 bp. For nested PCR, primers LSNVnF 5′ GCG CAA GAG TTC TCA GGC TT 3′ and LSNVnR 5′ATC ACC GCA GGC TAA TAT AG 3′ internal to the 200 bp fragment were designed from the sequence (GenBank Accession No. DQ127905) to yield an amplicon size of 140 bp. Reactions were performed in 30 µl RT-PCR buffer containing 10 pmol of each primer and 2 µl of RNA template. The thermocycling conditions consisted of RT at 42°C for 1 h, denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1 min, ending with an additional elongation step of 10 min at 72°C. The RT-PCR products were then analyzed by electrophoresis on a 2% agarose gel. A cloned LSNV fragment (kindly provided by T. W. Flegel, Thailand) was used as the positive control.

RESULTS AND DISCUSSION

Of the 56 samples analyzed in this study (Table 1), 28 were positive for WSSV, 14 for MBV and 12 for HPV. All these samples were also analyzed by RT-PCR for the detection of LSNV, and 3 were found to be positive (Fig. 1). Interestingly, none of the swollen hindgut syndrome postlarval samples was positive for LSNV by

Table 1. *Penaeus monodon*. Numbers of samples analyzed for white spot syndrome virus (WSSV), monodon baculovirus (MBV), hepatopancreatic parvovirus (HPV), and Laem-Singh virus (LSNV) which displayed signs of loose shell syndrome (LSS), swollen hindgut syndrome (SHG), or were normal or putatively normal

Sample details	No. of samples
Samples with loose shell syndrome (Kundapura[a])	4
Samples with swollen hindgut syndrome (post larvae) (Kakinada[b])	13
Samples of putatively normal post-larval stage	21
Samples with swollen hindgut syndrome (Kundapura)	5
Normal adult samples	13

[a]Southern west coast of India
[b]Southern east coast of India

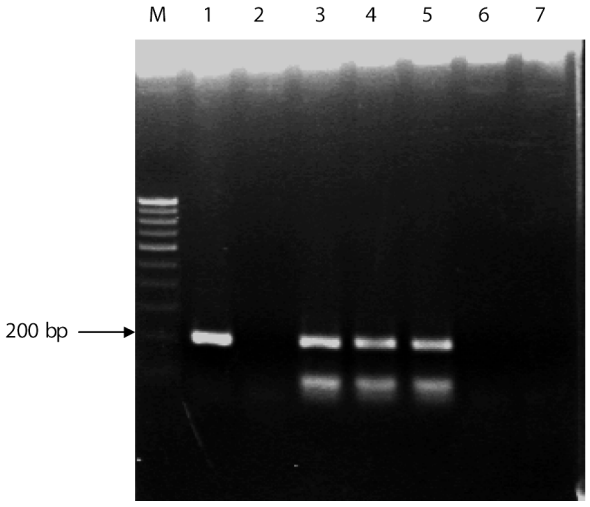

Fig. 1. *Penaeus monodon*. First step RT-PCR for detection of LSNV. M: 100 bp DNA ladder Plus (Gene Ruler TM genetix) . Lane 1: Positive control; Lane 2: Negative control; Lanes 3 – 5: samples with LSS positive for LSNV; Lane 6: Normal adult shrimp sample; Lane 7: Normal postlarvae

RT-PCR. All 3 LSNV-positive samples were confirmed by sequencing the PCR products. BLAST (Altschul et al. 1997) analysis of the sequences revealed 98% homology (EF593037) with the sequences of LSNV reported by Sritunyalucksana et al. (2006). To our knowledge, this is the first report of LSNV from India.

All 3 samples positive for LSNV were adult shrimp showing signs of 'loose shell syndrome' (LSS). LSS-affected shrimp have hard or leathery shells (that are not soft), and shrunken tail meat, so that there is a gap between the shell and the muscle tissue (causing the 'loose shell'). They also reject feed and may have swollen hindguts that contain a whitish fluid. Moribund specimens exhibit bacterial and fungal infections and exterior algal fouling. The infected shrimps move to the pond margin and finally die. Studies conducted so far have not conclusively determined the etiological agent for this syndrome. The presence of LSNV in both normal and MSGS shrimp has been reported previously (Sritunyalucksana et al. 2006).

Of the 3 shrimp samples positive for LSNV, 2 were also positive for WSSV and MBV, and within these 2 samples, 1 was also infected with HPV. Previous studies have revealed multiple infections in growth-retarded *Penaeus monodon* cultivated in Thailand (Chayaburakul et al. 2004). According to these studies, HPV infection was a contributing factor, but not the overriding factor responsible for MSGS, even though *P. monodon* infected with HPV shows reduced growth (Flegel et al. 1999). Multiple viral infections were also reported in postlarval samples from hatcheries (Manivannan et al. 2002) and from adult shrimp (Umesha et al. 2006). In the present study, we could not relate the presence of LSNV to LSS, even though the 3 samples positive for LSNV showed symptoms of LSS. More studies are needed to understand the impact of LSNV on shrimp health and growth.

Acknowledgements. Financial support from the Department of Biotechnology, Government of India through the 'Programme Support in Fisheries Biotechnology' is gratefully acknowledged.

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Editorial responsibility: Timothy Flegel, Bangkok, Thailand

Submitted: March 28, 2007; Accepted: June 5, 2007
Proofs received from author(s): July 31, 2007