Isolation and Characterization of Bacteria Associated with Cultured *Penaeus monodon* Affected by Loose Shell Syndrome

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Abstract

Seventy shrimps affected by loose shell syndrome (LSS), a major disease causing mass mortalities in shrimp farms in north coastal Andhra Pradesh (India), were subjected to bacteriological analysis. Total bacteria and *vibrio* counts in the hemolymph were estimated; bacteria were isolated and identified; their pathogenicity and drug sensitivity were studied. Total bacteria ranged 1.28 x 10^2 to 5.8 x 10^5 cfu/ml while *Vibrio* ranged 0.7 x 10^2 to 2.1 x 10^5 cfu/ml. Six species of *Vibrio* were isolated from the hemolymph including *V. harveyi, V. alginolyticus, V. parahaemolyticus, V. anguillarum, V. splendidus*, and *V. vulnificus*. *Vibrio harveyi* was the most dominant with a prevalence of 100%. It was also the most virulent with a low LC_{50} value of 1 x 10^3 cfu/g. Histopathological study revealed changes typical of bacterial septicemia including invasion of bacteria into the hemolymph, formation of granulomas in the hepatopancreas and other organs, and extensive proliferation of connective tissue and necrosis of the hepatopancreas, gills, and ovarian tissues. Sensitivity to 22 antibiotics was tested. Most of the bacterial isolates were highly sensitive to chloramphenicol, ciprofloxacin, and norfloxacin while *V. harveyi* was resistant to as many as 16 antibiotic drugs including chloramphenicol.

Introduction

Penaeus monodon culture ponds in north coastal Andhra Pradesh (India) have been experiencing mass mortalities due to the emerging loose shell syndrome (LSS) since 1998. The disease is quite prevalent in East Godavari district and, together with the white spot syndrome (WSS), has caused severe economic losses. Diseased shrimps display clinical symptoms such as a loose shell, soft muscle, and a condensed melanized hepatopancreas. Other symptoms include loss of appetite, weak response to stimuli, and erratic swimming. The disease affects shrimps of all ages but juveniles more frequently.

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Culture ponds of all types, irrespective of stocking density, were affected and mortality ranged from moderate to high. Identification of the etiological agent and strategies to control the disease have remained most challenging. Earlier studies of LSS-affected shrimps include those of Mayavu et al. (2003) on histology, Gopalakrishnan and Parida (2005) on growth, production, and histopathology in ponds on the northern bank of Vellar Estuary, and Jayasree et al. (2006). The aim of the present study was to identify the *Vibrio* spp. associated with the disease and study their pathogenicity and implications on disease onset.

Materials and Methods

Sampling sites. LSS-affected shrimp were collected from culture ponds in Moolapeta (Kakinada), Bodasakurru (Amalapuram), and Mulakuddu (Visakhapatnam) in north coastal Andhra Pradesh. Stocking density in the ponds ranged 100,000-150,000/ha. Shrimp were sampled during the summer (April-June) when the pond temperature (28-32°C) and salinity (28-40 ppt) were high.

Isolation and identification of bacteria. Seventy diseased shrimps (5-30 g), including 30 from Kakinada, 20 from Amalapuram, and 20 from Visakhapatnam, were subjected to bacterial analysis. Hemolymph (0.2 ml) from each shrimp was plated on tryptone soya agar (TSA) and thiosulphate citrate bile sucrose agar (TCBS) for estimation of total bacteria and *Vibrio* counts as suggested by Lightner (1996). Dominant colonies were selected and repeatedly streaked on TSA to obtain pure isolates. The bacteria were identified by morphological, physiological, and biochemical tests (Baumann and Schubert, 1984). In addition, bacteria were grown on different concentrations of sodium chloride supplemented in TSA. The reactions of the bacteria isolates were compared with reference strains in the laboratory.

Histopathology: Moribund shrimps in acute stage of infection were fixed in Davidson's fixative and sectioned at 5 mm thickness and stained with Hematoxylin and eosin following standard methods (Bell and Lightner 1988). PCR (supplied by Bangalore Genei Pvt. Ltd. India) was conducted on gill tissue to detect the WSS virus.

Pathogenicity trials. Healthy juveniles (4.5 g) were experimentally infected to determine the pathogenicity of four *Vibrio* spp.: *V. harveyi, V. alginolyticus, V. parahaemolyticu*s, and *V. anguillarum*. Inocula of each species were prepared in triplicate on phosphate buffered saline (PBS) at concentrations ranging 1 x 10^1-10^7 colony forming units (cfu) per g. The solution (0.5 ml) was inoculated intramuscularly into 20 juvenile shrimp per replicate while shrimp inoculated with PBS alone served as the control. After inoculation, shrimp were maintained in fiberglass tanks in sterile sea water (15 ppt). Mortality was recorded up to seven days after inoculation and LC_{50} values were determined. Moribund shrimp were sacrificed and bacteria were isolated.

Drug sensitivity. Standard methods were followed to study the susceptibility of *Vibrio* spp. to commonly used drugs. Muller Hinton agar (MHA) was used as the medium on antibiotic discs supplied by Himedia, Mumbai (India). The diameter of the inhibition zone around the disc was measured after 24 h of incubation at 24°C and sensitivity was assessed using standard tables given by Himedia.

Results

External symptoms. Diseased shrimp were weak, sluggish, and easily identified by a loose shell, soft muscle, and condensed and melanized hepatopancreas. Their behavior differed from normal shrimp in that they stopped feeding, assembled near the edges of the ponds during early morning, and exhibited erratic movement. Mortality in affected ponds was initially low but reached 100% within ten days after the onset of the disease. The disease was observed throughout the year but prevalence was high in summer, i.e., April-June, when the pond salinity and temperature were high. The disease occurred as an acute epizootic, especially at Kakinada.

Bacteriological study. Total bacteria (TBC) and *Vibrio* (TVC) counts were high in diseased shrimp. Values were highest in sam-

ples from Kakinada (1.69 x 10^4-5.8 x 10^5 and 0.8 x 10^3-0.1 x 10^5 cfu/g, respectively), intermediate in samples from Amalapuram (1.28 x 10^2-3.4 x 10^4 and 1.1 x 10^2-1.8 x 10^4 cfu/g, respectively), and lowest in samples from Visakhapatnam (0.9 x 10^3-1.4 x 10^4 and 0.7 x 10^2-1.1 x 10^2 cfu/g, respectively). Six *Vibrio* species were morphologically and biochemically identified in isolates from diseased shrimp (Table 1). Of 196 isolated and identified *Vibrio* colonies, 70 were *V. harveyi*, 50 were *V. alginolyticus*, 50 were *V. parahaemolyticus*, and 24 were *V. anguillarum. Vibrio harveyi* was found in all diseased shrimp while the other three were found in 50-80% of the diseased shrimp. *Vibrio splendidus* and *V. vulnificus* were found in only two. Except for production of acid from carbohydrates, isolates responded similarly to biochemical tests.

Histopathology. Gills were pale and the hepatopancreas was greatly condensed and melanized in diseased shrimp. There were typical symptoms of bacterial septicemia including numerous granulomas of different sizes and development stages in the hepatopancreas (Fig. 1), extensive proliferation of connective tissue, formation of melanized granulomas, and necrosis of hepatopancreas tubules (Fig. 2). There was severely deteriorated tissue in the lymphoid organ, ovary, and gills with varying degrees of pycnosis and karyorrhexis. Granulomas were also observed in connective tissue and the heart (Fig. 3). Bacteria invaded the lumen of hepatopancreas tubules and cells lining the tubules separated from the basal lamina and became necrotic. There was massive proliferation of the connective tissues of the hepatopancreas, heart, and ovary and infiltration into internal parts, displacing cells (Figs. 4, 5). Hemocytes infiltrated into the hemolymph, hepatopancreas, heart, and gills. In the connective tissue around the hepatopancreas and heart, giant cells with a large amount of eosinophilic fluid filled the vacuole occupying the major part of the cell, forcing the cytoplasm and crescent shaped nucleus to one pole (Fig. 6). The PCR test was negative, indicating an absence of the WSS virus. However, three of the sixty examined shrimp had monodon baculovirus virus (MBV) in the hepatopancreas.

Pathogenicity trial. Table 2 shows the LC_{50} values of the four *Vibrio* species. There was no mortality in control shrimp.

Drug sensitivity. All isolates were sensitive to ciprofloxacin and norfloxacin (Table 3). In addition, *V. alginolyticus*, *V. parahaemolyticus*, and *V. anguillarum* were highly sensitive to chloramphenicol while *V. harveyi* resisted the drug. Sensitivity to other drugs differed from species to species. *Vibrio harveyi* was resistant to sixteen of the 22 tested antibiotics.

Discussion

Of the six *Vibrio* species in the hemolymph, the luminous *V. harveyi* was the most dominant and most virulent as evidenced by its occurrence in all 70 diseased shrimp and its low LC_{50} value. The overall symptoms of the diseased shrimp, especially the soft muscle, condensed and melanized hepatopancreas, and sluggish erratic movements, are similar to symptoms reported earlier for shrimp affected by LSS (Mayavu et al., 2003; Gopalakrishnan and Parida, 2005; Jayasree et al., 2006) and luminous vibriosis, a particularly pathogenic form of vibriosis caused by *V. harveyi* (Chen et al., 1992; Jiravanichpaisal et al., 1994; Lavilla-Pitogo et al., 1998). Histopathological changes, including hemocytic infiltration, granuloma formation, proliferation of connective tissue, and necrosis of hepatopancreas tubules, agree with changes reported in shrimps affected by luminous vibriosis (Chen et al., 1992; Lavilla-Pitogo et al., 1998). These observations show that the luminous *V. harveyi* is probably an etiological agent of LSS in shrimp. The luminous *Vibrio* has invaded most shrimp hatcheries in coastal Andhra Pradesh. Luminous *Vibrio* spp. commonly occur in coastal waters where they constitute dominant members of the autochthonous microbial flora. Hence their occurrence in culture ponds is not unexpected.

Biochemical characteristics of *V. harveyi* isolated from the LSS-affected shrimps coincide with those reported by earlier workers (Jiravanichpaisal et al., 1994; Abraham and

Table 1. Morphological and biochemical characteristics of *Vibrio* bacteria isolates from *Penaeus monodon* affected by loose shell syndrome.

Test	*V. alginolyticus*	*V. parahaemolyticus*	*V. anguillarum*	*V. splendidus*	*V. vulnificus*	*V. harveyi*
Gram stain	-	-	-	-	-	-
Growth on TCBS agar	+ (yellow)	+ (green)	+ (yellow)	+ (yellow)	+ (yellow)	+ (yellow)
Motility	+	+	+	+	-	+
Swarming	-	-	-	-	-	-
Oxidase reduction	+	+	+	+	-	+
Catalase reduction	+	+	+	+	-	+
O/F test	Fermentative	Fermentative	Fermentative	Fermentative	Fermentative	Fermentative
Gas from glucose	+	+	+	+	+	+
Arginine dihydrolase	-	-	+	-	+	-
Lysine decarboxylase	+	+	-	-	-	+
Ornithine decarboxylase	+	+	-	-	-	+
Methyl red test	+	+	+	-	-	+
Voges proskauer	+	-	+	-	-	-
Indole production	+	-	+	+	+	+
Nitrate reduction	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	+
Gelatinase	+	+	+	+	+	+
Esculin hydrolysis	+	-	-	+	-	-
Sensitivity to O/129 (10 µg)	+	-	-	+	+	+
Sensitivity to O/129 (150 µg)	+	+	+	+	+	+
Growth at 4°C	-	-	-	-	-	-
Growth at 42°C	-	-	-	-	+	-

Table 1. Con'd

Growth in peptone water with NaCl (%)						
0	-	-	-	-	-	-
0.5	+	+	+	+	+	+
1	+	+	+	+	+	+
3	+	+	+	+	+	+
6	+	+	+	+	+	+
8	+	+	-	+	-	+
10	-	-	-	-	-	-
Acid production from						
Arabinose	-	-	-	-	-	-
Cellobiose	-	+	+	-	-	+
Galactose	+	-	+	-	-	+
Glucose	+	+	+	+	+	+
Inositol	-	-	-	-	-	-
Lactose	-	-	-	-	+	-
Mannitol	+	+	+	-	-	+
Sorbitol	+	+	+	-	-	+
Sucrose	+	+	+	-	+	+
Urease	+	-	-	+	+	-
Hydrogen sulfide production	-	-	-	-	-	+

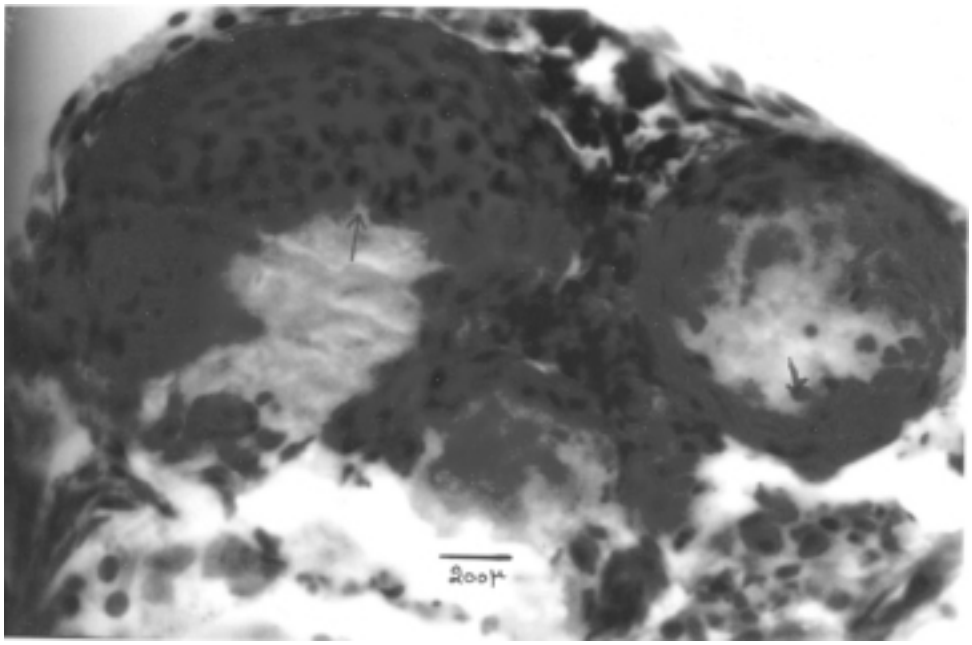

Fig. 1. Cross section of hepatopancreas of *Penaeus monodon* affected by loose shell syndrome, with granulomas of different sizes (arrows).

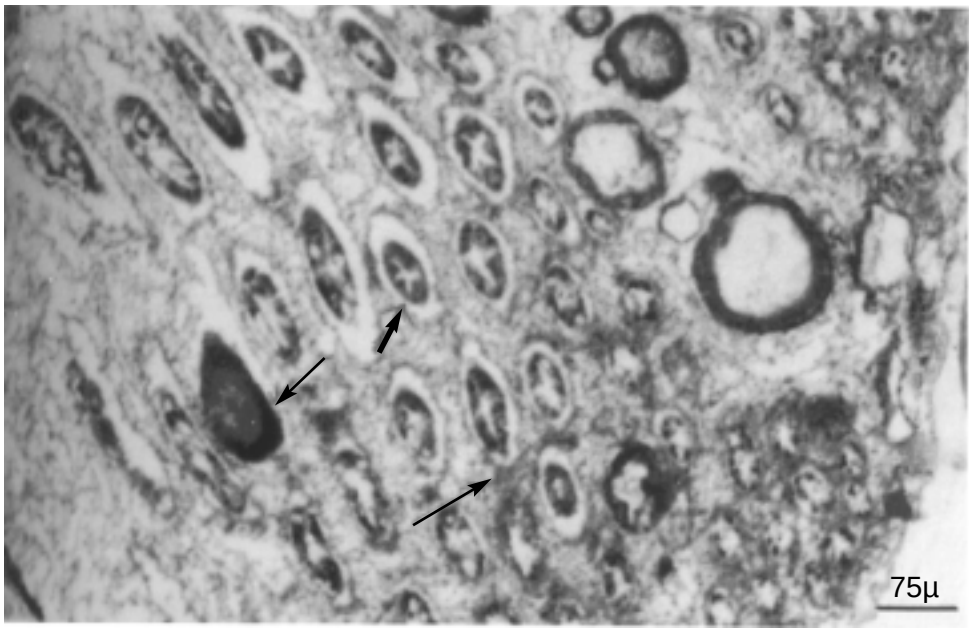

Fig. 2. Cross section of hepatopancreas showing infiltration of hemocytes into intertubular space (arrow head), necrosis (small arrow), and melanization of tubules (large arrow).

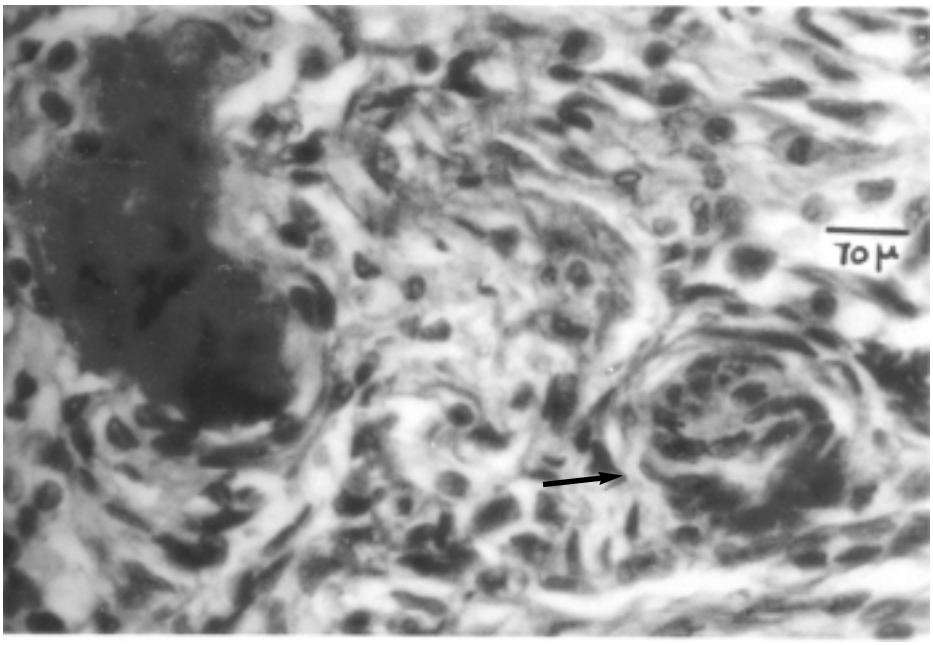

Fig. 3. Cross section of heart showing granulomas in initial stage of formation (arrow).

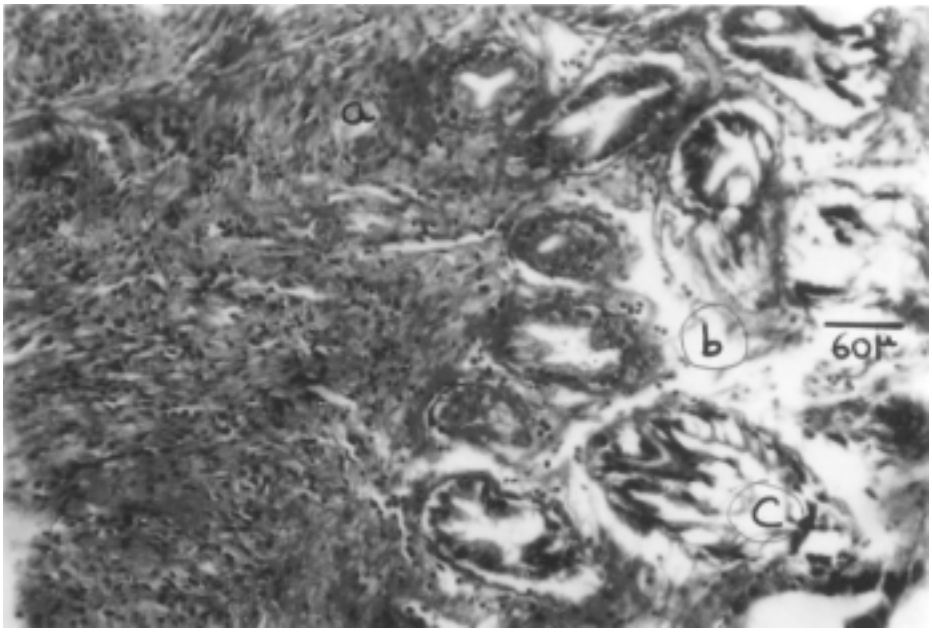

Fig. 4. Cross section of hepatopancreas showing (a) extensive proliferation of connective tissues, (b) invasion into intertubular space, and (c) necrosis of tubules.

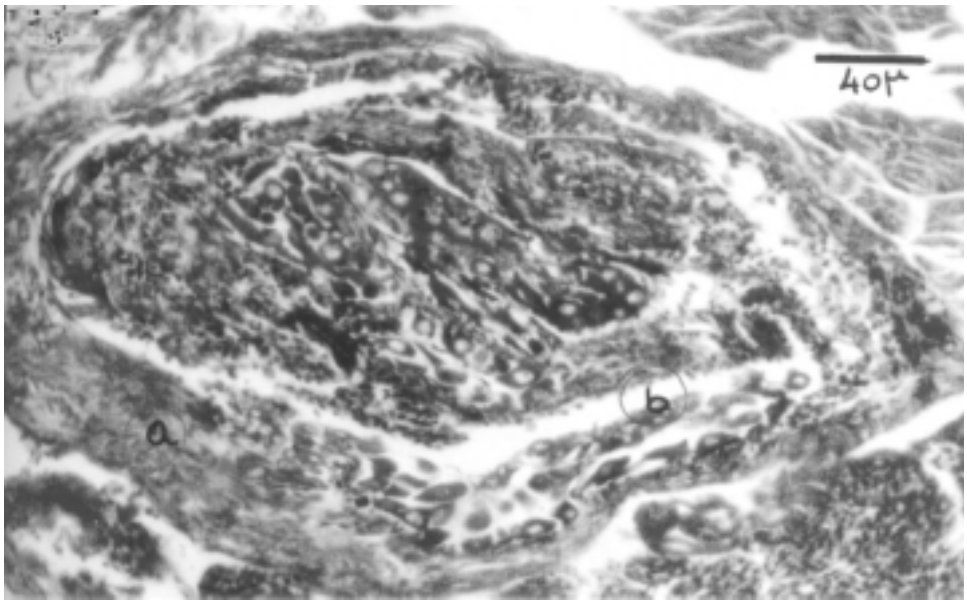

Fig. 5. Cross section of ovary of loose shell syndrome-affected *Penaeus monodon* showing (a) extensive proliferation of connective tissue and (b) degenerating ova.

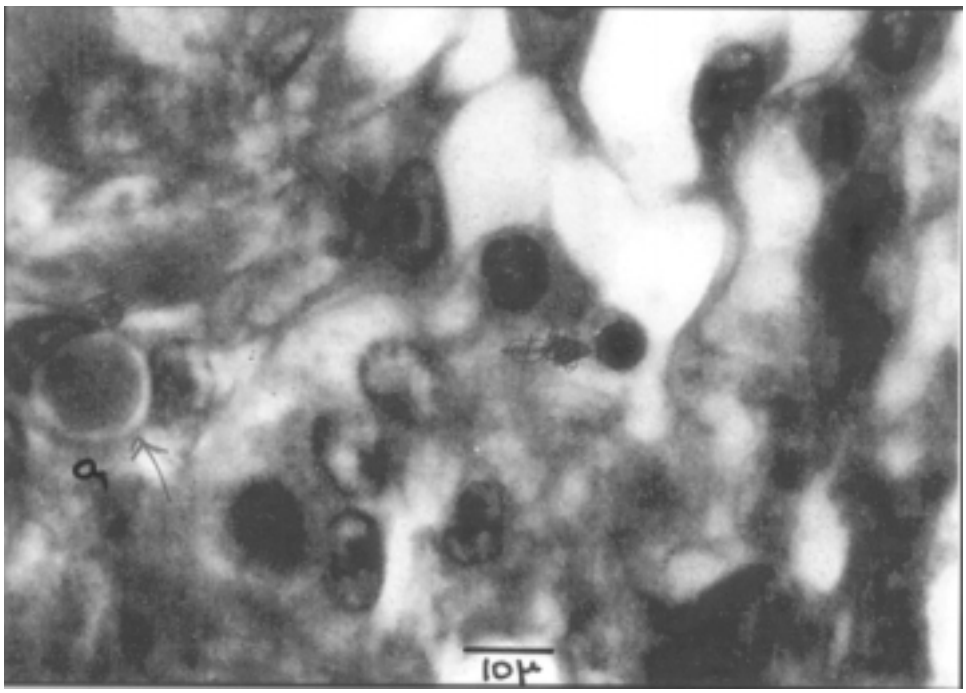

Fig. 6. Connective tissue showing giant cells (a) with fluid filled vacuoles occupying the major part of the cell and the nucleus pushed to one side of the cell (arrow).

Table 2. LC_{50} values of *Vibrio* spp. isolated from *Penaeus monodon* affected by loose shell syndrome.

Bacteria (cfu/g)	*LC_{50} value*
V. harveyi	1.0×10^3
V. alginolyticus	1.5×10^4
V. parahaemolyticus	0.2×10^5
V. anguillarum	1.0×10^5

Manley, 1995; Lavilla-Pitogo et al., 1998) except for slight differences such as utilization of carbohydrates. The LC_{50} value approaches that reported for virulent strains of *V. harveyi* (Abraham et al., 1997; Ruangpan, 1998). Luminous *Vibrio* can induce disease and cause mortality in *P. monodon, P. indicus*, and *P. merguiensis* (Sae-oui et al., 1987; Prayitno and Latchford, 1995). Although several factors may be involved in causing LSS in cultured shrimp (Mayavu et al., 2003; Gopalakrishnan and Parida, 2005), our study suggests that *V. harveyi* is an important con-

Table 3. Drug sensitivity of *Vibrio* spp. isolated from *Penaeus monodon* affected by loose shell syndrome.

Antibiotic	*V. harveyi*	*V. alginolyticus*	*V. parahaemolyticus*	*V. anguillarum*
Amoxicillin	R	R	R	R
Ampicillin	R	R	R	R
Cefazolin	R	R	R	R
Cephadroxil	R	S	I	R
Chloramphenicol	R	S	S	S
Ciprofloxacin	S	S	S	S
Clotrimazole	R	R	R	R
Cloxacillin	R	R	R	R
Co-Trimoxazole	R	R	S	S
Erythromycin	R	I	R	R
Furazolidin	I	R	I	R
Gentamycin	I	I	R	I
Metronidazole	R	R	R	R
Nitrofurazone	R	R	S	R
Norfloxacin	S	S	S	S
Oxytetracycline	I	I	I	I
Pefloxacin	R	S	S	I
Penicillin-G	R	R	R	R
Rifampacin	R	R	R	R
Streptomycin	R	R	R	R
Tetracycline	I	I	I	S
Trimethoprim	R	I	S	I

R = resistant, I = intermediate, S = sensitive

tributor to the onset of LSS and occurrence of mass mortalities in cultured *P. monodon*.

Apart from *V. harveyi*, three other species occurred in LSS-affected shrimp (*V. alginolyticus, V. anguillarum*, and *V. parahaemolyticus*) but their lower prevalence and virulence suggest that they constitute secondary invaders to the primary *V. harveyi* infection. Whether any virus is involved in causing LSS needs to be investigated. Our study produced no evidence of the WSS virus, although three of 60 shrimp subjected to histopathological study had MBV.

A wide range of antimicrobial compounds is used in *P. monodon* hatcheries and farms in India to control bacterial infection (Karunasagar et al., 1994; Pillai and Jayabalan, 1996; Abraham et al., 1997; Shome et al., 1999; Sahul Hameed et al., 2003). Many reports document the emergence of antimicrobial drug resistance in *Vibrio* spp., especially *V. harveyi* (Baticados et al., 1990; Karunasagar et al., 1994; Abraham and Manley, 1995; Sahul Hameed et al., 2003; Sengupta et al., 2003; Thakur et al., 2003). The present study revealed that *V. harveyi* also developed resistance to most antibiotics. Such development of antibiotic resistance has created novel problems for the management of shrimp farms and hatcheries.

Aquafarmers in Andhra Pradesh, aware of the luminous problem, are adopting new methods to protect shrimp ponds from luminous vibriosis. Chemotherapeutic control of the disease based on available drugs has been abandoned because of the development of resistant strains of bacteria (Baticados et al., 1990; Ruangpan and Kitao, 1992; Karunasagar et al., 1994; Moriarity, 1998) and restrictions imposed on their use for environmental and health reasons. Alternative methods for disease control include the use of reservoirs where the bacterial population is effectively reduced by chlorination. Also, technologies that depend on competitive advantage using beneficial bacteria, popularly known as bioaugmentation or probiotics, are gaining importance in shrimp aquaculture. Extensive work is being undertaken to identify suitable immunostimulants to enhance the activity of the nonspecific immune system and resistance to *Vibrio* infections. Since most bacterial infections are stress induced, the best way to control these infections is through environmental management. Traditional chemotherapeutic methods that are plagued by the development of resistant strains must be replaced by eco-friendly measures. Further research work needs to be carried out to protect the industry from this emerging disease.

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