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Distribution of luminescent *Vibrio harveyi* and their bacteriophages in a commercial shrimp hatchery in South India

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Abstract

Luminescent *Vibrio harveyi* is a natural microflora of marine and coastal water bodies and is associated with mortality of larval shrimp in penaeid shrimp hatcheries. It is also known that the bacteriophages occur virtually in all places where their hosts exist. In this study, distribution of luminescent *V. harveyi* and the bacteriophages affecting these hosts was examined in a commercial *Penaeus monodon* hatchery during three shrimp larval production cycles, including a cycle affected by luminescent bacterial (LB) disease outbreak.

Out of a total of 1195 samples drawn from seawater source, sand-filtered water, nauplius, zoea, mysis and post larval rearing tanks, maturation and spawning tanks, *Artemia* hatching tank and algal culture tanks processed using conventional microbiological techniques, 21.4% of the samples harboured luminescent bacteria. During the larval production cycle affected by LB disease (LBD), luminescent *V. harveyi* could be recovered from 52% of the hatchery samples, whereas during luminescent bacterial disease-free larval production cycle (LBDF), these bacteria could be recovered from only about 9% of the samples. The predominant source of luminescent bacteria was the brood shrimp and their rearing tanks in maturation and spawning facilities. 73% of the maturation and 80% of the spawning tank water samples harbored LB during LBD, whereas, only 20% and 32% of the maturation and spawning tanks respectively harbored LB during LBDF. LB could be isolated from 17% of the water samples in tanks from nauplius stage onwards with increasing counts that subsequently lead to LB disease.

Bacteriophages affecting the luminescent *V. harveyi* could be isolated from as many as 36% (21% and 43% of the samples analysed during LBDF and LBD respectively) of a total of 181 water samples drawn from various sources in the hatchery, using 27 luminescent *V. harveyi* hosts by agar overlay technique. The maturation tank water samples were found to be the predominant source of bacteriophages, followed by spawning tank water samples as observed with the LB. Sixty five bacteriophages, 13 during LBDF and 52 during LBD were isolated, which were grouped in to seven types based on their plaque morphology.

The study has indicated that the brooders, maturation and spawning facilities in the shrimp hatchery are the main source of luminescent *V. harveyi* and their bacteriophages and that occurrence of LB even in low counts during early larval stages can possibly lead to development of LB disease despite presence of bacteriophages in the larval rearing tanks.
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1. Introduction

The luminescent *Vibrio harveyi* has been reported to be one of the major causes of mass mortality of larval stages in penaeid shrimp hatcheries throughout the world (Sunaryanto and Mariam, 1986; Lavilla-Pitogo et al., 1990; Karunasagar et al., 1994; Jiravanichpaisal et al., 1994; Pizzutto and Hirst, 1995;

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Liu et al., 1996; Alvarez et al., 1998). Luminescent bacteria (LB) are ubiquitous in the marine environment and are abundant in sea water (Yetinson and Shilo, 1979), surface and gut of marine animals (O' Brien and Sizemore, 1979; Ruby and Morin, 1979). A number of reports exist on their distribution in the marine environment (Ramaiah and Chandramohan, 1993), shrimp grow out ponds and hatcheries (Karunasagar et al., 1994; Abraham and Palaniappan, 2004). In a recent study in Tamil Nadu, India, based on the analysis of a large number of samples from maturation and spawning tanks, larval rearing tanks, *Artemia* and algal culture tanks it was reported that, the gut of shrimp brooders was the predominant source of LB (Abraham and Palaniappan, 2004). However, the study did not relate the occurrence of LB with the outbreak of LB disease in the larval stages during the hatchery operation.

Control of luminescent bacterial disease using antibiotics was reported to be effective in laboratory trials (Baticados et al., 1990) while their efficacy in field conditions are reportedly very low (Moriarty, 1999). Further, use of antibiotics in the hatcheries has led to the emergence of resistant strains of bacteria (Karunasagar et al., 1994). Bacteriophages, the viruses that infect and kill their specific hosts, have been reported to offer scope as an alternative to antibiotics as therapeutic agents in controlling bacterial infections (Sulakvelidze et al., 2001) and the concept of phage therapy has been explored by some investigators in aquaculture (Nakai and Park, 2002). Bacteriophages can be found in virtually all places where their hosts exist (Ackermann, 1996) and the host cells are the main resource for their proliferation and life cycle (Weinbauer, 2004). Bacteriophages are also known to play an important role in prokaryotic predation and controlling bacterial populations in the aquatic ecosystems (Weinbauer, 2004; Proctor and Fuhrman, 1990). It is estimated that the viral load in the coastal ecosystems are of the order of 10^8 viruses per ml (Suttle, 2005) and about 10–20% of the heterotrophic bacteria are lost per day due to viral infection (Suttle, 1994). Bacteriophages specifically infecting bacterial pathogens of finfish such as *Lactococcus garviae* and *Pseudomonas plecoglossicida* have been reported earlier (Park et al., 1997; Park et al., 2000). Isolation of bacteriophages of *V. harveyi* has been recently reported by Pasharawipas et al. (2005) and Vinod et al. (2006). However information on the distribution of bacteriophages of *V. harveyi* and their role *in situ* in the shrimp hatchery ecosystem is scanty.

The objective of the present study was to understand the distribution of luminescent bacteria and their associated bacteriophages during shrimp larval production cycles in a commercial shrimp hatchery during various stages of seed production and to understand the role of *in situ* population of bacteriophages in containment of luminescent *V. harveyi* and LB disease.

2. Materials and methods

2.1. Sample collection, isolation and identification of *V. harveyi*

Samples were collected from a commercial *Penaeus monodon* hatchery located 20 km north of Pondicherry, south east coast of India, during three cycles of seed production, two of which were free from luminescent bacterial disease (LBDF) and one cycle had an outbreak of luminescent bacterial disease (LBD) in the mysis-PL stages. Water samples from seawater source, sand-filtered water, nauplius, zoea, mysis and post larval rearing tanks, maturation and spawning tanks, *Artemia* hatching tanks and algal (*Skeletonema* sp.) culture tanks were collected in sterile polypropylene containers. Swab samples of brooders' body surface, gills, intestine and faecal matter were also collected for microbiological analysis. Weighed samples of gills, intestine and faecal matter were processed to obtain LB counts per unit weight of the sample. Luminescent bacterial count was determined using Luminescence agar medium (Schneider and Rheinheimer, 1988) and *V. harveyi* selective medium (Harris et al., 1996) by serial dilution and spread plate technique. Luminescent colonies on Luminescence agar medium and dark green centered colonies with yellow halo on Harris' medium were purified on Trypticase Soya Agar (TSA) (Himedia, Mumbai, India) and subjected to a series of phenotypic and biochemical tests such as grams stain, motility, salt tolerance (0%, 3%, 6% 8% and 10%), amino acid decarboxylation (arginine, lysine and ornithine), production of indole, methyl red and acetyl methyl carbinol, and sugar fermentation (glucose, sucrose, arabinose, mannitol and cellobiose) (Smibert and Krieg, 1991). The isolates were identified using the keys described by Alsina and Blanch (1994), and Baumann and Schubert (1984).

2.2. Isolation of bacteriophages of luminescent *V. harveyi*

Water samples from seawater source, spawning and maturation tanks and larval rearing tanks were processed for isolation of bacteriophages of luminescent *V. harveyi* by overlay agar technique (Suttle, 1993). Twenty-seven isolates of luminescent *V. harveyi*, were used as hosts for the isolation of bacteriophages. Ten ml of water samples collected from these sources in sterile polypropylene tubes were centrifuged at 10,400 g at 4 °C, and the supernatant was filtered through 0.45 µm filter (Millipore, Bedford, USA). One ml of this filtrate was used as phage sample and mixed with 0.1 ml of log phase cultures (OD_{600} = 0.3) of *V. harveyi* hosts grown in Tryptone soya broth (TSB, Himedia, Mumbai, India) and incubated at 30 °C for 30 min. After incubation, 5 ml of molten soft agar (TSB with 1.5% NaCl, 0.3% glycerol and 0.7% agar) held at 46 °C in a water bath was added, mixed and overlaid on TSA supplemented with 1.5% NaCl and 0.3% glycerol. The plates were incubated at 30 °C for 18–24 h and observed for the presence of plaques. The plaques were counted and expressed as plaque forming unit per ml (pfu ml^{-1}). 10–20 of these plaques were suspended in 1 ml sterile SM buffer (Tris HCl 0.05 M, pH 7.4, NaCl 0.1 M, $MgSO_4$ 10 mM, gelatin 1% w/v) (Carlson, 2005) and centrifuged at 10,400 g for 10 min at 4 °C and filtered through 0.45 µm filter. The filtrate was added to 10 ml broth culture of the respective host strain growing at log phase and incubated at 30 °C for 24 h. After incubation the suspension was centrifuged at 10,400 g for 10 min. The supernatant was filtered through 0.45 µm filter and aliquots were stored at −70 °C with 50% glycerol for further studies.

3. Results

3.1. Distribution of luminescent bacteria

Luminescent bacteria could be isolated from as high as 52% of the 338 samples comprising hatchery seawater source, brooders, maturation and spawning tanks, algal culture tanks and larval rearing tanks during the shrimp seed production cycle affected by LB disease during mysis stage. Recovery of LB was far lower (9%) among the 857 samples analysed during the two shrimp seed production cycles free from LB disease (Table 1). Very low counts of LB (20–230 cfu ml^{-1}) could be found in hatchery seawater source and sand-filtered water samples (4/25 and 1/25 respectively) during the cycle that subsequently developed LB disease, whereas, none of 45 each of these seawater source and sand-filtered water samples processed during the LBDF yielded any LB. The brooders were found to be the main source of LB as revealed by the rate of isolation of these bacteria during both LBDF and LBD (71–80% of gills and surface swab samples and 33–59% of the intestinal and faecal samples). However the LB counts in the gills, intestine and faecal matter during the LBD cycle was significantly high ($62–800 \times 10^4$ cfu ml^{-1}), compared to that during LBDF ($7.2–320 \times 10^4$ cfu ml^{-1}). A large percentage of water samples from the

Table 1
Occurrence of luminescent bacteria during luminescent bacterial disease-free larval production cycle (LBDF) and Luminescent bacterial disease-affected larval production cycle (LBD) in a commercial shrimp hatchery

Source	LBDF					LBD				
	No. processed	No. positive	Range no. × 10^4 cfu ml^{-1}	Average no. × 10^4 cfu ml^{-1}	Percentage of samples positive for LB	No. processed	No. positive	Range no. × 10^4 cfu ml^{-1}	Average no. × 10^4 cfu ml^{-1}	Percentage of samples positive for LB
I. Hatchery water source										
i. Seawater Source	45	0	–	–	0	25	4	0.002 to 0.023	0.13	16
ii Sand-filtered water	45	0	–	–	0	25	1	0.003	0.003	4
II. Brooders										
i. Surface	13	10	–	–	77	10	8	–	–	80
ii. Gills*	7	5	7.2 to 320	40	71	5	4	62 to 790	220	80
iii. Intestine*	6	2	1.2 to 180	30	33	10	4	70 to 560	140	40
iv. Feacal matter*	4	2	8 to 19	13	50	17	10	120 to 800	320	59
III. Maturation and spawning										
i. Maturation tank water	5	1	52	52	20	11	8	17 to 290	260	73
ii. Spawning tank water	25	8	0.7 to 250	17	32	15	12	9 to 590	92	80
iii. Eggs	5	0	–	–	0	10	0	–	–	0
IV. Feeds										
i. Algal culture	30	0	–	–	0	12	1	0.2	0.02	8
ii. Artemia tank	60	0	–	–	0	0	0	–	–	–
V. Larval rearing tanks										
i. Nauplii	15	0	–	–	0	10	0	–	–	0
ii. Nauplii tank water	40	0	–	–	0	18	3	0.002 to 0.03	0.02	17
iii. Zoea	15	0	–	–	0	10	1	0.0011	0.11	10
iv. Zoea tank water	180	11	0.012 to 0.56	0.9	6	60	42	0.18 to 42	3	70
v. Mysis	20	2	0.18 to 0.28	0.23	10	12	8	6.2 to 79	56	67
vi. Mysis tank water	100	11	0.80 to 32	1.4	11	40	26	1.6 to 29	12	65
vii. Post larvae -14	40	10	0.16 to 9.30	1.5	25	10	9	1.7 to 198	36	90
viii. Post larval tank water	202	18	0.12 to 8.20	4.2	9	38	35	7.2 to 67	26	92
Grand Total	857	80	–		9	338	176	–	–	52

*cfu g^{-1} of sample.

maturation and spawning tanks (73% and 80% respectively) during the LBD cycle had significantly high counts of LB (17–290 × 10^4 and 9–590 × 10^4 cfu ml^{-1} respectively). The algal culture tanks did not harbour any LB during LBDF cycle, whereas only one of the 12 samples from algal culture tanks analysed during LBD affected cycle had LB.

LB could be recovered from 17% of nauplius tank water samples which subsequently developed luminescent bacterial disease during the mysis stage. Prior to the onset of LB disease, LB could be found in 70% of the tank water samples during the protozoeal stage with considerably high counts ranging from 0.18 to 42 × 10^4 cfu ml^{-1} (with averages of 3–56 × 10^4 cfu ml^{-1}). In the LBDF cycle, LB could not be isolated from the nauplii up to zoea stage. However, LB could be isolated from 6 to 25% of the tank water samples during the protozoeal through PL stages, although in low counts (averages of 0.2–4.2 × 10^4 cfu ml^{-1}).

3.2. Phenotypic characteristics of luminescent bacteria

Ninety seven luminescent bacteria isolated during the study were subjected to detailed phenotypic characterization. All the isolates were gram negative, oxidase positive. Ninety-four percent of the isolates grew well in 8% NaCl and none of the isolates could grow in 0% NaCl concentration. None of the isolates produced acetyl methyl carbinol. All the isolates fermented glucose and mannitol while 28% of the isolates could ferment sucrose. None of the isolates could produce acid by the fermentation of arabinose, lactose and inositol. 86% of the isolates produced indole and all the isolates tested were able to use cellobiose as the sole source of carbon. Among the 97 isolates, 89 belonged to *V. harveyi* and others to *V. splendidus* (3), *V. logei* (2), *V. fischeri* (2) and *Photobacterium* sp. (1).

3.3. Isolation of bacteriophages from water samples

Out of a total of 181 samples comprising water samples from seawater source, maturation tanks, spawning tanks and larval rearing tanks, 65 bacteriophages capable of infecting luminescent *V. harveyi* isolates were recovered (Table 2). Thirteen bacteriophages on eight *V. harveyi* hosts were isolated during LBDF cycle and 52 bacteriophages were isolated on 24 *V. harveyi* hosts during LBD (Table 2). Based on the morphology of the plaques, the phages were categorized in to seven types (Table 3). A total of 28 phages of plaque morphologies of types II, III, IV and VI could be isolated from various samples in the hatchery only during LBD.

Maturation tank water samples were found to be the main source of bacteriophages, 52% and 90% of these samples had bacteriophages

Table 2
Isolation of bacteriophages of luminescent *V. harveyi* during luminescent bacterial disease-free shrimp larval production cycle (LBDF) and luminescent bacterial disease outbreak affected cycle (LBD) from a commercial shrimp hatchery

Sl.No	Host	LBDF			LBD		
		Phages isolated	pfu ml^{-1}	Plaque Type	Phages isolated	pfu ml^{-1}	Plaque Type
1	Vh01	P01	138	I	P32	112	I
2	Vh09	P05	45	V	–	–	–
3	Vh10	P06	140	VII	–	–	–
4		P07	26	V	–	–	–
5	Vh11	P08	58	VII	P44	726	II
6	–	–	–	–	P49	164	III
7	–	–	–	–	P54	960	I
8	Vh13	P09	246	V	P48	518	II
9		P14	154	V	P60	532	V
10		P15	160	VII	P63	48	IV
11					P65	516	III
12	Vh14	P10	206	VII	P62	438	VI
13	Vh16	P13	118	I	–	–	–
14		P52	126	V	–	–	–
15		P59	230	VII	–	–	–
16	Vh21	P11	242	I	P37	560	V
17					P38	620	VII
18	Vh26	–	–	–	P12	710	V
19	Vh29	–	–	–	P35	820	VII
20		–	–	–	P46	830	III
21		–	–	–	P50	740	V
22		–	–	–	P57	169	II
23		–	–	–	P58	267	VII
24		–	–	–	P61	172	VI
25	Vh30	–	–	–	P36	167	III
26		–	–	–	P45	169	V
27		–	–	–	P47	226	VII
28		–	–	–	P51	176	II
29		–	–	–	P55	522	V
30		–	–	–	P56	396	VII
31		–	–	–	P64	165	V
32	Vh44	–	–	–	P16	218	V
33		–	–	–	P18	242	III
34	Vh45	–	–	–	P17	286	VII
35	Vh49	–	–	–	P19	166	III
36	Vh51	–	–	–	P02	182	II
37		–	–	–	P43	193	III
38	Vh54	–	–	–	P21	182	V
39	Vh57	–	–	–	P20	194	III
40	Vh84	–	–	–	P41	320	V
41		–	–	–	P42	418	V
42	Vh93	–	–	–	P23	456	III
43	Vh94	–	–	–	P26	326	IV
44	Vh95	–	–	–	P25	226	V
45	Vh97	–	–	–	P24	423	III
46	Vh99	–	–	–	P22	424	VI
47		–	–	–	P39	510	V
48	Vh100	–	–	–	P27	256	III
49		–	–	–	P28	412	IV
50	Vh101	–	–	–	P40	452	VI
51	Vh102	–	–	–	P03	690	III
52		–	–	–	P29	182	VII
53		–	–	–	P30	422	V
54		–	–	–	P33	329	III
55		–	–	–	P53	150	VII
56	Vh105	–	–	–	P04	184	IV
57		–	–	–	P31	30	VI
58		–	–	–	P34	600	III
Total	27	13	–	–	52	–	–

affecting *V. harveyi* during LBDF and LBD respectively, with a relatively higher pfu, compared to that in the other sources. The bacteriophage counts in the maturation tanks, prior to the incidence of LBD in larval shrimp were also found to be relatively higher (164–960 pfu ml^{-1}), whereas, their populations during the LBDF were relatively low and ranged from 26 to 246 pfu ml^{-1}. Similarly spawning tank water samples were also found to be important sources of bacteriophages of *V. harveyi* (Table 4). Bacteriophages of *V. harveyi* could also be recovered from the larval rearing tanks (LRTs) during LBD, although from a lesser percentage (7%) of the samples. We could not recover any bacteriophages from LRTs during LBDF. The bacteriophage count was found to be positively correlated with the luminescent bacterial counts in all these samples ($P=0.288$ at 0.05% significance level during disease outbreak and $P=0.809$ at 0.01% significance level during LBDF) (Fig. 1).

4. Discussion

The present study deals with the distribution of luminescent bacteria and their bacteriophages during various stages of shrimp larval production in a commercial hatchery. This study was carried out during February and May 2006, and LB disease was noticed in the month of May 2006 during mysis stage. Of the 1195 samples comprising brooders, water from maturation, spawning and larval rearing tanks, eggs, nauplii, zoea and mysis, luminescent bacteria could be isolated from 21% of the samples. However, an earlier similar study (Abraham and Palaniappan, 2004) reported a higher rate of isolation of luminescent bacteria (59.68%) from samples including source water, eggs, broodstock, larvae, larval rearing tank water, algal culture tanks, *Artemia* nauplii and swab samples from water distribution systems in *P. indicus* and *P. monodon* hatcheries of Tamil Nadu, India.

Near shore water was reported to be one of the main sources of *V. harveyi* infection in *P. monodon* hatcheries in the Philippines (Lavilla-Pitogo et al., 1992). However in the present study luminescent bacteria could be isolated from only 16% of source water samples during LBD and could not be isolated during LBDF in shrimp hatchery. The intake water was filtered through sand filters, followed by chlorination providing some

Table 3
Morphology of *V. harveyi* bacteriophages recovered during luminescent bacterial disease-free shrimp larval production cycle (LBDF) and luminescent bacterial disease outbreak affected cycle (LBD) from a commercial shrimp hatchery

Plaque type	Plaque morphology	LBDF	LBD	Total
Type I	Pinpoint, clear centered, irregular margin	3	2	5
Type II	0.5–1.5 mm, opalescent, irregular margin	–	5	5
Type III	1–2 mm, clear centered, irregular margin	–	14	14
Type IV	Pinpoint to 1 mm, opalescent, irregular margin	–	3	3
Type V	2–3.5 mm, clear centered, irregular, hazy margin	5	14	19
Type VI	1–1.5 mm, clear centered, entire margin	–	6	6
Type VII	1.5–2 mm, clear centered, regular, hazy margin	5	8	13
	Total	13	52	65

Table 4
Occurrence of bacteriophages of luminescent *V. harveyi* from samples in a commercial shrimp hatchery during luminescent bacterial disease-free shrimp larval production cycle (LBDF) and luminescent bacterial disease outbreak affected cycle (LBD)

S.No	Source	LBDF				LBD			
		No. processed	No. positive	Phage counts pfu ml $^{-1}$	Average pfu	No. processed	No. positive	Phage counts pfu ml $^{-1}$	Average pfu
1	Source water	20	0	0	0	20	2 (10)	30–150	90
2	Maturation tank	21	11 (52)	45–246	167	40	36 (90)	164–960	401
3	Spawning tank	10	2 (20)	26–126	76	30	12 (40)	112–726	385
4	Larval rearing tank	10	0	0	0	30	2 (7)	48–165	106.5
	Total	61	13 (21)	–	–	120	52 (43)	–	–

Figures in parenthesis in percentage.

advantageous effect in reducing the load of *V. harveyi* in the hatchery.

The shrimp brooders used in the hatchery were obtained from offshore water of Andamans and southern Bay of Bengal, and were found to be the main source of luminescent bacteria as revealed by their occurrence in 77% and 80% of the surface samples and 71% and 80% gill samples during LBDF and LBD respectively. Formation of biofilm on the surfaces in hatchery systems appears to be an important trait of luminescent bacteria helping them in persisting in the hatchery systems (Karunasagar and Otta, 1996; Abraham and Palaniappan, 2004). The gut content of brood shrimp were also found to be an important source of luminescent bacteria as revealed in earlier studies (Hasting and Nealson, 1981). *V. harveyi* are known to enter and traverse the digestive tract and survive the digestive process of marine fishes and proliferate extensively in the faecal matter and distribute into the surrounding sea water (Ruby and Morin, 1979). After excretion, the luminescent bacteria proliferate in the faecal matter and with the help of other organic matter such as the uneaten feed in the maturation and spawning tanks.

Although the occurrence of luminescent bacteria was more frequent in the maturation and spawning tanks, we could not recover any luminescent bacteria from eggs and nauplii. The eggs were washed with iodine and formalin soon after collection as a management practice in the hatchery, which appear to help a great deal in their containment after spawning. The fact that LB could not be recovered from eggs and nauplii poses questions on their occurrence in the nauplii tank water samples (during the seed production cycle affected by LB disease) and zoea stage onwards (during the seed production cycle free from LB disease) and subsequent larval stages. This may be possibly due to presence of low counts of LB in eggs and nauplii, which could not be detected by the methods used (0.1 g of egg/nauplii biomass and 1 ml of water samples used for isolation of LB by serial dilution and spread plate technique). Another plausible reason could be existence of LB in "viable but non-culturable" state (Roszak and Colwell, 1987) followed by their resuscitation in the tanks during the subsequent larval developmental stages (Ramaiah et al., 2002).

Analysis of samples in the post-hatching sections has yielded interesting results. Total mortality of shrimp larvae was observed in larval production cycle affected with luminescent bacterial disease by PL6 stage. Survival rate at PL14 was 80% during LBDF. During the larval production cycle affected by luminescent bacterial disease, colonization of protozoeal stages with luminescent bacteria preceded their occurrence in the LRT water samples during the nauplius stage. Further when we examine the two situations, viz., LBD and LBDF, occurrence and multiplication of

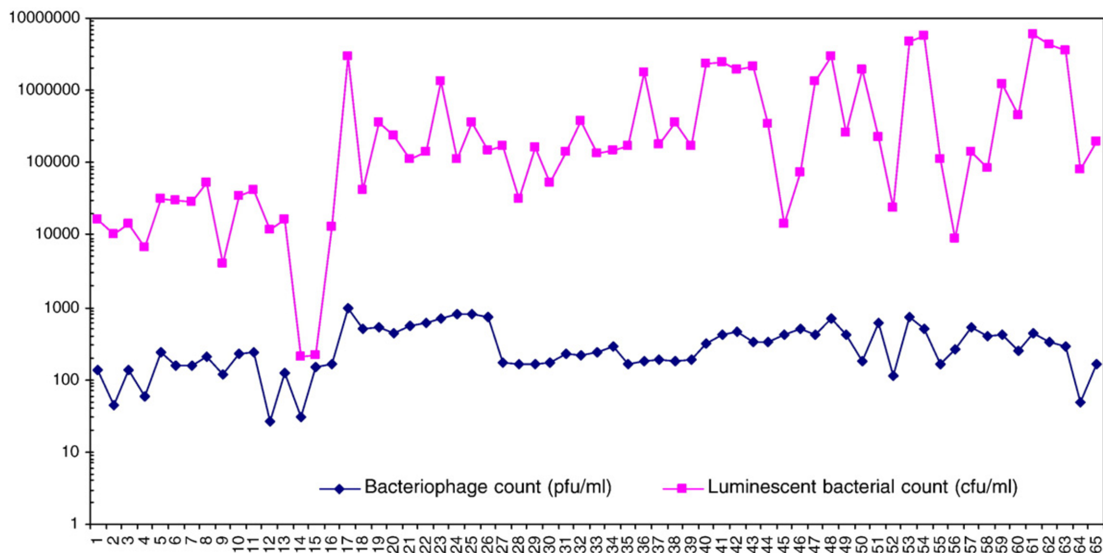

Fig. 1. Relative count of luminescent *V. harveyi* and associated bacteriophages in shrimp hatchery samples.

luminescent bacteria occurred in an exponential phase during the LBD, with corresponding drop in survival of affected larval shrimp. The observation suggests that the occurrence of luminescent bacteria during nauplii stage in the rearing tank water could be an indicator of impending luminescent bacterial disease outbreak in the larval rearing tanks and possibly help in the prognosis of LBD.

Although earlier studies reported the occurrence of luminescent bacterium in *Artemia* and *Artemia* hatching water (Abraham and Palaniappan, 2004; Vaseeharan and Ramasamy, 2003) we could not recover any luminescent bacteria from these samples. Similarly, the algal cultures did not contribute luminescent bacteria to the larval rearing tanks as reported by Lavilla-Pitogo et al. (1992). Abraham and Palaniappan (2004) suggested that the low incidence of luminescent bacteria in *Chaetoceros* cultures may be due to the antibacterial activity of this diatom.

The observations made in the present study that, luminescent *V. harveyi* could be isolated during all the three cycles of seed production, particularly during protozoea and PL stages, and why the disease outbreak occurred in only one seed production cycle remains to be explored further. Manefield et al. (2000) suggested that virulence associated with extracellular protein and luminescence in *V. harveyi* are co regulated, and their intracellular signal antagonist such as halogenated furanone inhibited concurrent expression of luminescence and toxin production. However, the results obtained in this study do not suggest that luminescence and virulence may be interdependent in *V. harveyi*.

Sixty five phages capable of infecting *V. harveyi* were recovered during the three larval production cycles. The shrimp broodstock maturation tanks were also found to be the main source of bacteriophages of luminescent bacteria. Recovery of bacteriophages of luminescent *V. harveyi* recorded in the present study in maturation and larval rearing tanks suggests existence of a mechanism of natural biocontrol of luminescent bacteria in shrimp hatchery systems. Bacteriophages of *V. harveyi*, belonging to Siphoviridae family have been isolated and described by Pasharawipas et al. (2005) and Vinod et al. (2006). It was also reported that the shrimp larval survival in the presence of the bactriophage was enhanced by 80% and brought about a decline in the luminescent *V. harveyi* in the hatchery tanks (Vinod et al., 2006). Occurrence of bacteriophages and luminescent *V. harveyi* observed in the present study indicates that there exists a tremendous scope in harnessing bacteriophages for biocontrol of luminous vibriosis in the shrimp hatcheries. It has been reported that there exists a high abundance of free viruses in the aquatic ecosystem (Bergh et al., 1989) and are considered important agents of mortality of bacteria and even influence microbial community composition in the natural marine ecosystem (Fuhrman and Schwalbach, 2003). This report is substantiated by a positive correlation between the bacteriophage counts and LB counts in the hatchery water samples. Despite this positive correlation and the presence of bacteriophages in the hatchery systems observed in the present study, luminescent bacterial disease-affected larval shrimp during one cycle. The luminescent strains causing such outbreak in larval stages in the hatchery are possibly contaminants from the maturation and spawning facilities. Option of inoculation of larval rearing tanks with bactriophages isolated on luminescent bacterial hosts recovered from maturation and spawning facilities and early larval stages, as phage therapy remains to be explored for combating luminescent bacterial disease problems in shrimp hatcheries.

Virulence of a number of medically important bacterial pathogens such as *V. cholerae*, *Clostridium botulinum, C. diphtheriae* etc., has been proved to be mediated by bacteriophages (Wagner and Waldor, 2002). Similarly, *V. harveyi* myovirus like (VHML) particles are reported to be associated with virulence in *V. harveyi* (Munro et al., 2003). Nine (5 isolates recovered from infected larvae and 4 from water samples) of *V. harveyi* isolates obtained during the LB disease outbreak in the present study were screened for VHML by polymerase chain reaction (PCR) (Payne et al., 2004) (data not given) and none of these isolates produced the 900 bp product specific for tail sheeth encoding gene of VHML (instead, a 350 bp fragment could be amplified). Occurrence of luminous vibriosis in larval shrimp by LB isolates that lack VHML suggests possibilities of involvement of other bacteriophages similar to VHML in the virulence of *V. harveyi*. This argument may be substantiated by the observations that phages with near identical genomes are rarely isolated from independent sources in nature (Casjens et al., 1992). Hence, further studies would be required to explore the role of bacteriophages in the virulence of *V. harveyi*.

5. Conclusion

The study has indicated that the shrimp brooders are the main source of luminescent bacteria in the shrimp hatcheries. Good management practices such as egg washing helps in the elimination of luminescent bacteria in the larval rearing tanks. Presence of luminescent *V. harveyi* during nauplius and protozoeal stages could indicate impending luminescent bacterial disease outbreak in the subsequent larval stages of shrimp in the hatchery. The study has also indicated that the bacteriophages occur predominantly in the shrimp broodstock maturation and spawning facilities in the shrimp hatchery and co-exist with the luminescent *V. harveyi*. Reasons for the outbreak of luminescent bacterial disease in the larval shrimp despite presence of bacteriophages in the larviculture systems remain to be explored.

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