Biocontrol of Bacterial Pathogens in Aquaculture with Emphasis on Phage Therapy

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ABSTRACT

Loss due to diseases is a major problem in both shrimp farm and hatcheries and to control diseases, a number of antibiotics, sanitisers and chemicals are being used. This results in adverse environmental effects, emergence of antibiotic resistance and persistence of chemical residues in animal tissues. Considering the negative impact of antibiotics and other chemotherapeutants, biological control of pathogens would be a very useful strategy to prevent diseases in aquaculture and in this context, we looked into the potential for biocontrol of the shrimp pathogen, *Vibrio harveyi* which causes luminous bacterial disease in hatcheries and farms. A number of *Bacillus* spp were screened for their ability to produce anti-vibrio compounds. Several strains of *B. megaterium, B. licheniformis, B. coagulans, B. circulans* isolated from shrimp farm environments showed anti-vibrio activity. Bacteriophages capable of lysing several *V. harveyi* strains were isolated from shrimp culture environments and were found to be very efficient in reducing levels of *V. harveyi* in seawater microcosms. Application of bacteriophages to control luminous bacterial disease in shrimp hatcheries resulted in reduction of *V. harveyi* counts in water and in larvae and tremendously improved larval survival. The results show the potential of bacteriophages for therapy of luminous bacterial disease in aquaculture systems.

INTRODUCTION

One of the major constraints for the development of shrimp aquaculture is the mortality due to diseases (Lin, 1995; Subasinghe, 1997). According to a recent World Bank Report, global losses due to shrimp diseases are around US $ 3000 million (Lundin, 1996).

To control microbial diseases, a number of chemotherapeutic agents including antibiotics are used in shrimp farms. This has led to problems such as antibiotic resistance (Karunasagar *et al*., 1994). According to WHO fact sheet 194 (World Health Organisation Antimicrobial Resistance Fact Sheet 194, http://www.who.int/inf-fs/en/fact 194.html), the massive use of antimicrobials for disease control and growth promotion in animals increases selective pressure on the microbial world and encourages the emergence of resistant bacteria which can transfer their resistance genes to other bacteria. Another major concern associated with the use of antibiotics is the problem of residues which has already led to 'red alert' on

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imported shrimp in the European Union. Therefore, the environmental consequences of antibiotic use in aquatic environment are serious.

In this context, there is a need to have alternate strategies for pathogen control in aquaculture systems. Yasuda and Taga (1980) anticipated that bacteria would be useful both as food and as biological control agents for fish diseases and activators of the rate of nutrient regeneration in aquaculture. Several bacterial agents capable of inhibiting fish/shrimp pathogens have been reported in literature (Nogami and Maeda, 1992; Maeda and Liao, 1992; Gatesoupe, 1994; Austin *et al*., 1995; Riquelme *et al*., 1997 Sugita *et al*., 1998; Gram *et al*., 1999; Verschuere *et al*., 2000; Chythanya *et al*., 2002)

Another strategy is the use of bacteriophages. Polish and Soviet scientists have reported successful clinical use of bacteriophages for treatment of drug resistant suppurative infections of humans (Barrow and Soothill, 1997). Several successful results on phage therapy have been reported in various animal models (Soothill, 1992; Merril *et al*., 1996; Barrow *et al*., 1998). Bacteriophage therapy of infectious diseases in aquaculture has been suggested recently (Kumar, 2002; Nakai and Park, 2002). In this communication, we discuss our experience with bacteria and bacteriophages that have potential application as agents for biocontrol of *Vibrio* spp, which are important pathogens in aquaculture.

MATERIALS AND METHODS

Isolation and identification of Bacillus spp.

Samples of shrimp pond water, pond sediment, estuarine sediment and shrimp were collected from farms and estuaries along the coast of Karnataka. Aseptically collected samples were plated on Zobell Marine Agar. Opaque rough colonies that were likely to be *Bacillus* spp. were picked up, purified and stored in Zobell Marine Agar slants for conducting a battery of biochemical tests specified in Bergey's Manual of Systematic Bacteriology (Kreig and Holt, 1984) for specific identification.

Isolation and identification of luminous bacteria

Samples of estuarine water, estuarine sediment, creek water, shrimp pond water, pond sediment, hatchery water, live and dead post larvae (PL 15-20) were collected, plated on luminescent agar (West and Colwell, 1984) and luminous colonies were identified by a battery of biochemical tests (Karunasagar *et al*., 1994).

Testing anti-vibrio activity of Bacillus spp. by cross-streak method

An 18 h culture of Bacillus spp. was streaked as a 2 cm thick band across the diameter of tryptic soya agar with 1% NaCl (TSAS). After incubation for 24 h at 30°C, the growth was scraped with a sterile slide. The remaining bacteria were killed by exposure to 5 ml chloroform poured on the glass lid and left for 15 min by keeping the medium inverted over the lid. The plates were then air dried for about 10 min to remove any residual chloroform and four different vibrio cultures, *V. vulnificus, V. parahaemolyticus, V. alginolyticus and V. harveyi* were streaked perpendicular to *Bacillus* spp. band using sterile rod dipped in 18 h cultures. The plates were incubated for 18-24 h at 30°C. The linear zone of inhibition was recorded.

Isolation of bacteriophage

For isolating bacteriophages, water samples from both hatchery and shrimp farm were used. Five ml of water sample was centrifuged at 10,000 rpm for 10 min. One ml of the supernatant was added to 5 ml of 8 h old culture of *V. harveyi* grown in tryptic soya broth with 1% NaCl (TSBS) and incubated for 8 h at room temperature. One ml of this mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was filtered through 0.45 µ filter and the filtrate was used to test for the presence of specific bacteriophages.

Host bacteria was grown in TSBS for 10-12 h and poured on to TSAS plates to make a bacterial lawn and the plates were dried for 15 min to allow for the absorption of the culture by the agar plates. Ten microlitres of the filtrate (to be tested for the presence of bacteriophage) was placed on the bacterial lawn on the plates and incubated at room temperature (28 ± 2°C) for 6-8 h. The plates were observed for the zones of bacterial clearance at the spots where the filtrates were placed. To confirm that the lysis is actually due to bacteriophages, the process was repeated twice.

Testing ability of phage to lyse *V. harveyi* strains

The ability of phage to lyse 46 strains of *V. harveyi* isolated from various sources was studied. The host bacterium was grown on TSAS as a lawn and 10 µl bacteriophage was placed on the bacterial lawn as described above. The plates were incubated at room temperature (28 ± 2°C) and observed after 8 h.

Testing bacteriophage for therapy of luminous bacterial disease in laboratory microcosms

Three pre-sterilised plastic beakers containing 1L seawater were maintained at room temperature. Twenty healthy *P. monodon* postlarvae (PL18) were released in each beaker. Two of the beakers (A and B) were inoculated with 1 ml overnight culture of *V. harveyi* in TSBS and the third (C) served as control. The beakers were kept under aeration. On the initial day (day 1), 0.1 ml of bacteriophage containing 10^8 plaque forming units (pfu) per ml was added to the test beakers A and B. To beaker A, at 24 hr (day 2), the treatment was repeated by addition of 0.1 ml of the phage while no treatment was repeated in beaker B. In all the cases, the total bacterial count, total vibrio count and luminous bacterial count of water and larvae in the beakers were determined immediately after inoculation of *V. harveyi* and again at 24 hr and 48 hr. Zobell marine agar was used to estimate total bacterial count, TCBS to estimate total vibrio count and luminescent agar for estimation of luminous bacterial count. The larval survival was also monitored.

A shrimp hatchery with a production capacity of 100 million larvae situated in Visakhapatnam coast and experiencing luminous vibrio disease was selected for field trials. The larval tanks showing high intensity of luminescence and experiencing mortality were selected for treatment. One hundred ml phage containing 10^8 plaque forming units (pfu) per ml was added to 10 ton tank. Tanks with luminescence and mortality but not subjected to phage treatment served as controls. In all cases, the total bacterial count, total vibrio count and luminous bacterial count of water and larvae in the tanks were determined. Zobell marine agar was used to estimate total bacterial count, TCBS to estimate total vibrio count and luminescent agar was used for estimation of luminous bacterial count.

RESULTS AND DISCUSSION

As shown in Table 1, 28 strains of *Bacillus* spp. were tested for anti-vibrio activity. One strain did not inhibit any *Vibrio* spp. Four *Bacillus* strains did not inhibit *V. vulnificus*. All other strains showed inhibition of all the four *Vibrio* spp. to varying degree. The zone of inhibition ranged from 1.1 cm to 3.5 cm. It is interesting to note that the *Bacillus* spp. showing anti-vibrio activity were *B. licheniformis, B. firmes, B. coagulans, B. circulans, B. laterosporus, B. megaterium, B. subtilis*. The most common species were *B. megaterium* (13 strains) and *B. licheniformis* (11 strains).

Table 1. *Bacillus* spp. showing anti-vibrio activity.

Source	Species	Zone of inhibition after 12 hrs (in cms)			
		V. vul*	V. par	V. alg	V. har
Shrimp farm sediment	*Bacillus licheniformis*	2.0	2.2	2.2	2.1
	B. licheniformis	2.0	2.2	2.1	2.2
	B. licheniformis	1.4	1.6	1.7	2.0
Shrimp gut	*B. licheniformis*	2.0	2.3	2.0	2.1
Estuarine sediment	*B. licheniformis*	1.5	2.0	1.8	2.0
	B. firmus	2.1	2.2	2.1	2.3
	B.licheniformis	1.7	2.0	2.0	2.1
	Lactobacillus sp	1.5	1.5	1.2	1.0
Shrimp pond water	*B. licheniformis*	2.3	2.6	2.7	2.9
	B. coagulans	2.5	3.0	2.7	3.0
	B. circulans	2.7	3.5	3.0	3.0
Pond Sediment	*B. megaterium*	2.4	2.1	1.9	1.8
	B. coagulans	2.1	2.0	1.0	1.3
	B. laterosporus	-	1.0	-	1.1
	B. licheniformis	-	1.0	-	1.4
	B. megaterium	1.5	1.6	1.0	1.5
Pond sediment	*B. megaterium*	1.2	1.7	1.6	1.6
	B. megaterium	1.7	2.2	2.3	3.0
	B. megaterium	1.5	1.7	1.5	1.5
	B. coagulans	1.7	1.7	1.7	1.5
	B. licheniformis	1.5	1.6	1.5	1.8
Pond sediment	*B. coagulans*	2.0	2.5	2.7	3.2
	B. megaterium	3.2	3.0	3.0	2.7
	B. megaterium	3.0	2.7	3.0	3.5
	B. megaterium	1.9	2.3	2.4	1.5
	B. licheniformis	1.8	2.4	2.0	2.0
	B. licheniformis	2.3	2.5	3.0	2.5
Estuarine sediment	*B. subtilis*	1.3	2.1	2.0	1.8
	B. licheniformis	1.5	1.8	2.0	1.6
	B. megaterium	0.6	1.2	1.0	1.3
	B. megaterium	-	-	-	-
Pond water	*B. megaterium*	1.7	1.8	1.9	1.6
	B. megaterium	1.2	2.0	2.0	1.8
Pond water	*B. laterosporus*	2.0	2.2	2.1	2.0
Pond sediment	*B. firmus*	1.0	1.7	1.5	1.9
	B. laterosporus	1.1	1.8	1.6	1.8
	B. megaterium	-	1.0	1.0	0.9
	B. megaterium	-	1.2	0.7	1.0

**V.vul = V. vulnificus; V. par = V. parahaemolyticus; V. har = V. harveyi; V. alg = V. alginolyticus.*

There are many reports on the anti-vibrio activity of *Bacillus* spp. Production of bacteriocin or bacteriocin-like substances have been reported from *B. subtilis* (Jansen and Hirschmann, 1944), *B. thuringiensis* (de Borjac and Lajudie, 1974), *B. stearothermophilus* (Shafia, 1966), *B. licheniformis* (Bradley, 1967) *B. megaterium* (Ivanovics, 1962), *B. thermovorans* (Novotny and Perry, 1992) and *B. cereus* (Naclerio *et al*., 1993). Use of *Bacillus* spp. probiotic has been reported in piglets (Ozawa *et al*., 1981) and in black tiger shrimp, *Penaeus monodon* (Rengpipat *et al*., 1998). The latter study showed that *P. monodon* larvae fed with *Bacillus* S11 showed 100% survival after challenge with pathogenic *V. harveyi*, while only 26% control animals survived. The gut flora of control animals were predominated by *Vibrio* spp., while the flora in treated animals had predominantly *Bacillus* spp. Sugita *et al*. (1998) reported production of antibacterial substances by *Bacillus* strain NM12. They tested activity against *V. vulnificus*. The results of the study presented here in Table 1.show clearly that several Bacillus spp. show anti-vibrio activity (particularly against *V. harveyi*) and these have potential application as agents of biocontrol in aquaculture.

In this study, a bacteriophage isolated from hatchery water was found to be lytic for *V. harveyi*. All the 46 *V. harveyi* isolates from different sources such as shrimp farm water, sediment, shrimp hatchery water, shrimp larvae, both live and dead (from a hatchery where there was mortality due to luminous bacteria), estuarine water, creek water etc., were found sensitive to the bacterriophage (Table 2). To test the efficacy of the baceteriophage to reduce levels of luminous bacteria in hatchery systems, pilot experiments were conducted in the laboratory in sterile beakers to which filter strerilised sea water and *V. harveyi* cells were added. In this type of laboratory microcosms, the bacteriophage addition brought down the levels of *V. harveyi* by about 2-3 log units. This suggested the potential of this bacteriophage as a biocontrol agent.

Table 2. Lytic activity of phage on *Vibrio harveyi* isolated from different sources.

Source	**Ratio of phage sensitive isolates to number tested**
Sediment from estuary	2/2
Estuarine water	4/4
Creek water	3/3
Shrimp farm water	6/6
Hatchery water	8/8
Shrimp farm sediment	3/3
Dead larvae	5/5
Live larvae	15/15
Total	**46/46**

Results in Table 3 show the potential of the bacteriophage for therapy of luminous bacterial disease in a laboratory microcosm. Addition of two doses of phage at 24 h interval resulted in three log reduction in the level of *V. harveyi* and 80% survival in the larvae. In the control beaker, the *V. harveyi* levels were maintained and larval survival was only 10% at 48 h. In the beaker where phage treatment was done only in one dose, there was one log reduction in *V. harveyi* number and 40% survival in the larvae. These results suggest that phage therapy clearly improves larval survival in the presence of *V. harveyi*.

Table 3. Bacteriophage therapy in laboratory microcosm.

Beakers	Time interval	Dose of phage	TPC (cfu/ml)	LBC (cfu/ml)	Larval Survival (%)
A Test	Initial	100 µl	1.37×10^6	7.80×10^5	100
	After 24 h	100 µl	1.21×10^6	4.29×10^4	100
	After 48 h	Nil	9.80×10^5	1.20×10^2	80
B Test	Initial	100 µl	1.02×10^6	1.36×10^6	100
	After 24 h	Nil	1.29×10^6	9.30×10^5	80
	After 48 h	Nil	7.30×10^6	8.90×10^5	40
C Control	Initial	Nil	4.29×10^6	1.78×10^6	100
	After 24 h	Nil	9.20×10^6	4.68×10^5	75
	After 48 h	Nil	3.90×10^6	1.03×10^5	10

In the field trial, addition of bacteriophage resulted in the reduction of luminous bacterial count from 10^6/ml to 10^3/ml after 48 h and to undetectable levels in 72 h. The larval survival was 89%. Thus, the laboratory experiments and the field trials indicate the therapeutic potential of the bacteriophage in hatchery system.

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