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ASSOCIATION OF ASYMPTOMATIC MORTALITIES IN CULTURED WHITE SHRIMP, *PENAEUS INDICUS* WITH DOMINANCE OF NON-LUMINESCENT *VIBRIO HARVEYI* BIOTYPES IN MARICULTURE ENVIRONMENT

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SUMMARY

Asymptomatic mortalities among cultured *Penaeus indicus* adults and postlarvae were recorded in summer season. Two non-luminescent *Vibrio harveyi* biotypes (*V. harveyi*- 1 and *V. harveyi*- 2) were dominantly identified along with other bacterial species mainly *Pseudomonas fluorescencs* , *Aeromonas hydrophila* and *Staphylcoccus* species . The incidences of recovery of *V. harveyi*- 1 ,*V. harveyi*- 2 *P.fluorescencs, A. hydrophila* and *Staphylcoccus* spp. in infected adults and postlarvae reached 63.3 % , 26.7%, 6.7 %, 3.3 %, 0 % and 76.7%, 53.3%, 10%, 6.7%, 13.3 %, respectively.

The disease condition was found to be associated with the existance of stressful environmental role expressed by the recorded increase in values of water quality parameters especially ammonia and nitrite in affected ponds. However, the incidences of recovery of . *harveyi*- 1 ,*V. harveyi*- 2 *P.fluorescencs, A. hydrophila* and *Staphylcoccus* spp. in alfected pond water samples reached 93.3 %, 76.7 %, 13.3 %, 6.7 % and 23.3 %, respectively.

Experimental pathogenicity of *V. harveyi*- 1 ,*V. harveyi*- 2 in healthy shrimps confirmed the naturally existed disease condition. Recovered *V. harveyi* biotypes were shown to be sensitive to oxytetracycline, chloramphenicol, enrofloxacin, nalidixic acid and oxolinic acid while they were resistant to ampicillin, novobiocin, kanamicin, Gentamycin and sulfonamide. Recommendations and preventive measures are also discussed.

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INTRODUCTION

Vibriosis is one of the major worldwide serious threats affecting fish and shellfish mariculture caused by various *Vibrio* species (Lightner, 1988: Austin and Austin, 1993; Hjeltnes and Roberts, 1993). *Vibrio harveyi* is naturally existing in warm marine waters as well as on the surface and in gut of marine aquatic animals (Ruby and Morin, 1979).

Vibriosis in penaeids is generally influenced by factors such as stress, environmental failures and high counts of potentially pathogenic bacteria (Chen, 1992; Nash et al., 1992, Mohney el al., 1994, and Ruangpan et al., 1995). High mortalities of the tiger prawn (*P. monodon*) and larvae as well as juveniles associated with luminous *Vibrio harveyi* have been observed in hatcheries and farms in India (Karunasagar et al., 1994). Thailand (Jiravonichpaisal et al., 1994), Australia (Pizzutto and Hirst, 1995), Taiwan (Chen et al., 1992), Indonesia (Sunaryanto and Mariam, 1986) and Philippines (Baticados et al., 1990).

The presence of white spots in the carapace was the major clinical finding in recent outbreaks caused by vibrio species infections in cultured peneid shrimps (Takahashi et al., 1994 and Yu, 1995). However, outbreaks caused by *V. harveyi* among cultured *P. monodon* without overt gross signs were also recorded (Liu et al., 1996).

The use of antibiotics has been shown to be quite effective in controlling shrimp vibriosis at laboratory experimental trials (Baticados et al., 1990; Rukyani et al., 1992). However field application of antibiotics has not been very satisfactory (Chanratchakool ei al., 1995) where long term or improper usage of antibiotics may result in the development of antibiotic resistant strains.

This paper reports on the recovery of non-luminescent *V. harveyi* from cultured *P. indicus* adults and postlarvae as well as from rearing pond waters after some mortalities characterized by absence of gross signs of infection, Prevalence, characteristics, antibiotic sensitivity of isolated *V.harveyi* and significant water quality parameters as well as experimental pathogenicity were also investigated.

MATERIALS AND METHODS

1. Sampling :

A. Shrimp samples :

Thirty samples of moribund *P. indicus* adults and thirty samples of postlarvae (25 larvae / sample) were collected from some affected ponds of a commercial shrimp farm on the Red Sea coast in summer season suffering from asymptomatic mortalities. Twenty samples from each of adults and postlarvae were collected from normal healthy ponds and served as control. Samples were clinically examined

according to the method described by Austin and Austin (1989).

3. Water samples :

Thirty water samples aseptically collected from affected ponds as well as twenty samples from normal ponds were physico-chemically analysed for the following parameters: dissolved oxygen, salinity, pH, temperature, ammonia and nitrite by using the standard methods (Dass, 1989).

2. Bacteriological examination :

- Affected and control samples of *P. indicus* adults were dissected (Austin and Austin 1989), where bacteriological samples were aseptically taken from hepatopancreas and streaked on nutrient agar (Difco), MacConkey agar (Difco) and Tryptic Soy agar (Oxoid) supplemented with 2% NaCl.

- Affected and control samples of *P. indicus*, postlarvae were separately washed three times with sterile seawater, placed in sterile test tubes containing 5 ml sterile seawater and macerated with sterile glass rod. Samples were cultured using the same media mentioned before.

- Affected and control pond water samples were cultured on the same media mentioned above.

All plates were incubated at room temperature (25°C) for 24 hour. The recovered isolates were identified using standard morphological, physiological and biochemical characters as described by Krieg and Holt (1984), Lennette et al. (1985) and API 20 E system (Bio Merieux, France).

3. Antibiograms of the recovered *V. harveyi* biotypes:-

Predominant recovered *V. harveyi* biotypes were grown on TSA (supplemented with 2 % NaCl) for 24 h at 25°C. The bacteria were suspended in sterile PBS and diluted to a turbidity equivalent to Mac Farland No. 0.5 standard solution (0.5 ml BaS04 + 99.5 ml 0.36 N HCl). 0.2 ml of bacterial suspension was spread onto Muller-Hinton agar (Difco) and then antibiotic discs were added (Koneman et al., 1997). The used antibiotic discs (Bio Merieux, France) were oxytetracyline, chloramphenical, ampicillin, novobiocin, oxolinic acid, nalidixic acid, enrofloxacin, kanamycin, gentamycin and sulphonamide. The plates were incubated for 18 h at 25°C and the inhibition zones were measured and compared to the standards recorded by Bio Merieux, France.

4. Experimental pathogenicity:

A. Pathogenicity of *V. harveyi*- 1 ,*V. harveyi*- 2 to *P. indicus* postlarvae:

Experimental pathogenicity of representative biotypes of *V. harveyi*- 1 ,*V. harveyi*- 2 to postlarvae was carried out according to the method described by Prayitno and Latchford (1995), briefly,Seven flasks (two liter each) containing

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1 liter of sterile filtered sea water were stocked at a density of 80 postlarva each . Two strains of *V. harveyi*- 1 ,*V. harveyi*- 2 were cultured in TSA (supplemented with 2% NaCl), incubated at 28°C for 36 h., harvested and suspended in PBS .

Three flasks were used for each strain where each flask was inoculated with bacterial suspension at a final concentration of 10^2 cfu / ml. The seventh flask was inoculated with 30 ml sterile PBS and left as control. Flasks were continuously examined for disease signs, mortality and bacterial reisolation through out 7- day experimental period .

B. Pathogenicity of *V. harveyi*- 1 ,*V. harveyi*- 2 to *P. indicus* adults

Seven groups of *P. indicus* adults (20 each) weighing about 12 ± 2 g were used. Three groups were used for each of *V. harveyi*- 1 ,*V. harveyi*- 2 strains. The seventh group was left as control. The experiment was carried out as mentioned before.

RESULTS AND DISCUSSION

The rapid expansion of intensive shrimp mariculture and the upgrading of traditional shrimp farms worldwide have been encountered by the increase incidences of diseases and epizootics. The domination of *Vibrio* specie; infections in shrimp mariculture (Austin and Austin, 1993) has created that need towards understanding the epizootiology, pathogenesis and mechanism; by which disease course occurs that might be of significance in disease prevention and control programs.

Clinical examination *P. indicus* adults and postlarval samples from ponds with asymptomatic mortalities showed an absence of diagnostic signs and postmortem changes indicating vibriosis infection (Fig., 1). Mortality rates in infected ponds reached 14% and 19% in adults and postlarva respectively.

The hepatopancreas of shrimp is reportedly the main target organ of most bacterial pathogens (Chen , 1992). In the present study, several bacteria species of clinical importance including *V. harveyi*- 1 , *V. harveyi*- 2 , *P. fluorescencs, A. hydrophila and Staphylcoccus* spp were isolated from hepatopancreas of infected *P. indicus* adults, macerated whole bodies of postlarvae and affected rearing pond water (Table, 1). The prevalence of recovered bacterial species clarified the dominance of non luminescent *V. harveyi*- 1 ,*V. harveyi*- 2 biotypes both in affected and non-affected control samples (Tables, 1 & 2 and Fig., 2). In this regard, Prayitno and Latchford (1995) documented significant symptomatic mortalities in cultured *P. monodon* larvae caused by luminous *V. harveyi*. However, Lavilla-Pitogc et al. (1990) and Liu et al. (1996) recorded asymptomatic outbreaks of high mortalities among and cultured kuruma prawn *P. japonicus* cultured Juvenile tiger shrimp *P. monodon* respectively, caused by luminous *V. harveyi*. These results clearly reflect the

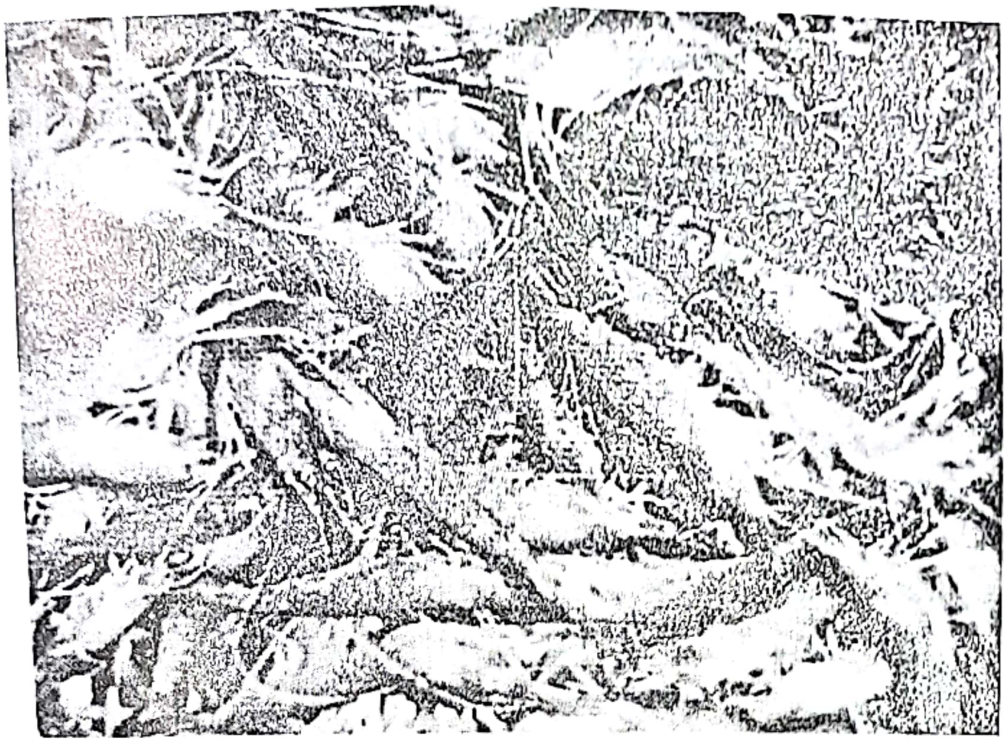

Fig. (1): Absence of gross signs of infection in died *P. indicus*

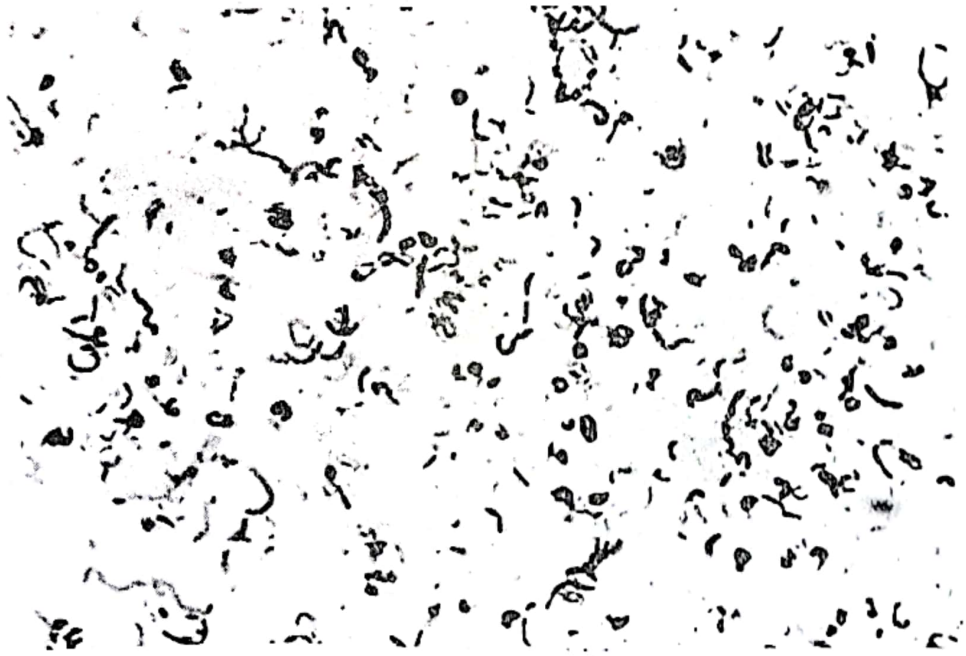

Fig. (2): Microscopic appearance of *V. harveyi* showing characteristic Gram-negative curved rods

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consistant existance and association of *V. harveyi* with shrimp as well as shrimp mariculture environment.

The incidences of recovery of *V. harveyi*- 1 ,*V. harveyi*- 2, *P. fluorescencs*, *A. hydrophila* and *Staphylococcus* spp. in total infected samples reached 77.8%, 52.2%, 10%, 5.6% and 12.2%, respectively whereas in total control samples reached 31.7%, 20%, 5%, 3.3% and 8.3% (Table 1) respectively. The higher incidence and prevalence of *V. harveyi* biotypes; (*V. harveyi*- 1 ,*V. harveyi*- 2) may reflect a degree of host specificity and/or the environmental selection for the survival, multiplication am domination of *V. harveyi* in mariculture elements based upon its virulence characteristics, competitive mechanisms to other existing bacterial species and tissue tropism evoked during host-pathogen interaction .

The recovery of *V. harveyi* biotypes from hepatopancreas of of adults with asymptomatic mortalities but not from control samples (Table. 1) confirmed the findings that internal organs in normal health conditions are bacteriologically sterile (Koneman et al., 1997) and strongly suggested the involvement of subclinical disease course caused by low virulence *V. harveyi* strains rather than the occurrence of carrier state or latent form of infection. However Leano et al. (1998) recorded consistent recovery of luminescent vibrios from pond-reared *P. monodon* juveniles with and without disease signs. The recovery of *V. harveyi* biotypes from both infected and control postlarval samples (Table 1) could be attributed to the use of macerated whole bodies of postlarvae for bacteriological examination where *Vibrio* species are naturally existing and dominating bacterial flora on the surface and in gut as also recorded by Ruby and Morin, (1979). Meanwhile, Lavilla-Pitigo et al. (1990) recovered luminescent *V. harveyi* from infected *P. monodon* larvae and hatchery rearing waters but not from uninfected ones.

Pseudomonas fluorescens and *A. hydrophila* are commonly existing pathogenic bacteria for freshwater aquatic animals and culture environment (Post, 1987 and Abdel-Aziz, 1988). However, their existence is not commonly associated with disease in marine aquatic animals (Roberts, 1989). Also, the low incidence of recovery of these bacteria in total infected samples (10% and 5.6% respectively) in contrast to the dominant incidences of recovery of *V. harveyi* biotypes (77.8% and 52.2 % for *V. harveyi*- 1 ,*V. harveyi*- 2 respectively, Table 1) may strongly support the environmental selection and domination of *V. harveyi* in mariculture and its considered significant existence as pathogenic bacteria for cultured shrimp and possibly other marine aquatic animals

Recovered Staphylococcus species from Postlarvae 15 and pond water samples (Table. 1) were shown to be Gram-positive cocci, non-motile, catalase positive and oxidase negative, aerobic and facultative anaerobic, non-haemolytic and co-

agulase negative organisms. Staphylococcus species are generally encountered as saprophytic non-pathogenic bacteria to aquatic animals in freshwater and mariculture environment (Austin and Austin, 1989). However their recovery in this study (Table 1) could be related to the specific character of salinity tolerance (Lennette et.al. 1985) as well as the hygienic quality of rearing pond water.

The biochemical and growth characteristics of recovered bacterial species (Table. 2) agreed with that recorded by Krieg and Holt (1984), Lennette et al. (1985) and Standards of API 20E system recorded by Bio Merieux. France. However, the characteristics of recovered *V. harveyi*- 1 ,*V. harveyi*- 2 revealed that these biotypes were non-luminescent, the result that might contradict considerable references describing the recovery of luminous V. harveyi from shrimp culture (Lavilla-Pitogo et al., 1990; Liu et al., 1996 and Leano et al., 1998). Interestingly, luminescence is not an absolute character for *V. harveyi* where non-luminescent V.harveyi strains do existing (Balows, et al., 1992).

The pathogenesis of vibrio species infections in marine aquatic animals is multifactorial. Variable factors related to the host, environment and the pathogen itself may work in concert to define the nature and extent of the triggered course of infection (Snieszko, 1974). In this regard, data presented in Table (3) revealed increase levels of some water quality parameters in affected rearing pond water specifically ammonia and nitrite. The increased values of these parameters in addition to the possible synergestic interactions of other culture parameters (Snieszko, 1974) may result in stressing and immunocompromising shrimps as well as enhancing environmental selection of V. harveyi in such rearing pond water (Post, 1987). Consequently invasion of *V. harveyi* to the organs and tissues of shrimps may occur resulting in the development of the recorded disease course that might be regulated by the overall interaction of host, pathogen and environment. Sunaryanto and Mariam (1986) reported that environmental factors such as organic load, temperature, salinity and pH and might be involved in triggering disease outbreaks caused by luminous bacteria. However, Roberts (1989) recorded that ammonia levels above 0.02 mg/liter are not recommended and its presence is always representing a potential danger to fish health especially in intensive mariculture systems with pH range 7.8-8.2. To the same extent Williams (1971) recorded that lower levels of ammonia in salmonid culture system can cause branchial hyperplasia as well as nitrite production by partial oxidation of ammonia resulting in severe losses.

The results of experimental pathogenecity (Table 4) indicated that exposure of *P. indicus* adults to *V. harveyi*- 1 ,*V. harveyi*- 2 induced mortalities reached 35% and 30% respectively, meanwhile, exposure of post-larvae to *V. harveyi*- 1 ,*V. har-*

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veyi- 2 resulted in mortalities reached 46.25% and 28.75% respectively through out 7- day experimental period. Absence of diagnostic signs and lesions of *V. harveyi* infection was recorded among died shrimps,however pure culture of *V. harveyi*- 1 ,*V. harveyi*- 2 were reisolated from hepatopancreas of moribund adults as well as macerated postlarval samples, confirming the results recorded in natural infection. On the other hand, no mortality was observed in controls exposed to PBS. while 3.75% mortality was recorded in post larval control group that could be considered a normal finding especially in such critical survival stage of poatlarvae (Dass, 1989). Prayitno and Latchford (1995) recorded 49.5 % mortality among clinically infected *P. monodon* postlarvae after 48 h of experimental exposure to *V. harveyi* at concentration of 10^4 /ml. Variations in these results could be attributed to the virulence of the involved *V. harveyi* strain.

The recovered *V. harveyi* biotypes in this study exhibited antibiotic sensitivity to oxytetracycline, nalidixic acid, oxolinic acid, chloramphenicol and enrofloxacin and resistance to sulphonamide, novobiocin, kanamicin, gentamycin and ampicillin. These results may suggest the possibility of using antibiotics in disease control. However, several problems associated with antibiotic usage including potential environmental and human health hazards (Kerry et al., 1995 and Capone et al., 1996) and the possible development and spread of antibiotic resistant strains (Karunasagar et al., 1994) were documented. On the other hand, Fagbenro-Byron and Ghaly (1950) and Ramesh et al. (1989) have shown that environmental factors such as temperature, salinity, pH and organic load might be involved in triggering disease outbreaks through its direct influence on the growth of luminous bacteria and possibly its virulence.

Therefore, other methods of disease control are urgently needed, and an understanding of virulence mechanisms and environmental factors controlling pathogens is of primary importance in developing such alternative controls. Consequently, the key role played by environmental factors in the occurrence and pathogenesis of *V. harveyi* infection in shrimp farms may allow the development of new strategy for disease prevention and control that rely on maintenance of master culture environmental parameters at maximum acceptable levels rather than the traditional usage of antibiotics.

The results and findings of the present study may draw attention to the following recommendations and preventive measures:

1. Maintenance of good husbandry practices and proper nutrition. These two important factors might help in reducing stress.
2. It is advisable to strictly adhere to quarantine practices for all live and newly acquired shrimps to the culture activities.
3. It is advisable to adhere to strict sanitation procedures prior to and during the rearing of larval

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stages of growth.

4. Controlling the existence of high counts of pathogenic vibrios in hatchery using ultraviolet irradiated water as well as employing a series of good quality filtration system.
5. It is advisable to use only previously chlorinated water during spawning and rearing to ensure a healthy rearing environment for newly hatched and developing larvae.
6. Periodical siphoning-out sediments and debris from the tank bottom that could serve as a good substrates for vibrios survival and multiplication .
7. Infected stock should be disinfected before finally discarding followed by a complete clean-

Table 1: Prevalence of predominant recovered bacterial isolates

Source of Samples	Nature of Samples	No. of Samples	Recovered Bacterial Species (No. and Percent)				
			V.h.1	V.h.2	P.fl.	A.h.	S.app
Adults hepatopancreas	Infected	30	19 (63.3)	8 (26.7)	2 (6.7)	1 (3.3)	Nil
	Control	20	Nil	Nil	Nil	Nil	Nil
Postlarvae macerated whole bodies	Infected	30	23 (76.7)	16 (53.3)	3 (10)	2 (6.7)	4 (13.3)
	Control	20	12 (60)	7 (35)	2 (10)	1 (5)	3 (15)
Pond water	Affected	30	28 (93.3)	23 (76.7)	4 (13.3)	2 (6.7)	7 (23.3)
	Control	20	7 (35)	5 (25)	1 (5)	1 (5)	2 (10)
Total	Infected	90	70 (77.8)	47 (52.2)	9 (10)	5 (5.6)	11(12.2)
	Control	60	19 (31.7)	12 (20)	3 (5)	2 (3.3)	5 (8.3)

V.h, = Vibrio harveyi
P. fl = Pseudomonas fluorescens
A.h = Aeromonas hydrophila
S. spp = Staphylococcus species

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Table (2): Biochemical and growth characteristics of predominantly recovered *V. harveyi* biotypes

Characteristics	Recovered biotypes	
	V.h.1	V.h.2
Gram Stain	-	-
Motility	+	+
P-galactosidase (ONPG)	+	+
Arginine dihydrolase (ADH)	-	-
Lysine decarboxylase (LDC)	+	+
Omithine decarboxylase (ODC)	+	+
Tryptophane deaminase (TDA)	-	-
Gelatinase (GEL)	+	-
Cytochrome oxidase (OX)	+	+
Urease (URE)	-	+
H2S (H2S)	-	-
Indole (IND)	+	-
Acetoin (VP)	-	-
Citrate (CIT)	-	+
Glucose (GLU)	+	+
Mannitol (MAN)	+	-
Inositol (INO)	-	-
Sorbitol (SOR)	-	
Rhamnose (RHA)	-	-
Sucrose (SAC)	+	-
Melibiose (MEL)	-	-
Amygdalin (AMY)	-	-
Arabinose (ARA)	-	-
Differential characteristics :		
Luminescence	-	-
Diffusible greenish yellow pigment production	-	-
Sensitivity to 0 /129 (150µg)	+	+
Growth on nutrient agar		-
Growth on TSA + 2 % NaCl	+	+
Growth on TSA + 7 % NaCl	+	+
Growth on Cetramide		-
Growth at 4°C		-

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Table (3) : Quality parameters of shrimp rearing pond waters.

Water Quality Parameter	Affected pond water*	Normal Pond water **
Temperatue (°C)	27-35	25-32
Salinity (ppt)	36-40	34-36
PH	7.2-7.5	7.5-8.5
Dissolved Oxygen (ppm)	4.3-5.6	5.4-6.8
Ammonia-NH_3 (ppm)	0.22-0.43	0,02-0.08
Nitrite-NO_2 (ppm)	0.132-0.624	0-0.118

* Range of water quality parameter for 30 infected samples

** Range of water quality parameter for 20 control samples.

Table (4): Mortality percentage of *P. indicus* postlarvae and adults experimentally infected with exposure to *V. harveyi*-1 and *V. harveyi*-2.

Experimental groups	Mortality percent (%)	
	Postlarvae	Adults
V. harveyi-1 (infected)	46.25	35
V. harveyi-2 (infected)	28.7530	30
PBS (control)	3.750	0

* Mean of 3 replicates

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up and disinfection of hatchery after every larval rearing period,

8. Daily water exchange of 80 - 90 % is advisable to reduce the load of harmful vibrios.

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