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ORIGINAL ARTICLE

Phenotypic and molecular typing of *Vibrio harveyi* isolates and their pathogenicity to tiger shrimp larvae

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Abstract

Aims: The objective of the present study was to identify the biotype(s) and molecular type(s) of *Vibrio harveyi* associated with pathogenicity in tiger shrimp (*Penaeus monodon*) larvae.

Methods and Results: Five luminescent and four nonluminescent *V. harveyi* isolates were subjected to phenotyping and random amplified polymorphic DNA (RAPD) fingerprinting, and pathogenicity testing to *P. monodon* mysis. Four isolates induced 34–41% mortality of *P. monodon* mysis when challenged at the rate of 10^6 CFU ml^{-1} within 60 h. Sucrose-fermenting biotypes of *V. harveyi* appeared to be associated with pathogenicity to larval shrimp. Higher temperature and salinity appeared to play a role on the onset of vibriosis and mortality in the challenged larval shrimp. Pathogenic isolates of *V. harveyi* could be demarcated as revealed by their clustering in the dendrogram constructed based on the RAPD fingerprints.

Conclusions: Nonluminescent *V. harveyi* also appear to be important aetiological agents of vibriosis of shrimp larvae. Sucrose-fermenting biotypes are likely to be pathogenic. High temperature may trigger onset of vibriosis.

Significance and Impact of the Study: Biotyping of *V. harveyi* isolates and looking for traits, such as ability to ferment sucrose may be helpful in identifying the pathogenic forms, and such approach requires to be investigated further with larger number of isolates.

Introduction

Vibrio harveyi has been widely recognized as a primary pathogen of many commercially cultured invertebrate species the world over, such as the black tiger shrimp (*Penaeus monodon*), with mortality of larval stages, protozoea to postlarvae in the hatcheries often reaching up to 100% (Lavilla-Pitogo *et al.* 1990; Song and Lee 1993; Karunasagar *et al.* 1994; Pizzutto and Hirst 1995; Liu *et al.* 1996a; Vandenberghe *et al.* 1998).

Random amplified polymorphic DNA polymerase chain reaction (RAPD–PCR) and M13 DNA fingerprinting have been reported to be useful in identifying the virulent and pathogenic isolates of *V. harveyi* (Pizzutto and Hirst 1995; Pujalte *et al.* 2003; Hernandez and Olmos 2004). However, information on identification of pathogenic strains using simple tests, such as phenotypic traits still needs to be generated. Further, information on the role of physico-chemical conditions of rearing on the onset of vibriosis caused by *V. harveyi* is also scanty. Hence, the present study was carried out to identify the biotypes and the molecular types of *V. harveyi* associated with pathogenicity and to understand the role of physico-chemical conditions of rearing on the pathogenicity in tiger shrimp larvae.

Materials and methods

V. harveyi isolates

Shrimp larvae affected with luminescent bacterial disease, commercial shrimp hatchery water samples, seabass

fingerlings, lobster and rotifers were processed for occurrence of *V. harveyi* using tryptose soy agar (TSA) fortified with 1% sodium chloride and 0·3% glycerol and a selective medium (Harris *et al.* 1996). Characteristic colonies on Harris' medium (small light green colonies with dark green centres and yellow halo) and luminescent colonies on TSA were phenotypically characterized according to the protocols of Smibert and Krieg (1991) and identified (Bauman and Schubert 1984; Alsina and Blanch 1994).

Production of virulence factors

Production of proteases and lipases by the isolates was tested by conventional methods (Smibert and Krieg 1991). Haemolysin production was tested in culture supernatants (Rowe and Welch 1994). Siderophore production was demonstrated by growing the isolates on Chrome Azural S (CAS) agar (Schwyn and Neilands 1987).

Pathogenicity to larval shrimp

Live *P. monodon* mysis were acquired from commercial shrimp hatcheries in plastic bags supplemented with oxygen and transported to the laboratory. Two litre flasks containing 1 l of sterile seawater (seawater autoclaved at 121°C for 15 min and cooled to ambient temperature) were stocked at a density of 100 mysis l^{-1} and fed on a diet of *Chaetoceros* and artificial feed and the system was constantly aerated. The mysis were allowed to acclimatize to the laboratory conditions for about 4 h. The salinity of seawater was 32–36 ppt, pH 8·0 and the temperature was maintained at 28 ± 1°C.

Isolates of *V. harveyi* were cultured in tryptose soy broth (TSB) supplemented with 1% NaCl for 18 h at 37°C in an orbital shaker incubator at 160 rev min^{-1}. The cells were harvested by centrifugation at 6000 ***g*** for 10 min. The bacterial pellet was rinsed twice using sterile phosphate-buffered saline (PBS; pH 7·4). The concentration of the cell suspension was determined by using spectrophotometer (Smartspec, Bio-Rad, Hercules, CA, USA). Shrimp mysis were challenged by adding this suspension to the experimental containers at a final concentration of 10^6 cells ml^{-1}. A control was maintained with same conditions without bacterial challenging. The number of dead mysis was counted at every 12 h up to 60 h.

Effect of salinity, temperature and pH on pathogenicity

In order to understand the effect of the rearing conditions, viz. salinity, temperature and pH on the pathogenicity of *V. harveyi*, Vh10 (identified as the most pathogenic, based on the above challenge experiment) to mysis of *P. monodon* was tested by bath challenging experiment in the similar manner as explained above. Pathogenicity was evaluated at selected salinities of 28, 30, 32 and 35 ppt; temperatures of 28, 30 and 32°C and pH of 7·6, 8·0 and 8·6. The mysis of tiger shrimp, acquired from the commercial hatchery, were brought slowly and steadily to the required salinity, temperature and pH over a period of 2 h and allowed to acclimatize for about 4 h prior to challenge. Each beaker was stocked with mysis at a density of 100 l^{-1}. The mysis were challenged at a concentration of 10^6 bacterial cells ml^{-1} of water. Data on the mortality rate were obtained up to 60 h by counting the number of dead larvae for every 12 h.

RAPD fingerprinting

Bacterial DNA was extracted (Wilson 1994) and RAPD–PCR was carried out by using four random primers (OPD 5, OPD 11, OPD 16 and OPD 20; Hernandez and Olmos 2004). PCR was carried out in 25 μl volumes containing 12·5 μl of 2X PCR master mix [AB Gene, Epsom, UK; 2X PCR mix contains, 1·25 units Thermoprime DNA polymerase, 75 mmol Tris-hydrochloride, 20 mmol ammonium sulfate, 1·5 mmol magnesium chloride, 0·01% (v/v) Tween 20, 0·2 mmol each of dATP, dCTP, dGTP and dTTP], 1 μl of primer (200 pmol), 1 μl of the template DNA (40 ng) and 10·5 μl of sterile MilliQ water.

The DNA in the reaction mixture was denatured at 95°C for 4 min and amplified by 35 cycles of denaturation at 92°C for 1 min, annealing at 37°C for 1 min and extension at 72°C for 2 min, and a final extension at 72°C for 3 min in a thermocycler (Eppendorf Mastercycler, Hamburg, Germany).

The amplified RAPD products were electrophoresed on 1·0% agarose gels at 65 V. λ-DNA double digest (*Hin*dIII and *Eco*RI) and 100 bp DNA ladder were included as the molecular weight markers (Genei, Bangalore, India). The gels were stained with 0·5 μg ml^{-1} of ethidium bromide and photographed under UV transillumination using gel documentation system (Bio-Rad).

Results

All the nine isolates were lysine decarboxylase- and ornithine dihydrolase-positive and arginine decarboxylase-negative, reduced nitrate, grew in 3% and 6% salt concentration, and fermented glucose with the production of acid only. None of the isolates could ferment arabinose, salicin, cellobiose or gluconate while five isolates fermented sucrose. Only two isolates utilized sodium citrate as the sole carbon source.

Table 1 Percentage mean mortality of *Penaeus monodon* mysis upon challenge with *Vibrio harveyi* isolates

	Time (h)			
Isolate number	12	24	48	60
Control	1	2	3	4
Vh1	1	2	4	6
Vh2	8	3	5	6
Vh4	2	3	4	4
Vh5	3	3	4	4
Vh6	1	2	6	34
Vh7	0	8	11	34
Vh8	1	2	4	39
Vh9	1	2	3	4
Vh10	1	11	23	41

Table 2 Effect of physico-chemical conditions on the pathogenicity of *Vibrio harveryi* Vh10 to *Penaeus monodon* mysis (percentage mortality)

		Time (h)			
Parameter	Value	12	24	48	60
Salinity (ppt)	28	2	5	8	12
	30	3	8	17	29
	32	8	13	28	38
	35	17	21	64	73
Temperature (°C)	28	2	7	21	29
	30	3	11	39	64
	32	3	9	68	85
pH	7·8	4	4	5	7
	8·0	8	9	11	13
	8·6	6	7	12	21

Production of virulence factors

All the isolates produced phospholiopases and gelatinases. Caseinase production was exhibited by Vh4 isolate alone. Lipases were not produced by any of the isolates. Isolates Vh4, Vh7, Vh8 and Vh9 showed protease activity. Haemolytic activity was exhibited by most of the isolates on blood agar. All the isolates produced siderophores as demonstrated by the production of orange halo around the colonies on CAS agar.

Pathogenicity to larval shrimp

Significant differences were observed in the mortalities of mysis of *P. monodon* compared with the controls. Four isolates including only one luminescent isolate Vh6 and other nonluminescent isolates Vh7, Vh8 and Vh10 caused mortality of *P. monodon* mysis ranging from 34% to 41% (Table 1).

Based on these results, being the isolate producing highest mortality (41%), Vh10 was selected for testing the role of salinity, temperature and pH of the larval rearing water on the pathogenicity of this isolate.

Effect of salinity, temperature and pH on pathogenicity

Among the different salinities tested for its role on the pathogenicity of *V. harveyi* Vh10, at higher salinity (35 ppt), mortality of mysis after 60 h was 73%. Similarly, higher temperature during challenge, viz. 28°C and 32°C, induced higher levels of mortality of mysis at the rate of 64% and 85% respectively. The range of pH tested did not show much effect on the mortality rate of *P. monodon* mysis. Only a maximum of 21% mortality was observed at pH 8·6 (Table 2).

RAPD fingerprinting

Nine isolates of *V. harveyi*, subjected to RAPD fingerprinting, produced up to five bands in the size ranging from 950 to 3700 bp with OPD 11 primer and 2–11 bands in the range of about 560–3650 bases with OPD 20 primer (Fig. 1). The banding pattern produced by the genomic DNA using the other two random primers OPD 5 and OPD 16 did not produce inferable results. The pathogenic isolates, Vh10, Vh7, Vh8 and Vh6 were clustered in phenons 3, 4, 5 and 6 at about 15% hierarchical level in the dendrogram generated from the RAPD fingerprints obtained using OPD 11 random primer. These isolates were grouped into phenons 1, 2, 3 and 4 in the dendrogram generated from the RAPD fingerprints obtained by using the random primer OPD 20 (figure not included).

Discussion

It was interesting to note that out of nine isolates of *V. harveyi*, only one luminescent isolate Vh6, induced significant mortality in larval shrimp in addition to three other nonluminescent isolates. Virulence factors, such as cysteine protease and extracellular alkaline metal chelator-sensitive proteases, phospholipases and haemolysins produced by *V. harveyi* were reported to be associated with pathogenicity to penaeid shrimp (Fukasawa *et al.* 1988; Lee *et al.* 1996; Liu *et al.* 1996b). In the present study, phospholipase and gelatinase activity was recorded in all the isolates as reported earlier by Baffone *et al.* (2001). Although *V. harveyi* Vh4 produced all the virulence factors tested *in vitro*, they produced only 14% mortality. On the other hand, *V. harveyi* Vh10, which produced only gelatinase in addition to phospholipase, haemolysin and siderophores caused 41% mortality after 60 h when challenged to mysis of

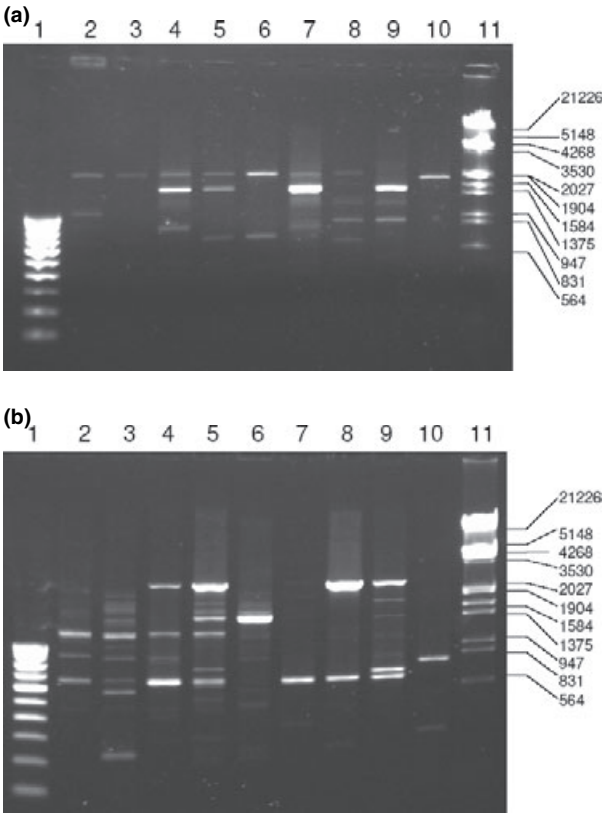

Figure 1 Random amplified polymorphic DNA (RAPD) fingerprint of the *Vibrio harveyi* isolates using random primers OPD 11 (a, AGC-GCC-ATT-G) and OPD 20 (b, ACC-CGG-TCA-C). Lane 1, 100 bp ladder; lane 11, λ-DNA double digest (*Hin*dIII and *Eco*RI); lane 2, Vh1; lane 3, Vh2; lanes 4 to 10, Vh4 to Vh10 respectively.

P. monodon. Three other isolates, Vh6, Vh7 and Vh8 similar to isolate Vh10 in elaborating virulence factors, also caused significant mortalities ranging from 34% to 39%. Five isolates, viz. Vh6, Vh7, Vh8, Vh9 and Vh10 fermented sucrose, and except isolate Vh9, all the other four isolates were pathogenic to *P. monodon* mysis. However, such biotype-associated pathogenicity, needs to be further evaluated using a large number of isolates.

Studies on the influence of environmental parameters on the pathogenicity of *V. harveyi* are very scanty. Among the three physico-chemical conditions of rearing tested for their influence on the pathogenicity in the present study, higher temperature and salinity appeared to play a role on the mortality of larval shrimp upon bacterial challenge. A study by Prayitno and Latchford (1995) has indicated that exposure of luminous bacteria prior to challenge to acidic pH significantly reduced the mortality of penaeid larvae. In contrast to the observations made in the present study, Prayitno and Latchford (1995) reported that exposure of luminescent bacteria to low salinities before challenge significantly enhanced mortality of the larvae. It could be inferred from these data that environmental factors do play a key role in the outbreak of bacterial disease in larval shrimp. Aguirre-Guzman *et al.* (2001) reported that shrimp larval susceptibility to the bacterial pathogens was age- and dose-related in the American white shrimp, *Litopenaeus vannamei* larvae. They reported that doses of 10^5 to 10^7 CFU ml^{-1} induced significant mortality of shrimp larval substages, protozoea I to postlarvae I.

In the present study, RAPD fingerprinting was able to distinguish pathogenic and nonpathogenic isolates to some extent. Hernandez and Olmos (2004) also had reported that virulent and nonvirulent *V. harveyi* could be distinguished by RAPD–PCR. Similarly, discrimination of epidemiologically related isolates involved in the disease outbreak in sea bass by molecular typing, phenotyping and virulence was demonstrated among *V. harveyi* isolates from the infected gilthead sea bream, and the European sea bass (Pujalte *et al.* 2003). Such clustering of the pathogenic isolates based on the RAPD fingerprints suggests that the technique may be developed as a useful tool in distinguishing the pathogenic and the nonpathogenic isolates.

The study has indicated that the pathogenicity of *V. harveyi* isolates may be associated with sucrose-fermenting biotypes. Higher temperature and higher salinity are likely to enhance the incidence of *V. harveyi*-associated disease in the larval shrimp.

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