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Original Article

Prevalence of *Vibrio alginolyticus* in Sediment Samples of River and Coastal Areas of Bangladesh

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***Vibrio alginolyticus* has been thought to be a halophilic marine bacterium that causes diarrhea, otitis media and wound infection through the consumption of raw or inappropriately cooked sea food. It is one of the main *Vibrio* pathogens affecting marine animals, such as marine fish, shrimp and shellfish which lead to large economic damage. Although there are reports on the presence of this organism in the coastal area of other countries, not so much work has been done on the isolation and characterization of this species in Bangladesh. The present study was, therefore, undertaken to isolate and characterize *V. alginolyticus* organisms isolated from the rivers (fresh water) and estuaries (brackish water) of Bangladesh. A total of 9 isolates of *Vibrio s*pecies were obtained from different water bodies (three from Meghna river, two from Shangu river and four from estuary) and provisionally identified as *Vibrio alginolyticus* following standard biochemical tests. All these 9 strains showed same pattern of antibiotic resistance to ampicillin, streotomycin, penicillin, but sensitive to nalidixic acid. In the virulence properties test, two isolates showed positive results for *toxR* gene and none of the isolates showed positive results for *tdh* gene. Challenge experiments in Singhi fish (*Heteropneustes fossi*) with the live cells and the culture filtrate prepared from the *V. alginolyticus* showed high mortality of the fish population. All these studies suggest the presence of pathogenic *V. alginolyticus* strains in the river water and estuarine bodies of Bangladesh and the extracellular toxin(s) of the *V. alginolyticus* might be one of the causes for fish mortality.**

***Keywords: Vibrio alginolyticus*, *toxR* gene, *trh* gene, *tdh* gene**

Introduction

Vibrio alginolyticus is a Gram negative bacterium belonging to the family *vibrioceae* that cause cholera. It is a moderately halophile or salt requiring organism that lives in marine and estuarine environments [1]. It causes gastrointestinal illness in humans after ingestion of contaminated sea water and sea foods[2]. Moreover, *V. alginolyticus* is able to cause extra-intestinal diseases, such as otitis media and wound infection to human[3] and it is an epizootic pathogen to several aquatic animals[4,5]. In South China, *V. alginolyticus* has been reported to be the dominant causative agent of high mortality outbreaks of vibriosis in large yellow croaker, sea bream, grouper, kuruma prawn as well as in larvae of several fish and shellfish species[6, 7]. This organism persists as a health hazard in the Far East, where it was originally isolated[8] and also as a source of human disease or as an environmental contaminant along the North American, African, and Mediterranean coasts[9,10,11]. This bacterium causes approximately half of all food poisoning cases in Taiwan, Japan and several Southeast Asian countries[2,12]. Strains of *V. alginolyticus* are among the most common organisms isolated from diseased fish with clinical signs of bacterial septicemia[13,14]. *V. alginolyticus* has also been reported to be responsible for ulcer disease of the gilthead seabream, sparus aurata, turbot, crimson snapper and sea mullet[15].

V. alginolyticus was originally classified as a biotype of *V. parahemolyticus*, the two of which are genetically quite similar. They can be differentiated phenotypically on the basis of fermentation of sucrose by *V. alginolyticus*[2]. Some differences in taxonomic traits between clinical and environmental isolates of *V. alginolyticus* have also been suggested. Several virulence factors, including iron uptake system, extracellular polysaccharide and protease have been suggested to be the major contributors to pathogenicity in this species[3,4,7]. However, there are not much reports on the pathogenicity of the *V. alginolyticus* isolated from different sources in and around estuaries. Therefore, this work was undertaken to investigate the occurrence of *V. alginolyticus in* the estuaries as well as rivers in Bangladesh and to compare the phenotypic and genotypic traits, as well as the pathogenicity of these organisms.

Materials and Methods

Sample collection and processing

Sediment samples were collected from June to September, 2008 from three coastal area (Estuary, Shangu and Karnaphuli) and

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six rivers (Buriganga at Dhaka, Shitalakhya at Narayanganj, Turag at Dhaka, Padma at Rajshshi, Jamuna at Shirajganj, Meghna at Comilla, and Padma at Maoa) of Bangladesh.

Sediment samples were collected from 10 cm depth of the shore by the help of sterile scoop. Approximately 100 grams of samples were aseptically collected in sterile plastic bags. Samples were stored at room temperature until processing in the laboratory. The salinity of the sample was measured in the laboratory by a refractometer.

Alkaline peptone water (APW) was used to facilitate the growth of *Vibrio* spp. One gram of sediment sample was mixed with 9 ml of APW supplemented with 2% NaCl and incubated at 37 °C for 6 h. After incubation, the enriched culture was shaken and one loopful of the culture was streaked on to X-VP media (X-VP agar; Nissui Co. Japan).

Screening of suspected V. alginolyticus colonies

White colonies on X-VP agar plates were selected as suspected *V. alginolyticus* after overnight incubation at 37 °C. Five to ten suspected *V. alginolyticus* colonies were picked up and subcultured onto fresh X-VP plate for pure culture. Again suspected *V. alginolyticus* isolates were streaked onto thiosulphate citrate bile salt sucrose agar (TCBS agar; Nissui Co., Japan). Following overnight incubation at 37 °C, isolates those produced mucoid, raised, yellow, 2-3 mm colonies were selected as *V. alginolyticus* and were readily transferred to Luria- Berrtani (LB) agar containing 3% NaCl.

Biochemical characterization

Biochemical tests were performed following standard methods[16]. Kligler's iron agar, lysine decarboxylase, arginine dihydrolase, ornithine decarboxylase, oxidase, citrate utilization, motility, indole production, methyl-red, Voges-proskauer, nitrate reduction, swarming motility and salt tolerance tests were used for the biochemical characterization of *V. alginolyticus*. In addition growth at 40°C was also examined.

Antibiotic susceptibility test

Ampicillin (10µg), Nalidixic acid (30µg), Novobiocin (30µg), Penicillin (10µg), Tetracyclin (30µg), and Streptomycin (10µg) antibiotics were used against all 9 environmental isolates according to the modified Kirby-Bauer method[17].

Determination of hemolysis activity

V. alginolyticus isolates were screened for hemolysis on sheep blood agar plate followed by incubation at 37 ^{o}C for 18 h.

*PCR amplification of the tox*R *and tdh gene*

Stock cultures of *V. alginolyticus* were maintained in liquid preservation medium (pH 8.8) containing 0.5% APW and 2.5% NaCl and revived on the LB agar plates. A single luminescent colony of each culture was inoculated in tryptic soy broth with 1% NaCl and incubated overnight at 37 ^{o}C. Genomic DNA was extracted and purified according to GuSCN DNA extraction method[18]. Briefly, 2-3 colonies of the bacteria were picked and suspended in 200 µl PBS, centrifuged at 13000 rpm for 5 min. The pellet was resuspended in 200 µl of TE buffer containing 1 mg/ml lysozyme and 1 mg/ml acromopeptidase and incubated at 37^{o}C for 1 hr. After incubation, 300 µl of 4M GuSCN (containing 2% w/v Tween-20) was added, mixed well and centrifuged at 13000 rpm for 5 min. The supernatant containing the DNA was purified using isopropanol and were its concentration and purity were checked using a spectrophotometer at 260/280 nm (Shimadzu 1601, Kyoto, Japan) and was finally stored at "20^{o}C until further use.

Isolates of *V. alginolyticus* strains used in this study were tested based on PCR targeting the *tox*R and *tdh*. PCR primers were used for targeting a 399 bp *tox*R (TOXR-1:AGCCCGC TTTCTTCAGACTC and TOXR-2:AACGAGTCTTC TGCATGGTG) and 251 bp *tdh* (TDH-3:CCACTACCACTC TCATATGC and TDH-4: GGTACTAAAT GGCTGACATC). PCR was carried out in a 25µL reaction mixture consisting of 2.50 µL *Taq* universal 10X buffer (100 mM Tris- HCl pH 8.3, 20 mM MgCl2, 500 mM KCl, 0.1% gelatin), 2 µL of dNTPs (2.5 mM of each), 0.5 µL of each primer (25 pmol of each), 0.125 µL of *Taq* polymerase (0.75 U of *Taq* polymerase), 17.375 ìL sterile deionised water and 2 µL of template DNA. Amplification was performed using a thermo cycler system (MJ Research, Watertown, USA) for 5 minutes at 95 °C, 35 cycles of 1 minute at 95 °C, 1 minute at 55°C, and 1 minute at 72 °C. The PCR products were resolved on 2% agarose gels containing 0.5 µg/mL ethidium bromide and documented using a gel documentation system (Bio-RAD, USA).

Preparation of live cells as inoculum

V. alginolyticus cells were incubated in Luria Bertani (LB) broth with 2% NaCl for 18 h on a rotary shaker at 37 °C and after incubation the density of cells were ~3×10^{8} cfu/ml which was determined by comparing with that of McFarland standards.

Preparation of culture filtrates as inoculum

V. alginolyticus cells were grown in 100 ml Brain Heart Infusion (BHI) broth for about 22 hours and the cells were harvested by centrifugation at 6000 rpm for 5 mins. The supernatant was filtered through a 0.45 µm syringe filter and kept at -20^{o}C.

Challenge of V. alginolyticus against Heteropneustes fossi

One hundred sixty Singhi fish (*Heteropneustes fossi*) of 10 to 18 gm body weight and 10 to 15cm length were kept for two days in the environment of aquarium for adaptation. 160 fish were equally distributed randomly in 16 aquariums (12-16 litre each). One aquarium was maintained as control and three each of the aquariums were inoculated with 10 ml volume of three different dilutions (~10^{4} to 10^{6} cfu/ml) of two different *V. alginolyticus* organisms (M3 isolated from river and E5 isolated from estuary). After five days, two fish from each aquarium (marked as smaller) were transferred to new corresponding

aquariums containing healthy fish (marked as longer). At least two fish from each aquarium were taken out from each aquarium on day 5 (or any fish which died due to infection) and were checked for the presence of *V. alginolyticus* in different organs of the fish (skin, lung and intestine). Fish in two aquariums were inoculated intra-peritoneally with live *V. alginolyticus* organisms (M3 and E5 series, 100 µl, 10^5 cfu/ml) and observed for 14 days after bacterial inoculation. On the other hand, fish of another aquarium were injected intra-peritoneally with culture filtrate (0.5 ml) prepared from the *V. alginolyticus.* All fish in different aquariums were observed for 14 days.

Results

Isolation of V. alginolyticus

Twenty five isolated strains were selected as suspected *V. alginolyticus* through enrichment of sample and by primary screening on X-VP (a special medium for *Vibrio* where *V. alginolyticus* showed characteristic white colored colonies) from 7 different sampling sites (Table 1) viz isolates numbers 8,3,4,1,4,3 and 4, from estuary (Chittagong), Karnaphuli (Chittagong), Shangu (Chittagong), Turag (Dhaka), Sitalakhaya (Dhaka), Padma (Maoa), and Meghna (Comilla) respectively. Salinity (%) was recorded 0.9, 0.1 and 0.1 in samples collected from estuary, Karnaphuli and Shangu respectively. The suspected *V. alginolyticus* colonies were found to be large, yellow, raised and mucoid having a diameter of 3-4 mm on the TCBS agar plates. Isolates that showed typical biochemical features of *V. alginolyticus* were further found to exhibit growth ability at 40 ^oC. Positive reactions for oxidase test, citrate utilization test, Voges-proskauer test, lysine and ornithine decarboxylases growth at 40^oC, nitrate reduction, swarming motility, alkaline red slant and acidic yellow butt in KIA test, indole production, motility, salt tolerance test and gave negative reaction to methyl red test, were identified as *V. alginolyticus*[19]. All the strains were sensitive to 150 µg vibrio-static 0/129 compound (2, 4-diamino-6, 7-diisopropylpteridine phosphate) but resistant to the concentration of 10 which is another unique feature of *V. alginolyticus*. Results of the biochemical tests are shown in Table 2.

Table 1. *Locations of different sampling sites of Vibrio alginolyticus*

Collection Period	Area of Sample Isolation	Temperature (^oC)		Salinity (%)	No. of positives
		Air	Water		
June, 2008	Estuary (Chittagong)	32	24	0.9	6
June, 2008	Kornaphuli(Chittagong)	31	25	0.1	3
June, 2008	Sangu (Chittagong)	34	25	0.1	4
June, 2008	Buriganga (Dhaka)	30	23	0	0
June, 2008	Turag (Dhaka)	32	24	0	1
June, 2008	SitaLakhya (Narayanganj)	32	22	0	4
June, 2008	Padma (Rajshahi)	34	26	0	0
August, 2008	Padma (Maoa)	31	22	0	3
July, 2008	Meghna(Comilla)	30	22	0	4
June, 2008	Jamuna (Shirajganj)	29	24	0	0

Table 2. *Results of biochemical reactions of the Vibrio alginolyticus isolate from river and brackish water environments*

Area of isolation	Estuary (Chittagong)				Shangu river (Chittagong)		Sitalakhya river (Narayanganj)	Meghna river (Comilla)		
Strain ID	E1	E3	E4	E5	Sg1	Sg3	S1	M1	M2	M3
Oxidase	+	+	+	+	+	+	+	+	+	+
Lysine	+	+	+	+	+	+	+	+	+	+
Arginine	-	-	-	-	-	-	-	-	-	-
Ornithine	+	+	+	+	+	+/-	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+	+	+	+
Indole	+	+/-	+	+	+	+/-	+	+	+	+
MR	-	-	-	-	-	-	+	-	-	-
VP	+	+	+	+	+	+	_	+	+	+
KIA Slant	A	K	K	K	K	K	K	K	K	K
Butt	A	A	A	A	A	A	A	A	A	A
H_2S	-	-	-	-	-	-	-	-	-	-
NO_3 reduction	+	+	+	+	+	+	-	+	+	+
Sensitivity to vibrio 10 µg	R	R	R	R	R	R	S	R	R	R
static agent 150µg	S	S	S	S	S	S	R	S	S	S

[Keys: K= Alkaline; A= Acidic; + = Positive; - = Negative; R= Resistant; S= Sensitive]

Antibiotic resistance

All the 9 isolates were tested for their susceptibilities against the six commonly used antibiotics and were found to be susceptible to nalidixic acid, resistant to ampicillin, streptomycin and penicillin and intermediate susceptible to tetracycline. Moreover, isolates each from Meghna (M1), Estuary (E5) and Shangu river (Sg2) showed intermediate susceptible to Novobiocin. Results are shown in Table 3.

Table 3. *Drug resistance pattern of V. alginolyticus against different antibiotics*

Sample	Strain ID	Susceptibility pattern					
		AMP	STR	NAL	NOV	PEN	TET
Meghna river	M1	R	R	S	I	R	I
	M2	R	R	S	R	R	I
	M3	R	R	S	R	R	I
Estuary	E1	R	R	S	S	R	I
	E3	R	R	S	R	R	I
	E4	R	R	S	R	R	I
	E5	R	R	S	I	R	I
Shangu river	Sg1	R	R	S	R	R	I
	Sg2	R	R	S	I	R	I

Key: AMP-Ampicillin (10µg), TET-Tetracycline (30µg), STR-Streptomycin (10µg), PEN-Penicillin (10µg), NAL-Nalidixic acid (30µg), NOV-Novobiocin (30µg), R-Resistant, S-Sensitive, I-Intermediate.

Hemolysis on blood agar

A total of 7 isolates, three from river (M2, M3, and Sg1) and 4 strains of brackish water (E1, E3, E4 and E5) were tested for the hemolytic activity in the blood agar plates. After overnight incubation at 37 ^{o}C, all 7 isolates of *V. alginolyticus* showed the zone of β-hemolysis on blood agar.

*Detection of tox*R *gene* and *tdh gene*

All isolates showing positive biochemical results were also checked for the presence or absence of *tox*R gene, using *tox*R specific primers, since *tox*R gene is well conserved among *Vibrio* species [20,21,22] . When template DNA from each isolate was subjected to amplification through PCR, two isolates (M3 and E5) yielded DNA bands (399 bp) specific for *tox*R region after 35 cycles of amplification (Figure 1). *V. alginolyticus* isolates were further subjected to PCR using *tdh*- specific primers as a reconfirmatory test for the presence of virulence gene *tdh*. However, all the isolates showed negative results for the presence of *tdh* gene after 35 cycles of amplification.

Challenge experiment

Cohabitation assay: In the first round of experiments, where water of three different aquariums containing 10 fish each and inoculated with different dilutions of *V. alginolyticus*, were found to infect different organs of the fish within 5 days after inoculation. In the second round of experiments, where two infected fish (marked as smaller) from each aquarium of first round were transferred to fresh aquariums containing 10 fish each, could infect the healthy fish (marked as longer). Different organs of the dead fish were checked for the presence of *V. alginolyticus* and it was observed that there was not much differences in the bacterial numbers in different organs of the infected fish in aquariums inoculated with different dilutions of *V. alginolyticus* organisms ($p<0.05$). All fish in the first round of experiments also died between 7 and 10 days. However, the average incidence of mortality of fish was found to be more severe in E5 series than the M3 series of *V. alginolyticus* organisms. Similar results were also obtained in the second round of experiments, where two infected fish from each of the three infected aquariums were transferred to fresh aquariums containing healthy fish. Here the average incidence of mortality of the fish was found to be 79.17% (for E5 series) and 70.83% (for M3 series), respectively, and these fish died between 8 and 14 days. Mortality of fish was more severe in E5 series than M3 series of *V. alginolyticus* organisms ($p<0.05$).

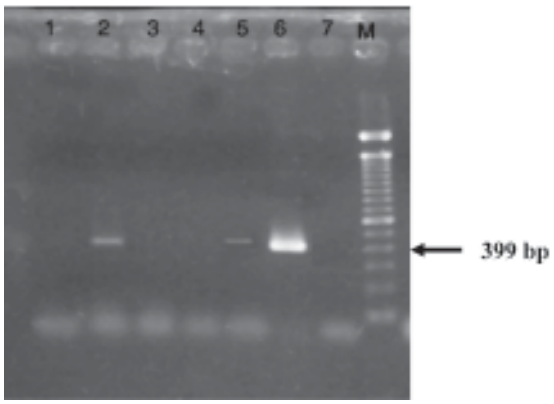

Figure 1. *Agarose gel electrophoresis of the PCR product. Template DNA from V. alginolyticus isolates were subjected to amplification through PCR, two isolates (M3, lane 2 and E5, lane 5) yielded DNA bands of 399 bp specific for toxR region after 35 cycles of amplification. Lane M- 100bp molecular weight marker; Lane 7- Negative control; Lane 6- Positive control strain VP11 (New clone of serotype O3:K6); Lane (1-5) - Test isolates from estuarine and river sediment sample (M2, M3. Sg1, E4, E5 respectively)*

Inoculation with live cells: All fish which were inoculated intraperitoneally with live *V. alginolyticus* organisms died between two and five days after inoculation. It was noted that fish which were inoculated with E5 series organisms died earlier than the fish which were inoculated with M3 series of *V. alginolyticus* organisms. All *V. alginolyticus* organisms isolated from different organs of the dead or infected fish were subjected to antibiogram profile to confirm the origin of the *V. alginolyticus*.

Inoculation with culture filtrate: All fish inoculated intraperitoneally with the culture filtrate of the E5 series *V. alginolyticus* organisms died within 2 days after inoculation. On the other hand, all negative control fish without any inoculation were found healthy till the end of the experiments.

Discussion

V. alginolyticus is a halophillic organism that normally inhabits marine and estuarine environments. This study was undertaken to isolate the *V. alginolyticus* organism, if any, in the rivers and estuaries of Bangladesh and characterize their virulence properties. Isolation and identification of the target bacterium is very crucial for its characterization and comparison and in this study, isolation required a pre-enrichment step followed by growth on selective media. According to Bergey's Manual of Systemic Bacteriology, isolates those showed positive reaction to oxidase test, citrate utilization test, acetone production, utilization of amino acids lysine, arginine and ornithinine, nitrate reduction, alkaline red slant and acidic yellow butt in KIA, positive indole production, swarming motility and tolerance up to 10% NaCl were identified as *V. alginolyticus.*

Antibiotic susceptibility is an important parameter for the characterization of organisms and *V. alginolyticus* has been reported to be resistant to ampicillin, methicillin, lincomycin, penicillin, and carbenicillin and susceptible to tetracycline, chloramphenicol, gentamicin, kanamycin, streptomycin, and neomycin[2,23,24]. However, all isolates showed sensitivity to nalidixic acid.

Virulent *V. alginolyticus* possess a number of enzymes such as hemolysin, collaginase, elastase, protease, etc. that play potential role in their pathogenecity [3,7,10]. In this study, a total of seven isolates, three from river (M2, M3, and Sg1) and four strains from brackish water (E1, E3, E4 and E5) showed zone of á-hemolysis on blood agar, indicating the production of hemolysin by these organisms. These isolates were then extensively studied for the presence of any toxin production controller gene such as *tox*R or toxin producing gene, like *tdh* or *trh.* All isolates showing β haemolysis on blood agar were also observed for the presence of *tox*R gene, using *tox*R specific primers. However, none of the isolates showed positive results for the presence of *tdh* gene, which confirmed the inability for the production of themostable direct haemolysin by the *V. alginolyticus* organisms.

In this study, the challenge experiment demonstrated that the *V. alginolyticus* organisms were found to infect different organs of the Singhi fish (*Heteropneus fossilis*) and all fish died between 7 and 11 days after the fish were kept in aquariums inoculated with different dilutions of the *V. alginolyticus.* Also, infected fish were able to cause disease in the healthy fish and the average incidence of mortality was found to be 79.2% for *V. alginolyticus* isolated from estuaries and 70.8% for *V. alginolyticus* isolated from rivers, respectively. However, in all cases, mortality was found to be more severe in *V. alginolyticus* isolated from the estuaries than the river isolates. Pathogenic nature of the *V. alginolyticus* were also revealed when live bacteria were inoculated in the fish intraperitoneally. Overall our results clearly indicate that extracellular toxin(s) might be one of the virulence factors of the *V. alginolyticus* responsible for fish mortality. Although the *V. alginolyticus* organisms were found to lack the pathogenic *tdh* gene, however, the challenge experiments strongly demonstrate the pathogenic nature of the *V. alginolyticus* in fish model. Characterization of the extracellular toxin(s) responsible for the fish mortality could be one of the future directions in *V. alginolyticus* pathogenicity research.

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