

Original Article

Isolation and Characterization of *Vibrio parahaemolyticus* Organisms from River Sources Around Barisal District, Bangladesh

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Vibrio parahaemolyticus is a halophilic bacterium and lives in coastal water and causes gastroenteritis in humans especially where the rate of seafood consumption is high. Although it is a marine organism, very less is known about its pandemic spreading in the fresh water environment across Bangladesh. The present study was, therefore, undertaken to isolate and investigate the virulence potential, as well as phenotypic and genotypic traits of *V. parahaemolyticus* strains occurring in the fresh water environments around coastal southern parts of Bangladesh. A total of 70 suspected colonies of *V. parahaemolyticus* were isolated from water, sediment and roots of water hyacinth samples collected from four different locations of Bishkhali river located in Barisal district of Bangladesh. Of the 70 colonies, fourteen isolates were confirmed as *V. parahaemolyticus*. PCR detection for the presence of *toxR*, *trh* and *tdh* genes among these seven isolates using primers specific for *toxR*, *trh* and *tdh* revealed that all seven were positive for *toxR* but negative for *trh* or *tdh* genes. The results indicate that the isolates under this study do not contain virulence properties that correlate to the ability of causing infection and diseases.

Key words: *Vibrio parahaemolyticus*, *toxR*, *trh*, *tdh*, virulence genes.

Introduction

Vibrio parahaemolyticus, a well-known human pathogen, is the leading cause of gastroenteritis due to the consumption of seafood, primarily raw or improperly cooked shellfish worldwide¹⁻². These organisms were first recognized as a potential food-borne pathogen by Fujino and coworkers in 1951 in Japan and caused approximately half of all food poisoning cases in Taiwan, Japan and several Southeast Asian countries³⁻⁴. It is also reported to be an important agent of travelers' diarrhoea and has also occasionally been associated with extra intestinal infections, including wounds⁵. Although *V. parahaemolyticus* is a halophilic bacterium and mostly occurred in sea water environment, however, these organisms have also found to occur in fresh water environments, where the salinity of the water is almost zero and the temperature is relatively low⁶.

Pathogenic strains of *V. parahaemolyticus* generally produce a thermostable direct haemolysin (TDH) that is associated with the Kanagawa phenomenon (KP)⁷ and/or thermostable direct haemolysin-related haemolysin (TRH). Both TDH and TRH are encoded by *tdh* and *trh* genes respectively⁸⁻⁹. Molecular epidemiological studies employing the DNA probes specific to the *tdh* and *trh* genes revealed that not only strains carrying the *tdh* gene but strains carrying a *trh* gene or both genes are strongly associated with gastroenteritis. Thus, both TDH and TRH are considered important virulence factors of *V. parahaemolyticus*¹⁰. One or both of these genes are detected in most clinical strains of

V. parahaemolyticus but are uncommon (about 1%) in environmental and food isolates. Since 1996, an increased incidence of gastroenteritis in many parts of the world has been associated with *V. parahaemolyticus* serotype O3:K6¹¹⁻¹². The association of this "new" O3:K6 serotype with large-scale food-borne disease outbreaks in Taiwan, Laos, Japan, Thailand, Korea and the United States between 1997 and 1998 suggest these organisms may have an unusual capacity to be transmitted by foods and/or to cause human infection and has pandemic potential¹³.

In Bangladesh, *V. parahaemolyticus* was identified as the etiologic agent in 3% of adult cases of dysentery¹⁵. Strains of different serogroups having genetic markers for the serogroup O3:K6 of *V. parahaemolyticus* were reported to have been isolated from hospitalized gastroenteritis patients in the Dhaka city of Bangladesh. Recent outbreaks of O3:K6 causing food poisoning in the sub-continent¹⁴, however, calls for scientific attention for environmental surveillance along the coastal regions as well as the fresh water environments of Bangladesh. Although it is a marine organism, very little is known about its pandemic spreading in the fresh water environment across Bangladesh. The present study was, therefore, undertaken to isolate and investigate the virulence potential, as well as phenotypic and genotypic traits of *V. parahaemolyticus* strains occurring in the fresh water environments around coastal southern parts of Bangladesh.

Materials and Methods

Sampling site and sampling method

This study on the isolation of *V. parahaemolyticus* was carried out for a period of six months (March to August, 2012) from the Bishkhali river located in the Barisal district. Samples were collected every three months from four different places of Bishkhali river and the salinity of the river water, which was found to be 0‰ in every time. Therefore, a total of 12 samples (water, sediment and water hyacinth) were collected each time from these sources.

Alkaline peptone water (APW) was used to facilitate the growth of the bacteria. For this, one ml of the sample (water, sediment or water hyacinth) was mixed with 9 ml of APW and was incubated at 37°C for 18 h. Ten fold serial dilution of the enriched samples were made and were subsequently streaked on to thiosulphate citrate bile salt sucrose (TCBS) agar plates. Following overnight incubation at 37°C, large, mucoid, raised, green colonies, were selected as suspected *V. parahaemolyticus* organisms.

Reference strain

V. parahaemolyticus strain A11 (new clone of serotype O3:K6, harboring *tdh* obtained from a patient in Aomori Prefecture in Japan in 2000) was used as a reference strain to compare the biochemical characteristics and gene analysis.

Identification of *V. parahaemolyticus*

Suspected isolates of *V. parahaemolyticus* were inoculated into triple sugar iron medium and nutrient agar media supplemented each with 0, 3, 6, 8 and 10% NaCl. Test strains showing alkaline slant and acid butt reactions in the triple sugar iron medium and no growth in 0% but growth in 3 to 8% NaCl, were identified as *V. parahaemolyticus*. The test strains were further confirmed by the presence of *toxR* gene. The reference *V. parahaemolyticus* strain A11 was used as the positive control in all the tests.

DNA template preparation

A single colony was picked and grown at 37°C for overnight in alkaline peptone water broth containing 3% NaCl. One ml of cell suspension was transferred to a microfuge tube and was centrifuged at 10,000 rpm for 5 min. After the supernatant was removed, the pellet was re-suspended in 1 ml of TE buffer and heated at 100°C for 5 min. After centrifugation at 10,000 rpm for 5 min, the supernatant was transferred to a new tube and was used as the template. The supernatant containing the genomic DNA was kept at -20°C before being assayed.

Detection of *toxR*, *tdh* and *trh* genes by Polymerase Chain reaction (PCR)

PCR detection of *toxR* gene in the purified genome DNA from bacterial isolates was performed with primer 1 (5'-AGCCCGCTTTTCTTCAGACATC-3') and primer 2 (5'-AACGAGTCTTCTGCATGGT-3'), D3 primer (5'-CCACTACCACTCTCATATGC-3') and D5 primer

(5'-GGTACTAAATGGCTGACATC-3') for *tdh* gene and R2 primer (5'-GGCTCAAAAATGGTTAAGCG-3') and R6 primer (5'-CATTTCCGCTCTCATATGC-3') for *trh* (*trh1*, *trh2*) gene. The resultant amplicons were 399 bp, 251 bp and 250 bp for *toxR*, *tdh* and *trh* genes respectively. The PCR mixture (25 µl) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01 mM EDTA, 0.1 mM dithiothreitol, 0.05% Tween 20, 0.05% Nonidet P-40, 5% glycerol, 0.2 mM each of the four deoxynucleoside triphosphates (dNTP mixture; Takara, Ohtsu, Japan), and 0.5 U of *Taq* polymerase (Takara Ex *Taq*; Takara, Japan). The amplification conditions were set at one cycle of 94°C for 5 min, followed by 35 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by one cycle of 72°C for 7 min. The PCR products were separated by electrophoresis in a 2% agarose gel, stained with ethidium bromide and recorded using a gel documentation system (Bio Rad, USA).

Results and Discussion

Identification of *V. parahaemolyticus* is usually achieved through a series of biochemical tests after their growth and isolation on a selective medium. Several specialized selective bacteriological culture media have been developed for isolating vibrios. CHROM agar is used as a selective medium for *V. parahaemolyticus*. *V. cholerae* and *V. alginolyticus* which ferment sucrose, produce yellow colonies on TCBS, while *V. parahaemolyticus* and *V. mimicus* being sucrose non-fermenter, grow as green colonies. It has been demonstrated that *toxR* gene, which has regulatory function in *V. parahaemolyticus*, is used for the specific identification *V. parahaemolyticus*.

On the TCBS agar plates, the suspected *V. parahaemolyticus* were found to be large, green, raised and mucoid colonies of approximately 2-3 mm in diameter. These separated colonies were then transferred to CHROM agar plate for further confirmation of the isolates as *V. parahaemolyticus*, where the colonies were found to be purple or violet colored. A total of 70 suspected colonies of *V. parahaemolyticus* were selected for biochemical tests.

The suspected 70 isolates were then subcultured onto alkaline peptone agar containing 3% NaCl and were subjected to extensive biochemical tests, like salt tolerance test and triple sugar iron test (TSI). Only fourteen isolates those showed growth on the NaCl concentration of 3%, 6%, 7% and 8% and could not grow in 0% and 10% were identified as *V. parahaemolyticus*¹⁶. The suspected fourteen isolates also showed the reactions similar to the positive control strain in the triple sugar iron tests and were re-confirmed as *V. parahaemolyticus*.

All the fourteen confirmed *V. parahaemolyticus* isolates showed positive results in PCR, using *toxR* specific primers and yielded DNA bands of 399 bp in agarose gel electrophoresis (Figure 1). However, all these isolates were found to be negative for the virulent *tdh* and *trh* genes.

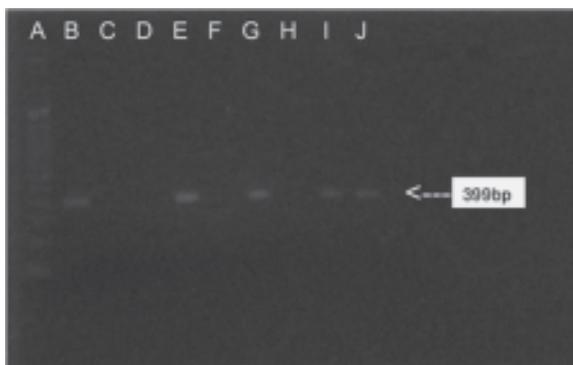


Figure 1. Agarose gel electrophoresis pattern of PCR amplicons of the seven confirmed *V. parahaemolyticus* isolates obtained with primers specific for *toxR*. Lane A, 100 bp DNA ladder; B to H, *V. parahaemolyticus* isolates; Lane I and J, positive control.

V. parahaemolyticus, a normal flora of the brackish and marine environment, has been considered as a common etiological agent of seafood-borne gastroenteritis and travelers' diarrhea in general¹⁻² and to some extent as extra intestinal infections⁵. The thermostable direct haemolysin (TDH) or Kanagawa phenomenon associated haemolysin has been considered as the major virulence factor due to the exclusive detection of TDH (encoded by *tdh* gene) among strains isolated from cases of gastroenteritis¹⁷⁻¹⁸. The *toxR* gene was found in *V. parahaemolyticus* and in other *Vibrio* species and its regulatory function was described²⁴. The *toxR* gene therefore appears to be well conserved among the *Vibrio* species.

In this study, all the fourteen confirmed *V. parahaemolyticus* isolates showed positive results in PCR using primer specific to *toxR* gene. However, none of these isolates gave positive results using primers specific to *trh* and *tdh* gene. This is also consistent with the biochemical results of the seven isolates, as none of the test isolates was found to be urease positive, which is thought to be directly correlated with the production of thermostable direct haemolysin related haemolysin (TRH) encoded by *trh* gene¹⁹⁻²⁰. This study only focuses strains isolated from Bishkhsli river of Barisal district, Bangladesh. Such an absence of virulent genes from the isolated strains of *V. parahaemolyticus* could therefore be correlated to their avirulent property. Previously this organism was isolated from water⁶, plankton²¹, and fish⁶ of freshwater environments. Miyamoto *et al.*, (1962) showed that *V. parahaemolyticus* could be isolated from seawater during the summer, but not during the winter²². Colwell (1973) reported that vibrios passed the winter in sediment and then are released from the bottom communities, attach to zooplanktons, proliferate as the temperature rises and the free *V. parahaemolyticus* in the water are released from plankton²³. However, it remains to be unknown how the numerous cells of *V. parahaemolyticus* present in the summer disappear when the water temperature drops.

Further studies can be designed to understand the factors contributing to the survival of *V. parahaemolyticus* in rivers of 0% salinity and to compare the clonal variation between clinical and environmental isolates belonging to the same serogroup. *V. parahaemolyticus* infections have been assumed to be of environmental origin, but little work has been done to confirm this assumption which needs further investigation.

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