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Pre-Enrichment of Estuarine and Fresh Water Environmental Samples with Sodium Chloride Yields in Better Recovery of *Vibrio parahaemolyticus*

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ABSTRACT

Vibrio parahaemolyticus organisms cause acute gastroenteritis in humans. These bacteria are natural inhabitants of both marine and estuarine ecosystems. In the present study, we investigated the effectiveness of a non-selective enrichment of sediment samples with sodium chloride prior to selective enrichment with alkaline peptone water for a better recovery of *V. parahaemolyticus*. Sediment samples were collected with or without 1% NaCl from the river Buriganga, located besides Dhaka city and about 400 km away from the Bay of Bengal, and from the estuary of the river Karnaphuli which flows into the Bay of Bengal. Very small number of *V. parahaemolyticus* (<30 MPN/g) were detected in the sediments of both river and estuary, where NaCl was not added. On the other hand, the number of *V. parahaemolyticus* increased to more than 40 times (1500 MPN/g) in the river and 32 times (960 MPN/g) in the estuary where NaCl were added. River sediment sample contained the serotype O9:K41 of *V. parahaemolyticus* and the estuarine sample contained O3:K41 and O3:KUT Our results suggest that a pre-enrichment of environmental samples with 1% NaCl helps *V. parahaemolyticus* to survive for at least 7 days until they are enriched with alkaline peptone water, for better recovery.

Keywords: *Vibrio parahaemolyticus*; Sodium Chloride; Estuary; Fresh Water

1. Introduction

Vibrio parahaemolyticus is a gram-negative, halophilic bacterium that inhabits in marine environments [1,2]. These organisms cause acute gastroenteritis in humans infected through the consumption of raw or inappropriately cooked sea foods [3]. Strains carrying the *tdh* gene, encoding the thermostable direct hemolysin (TDH), or the *trh* gene, encoding the TDH-related hemolysin (TRH), or both genes are considered as virulent strains, which can cause the gastroenteritis [2,4-6]. TDH elicits lethal activity in small experimental animals (*i.e.* production of vascular permeability in rabbit skin; cardiotoxicity; enterotoxicity when tested in the rabbit ileal loop model and mouse lethality) [7,8]. TDH is also cytotoxic to various cultured cells and is a pore-forming toxin. Its most extensively studied effect is the ability to cause hemolysis of erythrocytes from different mammalian species [9]. TRH is immunologically related but not identical to TDH. TRH stimulates fluid secretion in the rabbit ileal loop test which suggests a possible role for the toxin in inducing diarrhea [10]. TRH also shows increased rabbit skin vascular permeability and cardiotoxicity on cultured myocardial cells [11]. Moreover, both TDH and TRH induce chloride secretion in human colonic epithelial cells [12].

Many reports have been published on the isolation of *V. parahaemolyticus* from the environmental sources; most of them are marine or estuarine ecosystems, because of the organisms' indispensable requirement of NaCl for survival and growth [1,3]. Some researchers have also isolated *V. parahaemolyticus* from water, plankton or sediment samples of fresh water environments [13-15] which may suggest that the habitat of the halophilic *V. parahaemolyticus* could also be somewhere out of marine environment, where the requirement of NaCl for growth is probably substituted with some other compounds present in the fresh water [13,16].

Most of the environmental samples are selectively enriched first with alkaline peptone water (APW) and then plated on standard culture media for growth [17,18]. APW is the preferred enrichment medium for vibrios including *V. parahaemolyticus*. The high pH of the medium (pH close to 9) and NaCl concentration inhibit many other bacteria and favor vibrios [19]. The selective enrichment of the marine or estuarine environmental samples with APW has yielded in better recovery of *V.*

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parahaemolyticus [17,18,20]. The existing APW method has also been applied to detect *V. parahaemolyticus* in fresh water [20]. However, the isolation schemes have not been carefully studied yet and only enrichment in APW showed insignificant or low rate of isolation of *V. parahaemolyticus* from fresh water than marine water samples [21,22]. Also, a non selective enrichment of bacteria prior to selective enrichment was found to be more effective by researchers in detecting bacteria injured by various environmental stresses [23,24]. All these previous information made us interested to investigate the effectiveness, if any, of a non-selective enrichment of estuarine and fresh water environmental samples with sodium chloride for different periods of time prior to selective enrichment with APW for a better recovery of *V. parahaemolyticus* from those samples. This simple pre-enrichment with sodium chloride gave better recovery of *V. parahaemolyticus* from both estuarine and fresh water environmental samples, where the fresh water salinity was zero.

2. Materials and Methods

2.1. Study Area and Sample Collection

Sediment samples (approximately 100 g, 1 - 2 feet from the bank) were collected from two different sites. For fresh water environment, we collected samples from the river Buriganga, located by the side of Dhaka City, which is about 400 km away from the Bay of Bengal. Other samples were collected from the estuary of the river Karnaphuli, located in Chittagong District, which flows to the Bay of Bengal.

Temperature and salinity of each collection site were recorded. Each 100 g sediment sample was taken in plastic bags already containing 1 g of sodium chloride (NaCl) and mixed well, so that the final NaCl concentration of the sample becomes 1%. Control sediment samples were collected in bags containing no NaCl. All samples were kept at room temperature for 7 days until processed in the laboratory.

2.2. Estimation of *V. parahaemolyticus* in Sediment Samples

For the enumeration of *V. parahaemolyticus* in sediment samples, 3-tube 5-dilution Most Probable Number (MPN) method was used as described previously [25] with modification by Hara-Kuda *et al*., [26]. A 10 g of sediment sample was homogenized in 90 ml of APW (Nissui Co., Japan) in a stomacher bag and 10, 1, 0.1, 0.01 and 0.001 ml of the homogenate were inoculated into 10 ml of APW in triplicate and incubated at 35˚C to 37˚C for 18 h. One-milliliter portions of each APW culture were transferred into 10 ml of salt polymyxin broth (SPB; Nissui Co., Japan) and incubated at 35˚C to 37˚C for 18 h. Next, 1 ml of the SPB culture was inoculated into 10 ml of fresh SPB and incubated for 35˚C to 37˚C for 6 h. Then a loopful of final enrichment culture in SPB was plated onto CHROMagar™ *Vibrio* (CV) agar (CHRO-Magar, France). After incubation for 18 h at 37˚C, purple colonies on the CV agar plates were picked and subjected to standard biochemical tests for the identification of *V. parahaemolyticus*. The biochemical media used were triple sugar iron medium (TSI; Eiken Co, Tokyo, Japan), lysine-indole-motility medium (LIM; Nissui Co., Japan), and 1% of polypeptone broth (Nihon Pharmaceutical Co. Ltd., Japan) supplemented each with 0%, 3%, 6%, 8%, 10% NaCl. Test strains showing alkaline slant and acid butt reactions in the triple sugar iron medium, positive reactions for lysine decarboxylase production, indole production, motility in the LIM medium, and no growth in 0% but growth in 3% to 8% NaCl, were differentiated as *V. parahaemolyticus*. Finally, the test strains were identified by API-20E test strips (bioMériux, Inc.). Only tubes that confirmed the presence of *V. parahaemolyticus* were considered as positive tubes. The counts were determined from MPN table [25]. *V. parahaemolyticus* serotype O3:K6 was used as positive control to compare the biochemical characteristics of the test strains.

2.3. Serogrouping of the Isolated *V. parahaemolyticus* Strains

Serogrouping of the *V. parahaemolyticus* isolates was done using a commercially available *V. parahaemolyticus* antiserum test kit (Denka Seiken, Tokyo, Japan) by following the manufacturer's instructions. Briefly, the strains were first grown on APW agar containing 3% NaCl. Following overnight incubation at 37˚C, a loopful of inoculum was mixed with 1 ml normal saline. An aliquot of the cell suspension in normal saline was boiled for 2 h and used for serotyping, based on the O antigen used for serotyping, based on the O antigen. The remaining cell suspension (not boiled) was used for serotyping, based on the K antigen.

3. Results

3.1. Pre-Enrichment with NaCl and Quantitative Analysis of *V. parahaemolyticus* in Sediment Samples

In this study, we investigated the effectiveness of NaCl pre-enrichment of the estuarine and fresh water sediment samples prior to selective enrichment with APW for a better recovery of the *V. parahaemolyticus* from the sediment samples. **Table 1** shows the quantitative and qualitative recoveries of *V. parahaemolyticus* from both estuarine and fresh water sediment samples with or without pre-enrichment with NaCl. Sediment samples from Buriganga river, where the salinity is zero, showed promis-

ing results. The river sediment sample, which was pre-enriched with 1% NaCl, showed much higher MPN value on day 7 (MPN value 1500), when compared with the corresponding control sediment sample, without any NaCl pre-enrichment. Similar results were also found for the estuary samples, where the salinity was 2.0. Estuarine sediment sample with NaCl pre-enrichment produced an MPN value of 930 on day 7, where as the corresponding control sediment without any pre-enrichment produced only an MPN value of <30. All these results clearly indicate that a pre-enrichment with sodium chloride has profound effect in long time (at least up to 7 days) survival of the organisms in sediment samples of both estuary and river followed by enrichment in APW and recovery.

3.2. Serotype Distribution of *V. parahaemolyticus*

V. parahaemolyticus was isolated from sediment samples of upstream of river Buringanga and estuary of river Karnaphuli. Pre-enrichment followed by selective enrichement and inoculation on chromogenic agar medium were found to be reliable methods for isolation of *V. parahaemolyticus* from sediment samples. The two sampling sites examined differed for the isolated serotypes. The serotype O9:K41 was restricted in the river Buriganga whereas O3:K41 and O3:K un-typeable (KUT) were restricted in estuary of river Karnaphuli.

4. Discussion

Toxigenic *V. cholerae* O1 bacteria have been found to survive for extended period in water containing no nutrients [27]. During the reduced nutrient levels, such as those encountered in aquatic environments, *V. cholerae* O1 undergo physiological and morphological changes [28,29]. These changes may be related to viable but not culturable (VBNC) form of *V. cholerae* O1, which is induced by nutrient deficient environment [30]. In this state, the bacteria retain pathogenicity but their metabolic activities become minimal and they cannot be cultured on standard cultural media [31]. When the growth conditions become more favorable, the organisms can be readily revived [32]. This is probably why we enrich the environmental samples before we put them into standard cultural media for growth. Again, this might depend on the *Vibrio* species and salinity of the environment from where the organisms are isolated. It has been found that, *V. parahaemolyticus*, *V. alginolyticus* and *V. fluvialis*, species that tolerate higher salt concentrations are isolated from sites with high salinity. On the other hand, the study sites with much lower salinity, yielded almost exclusively *V. cholerae*, the species which prefer lower salinity [33]. Therefore, for *V. parahaemolyticus*, we need to take extra care in the initial isolation, especially when the sampling site is a fresh water environment and a pre-enrichment with sodium chloride probably helped them survive in the environment out of its natural habitat.

Recently, Schets *et al*., [20] reported the growth of different environmental *Vibrio* species using a culture method that included enrichments of the samples in alkaline buffered peptone water (ABPW) prior to cultivation on thiosulphate citrate bile sucrose (TCBS) agar. Enrichment steps included incubation in ABPW or ABPW without NaCl, at different temperatures and time periods. It was found that *V. parahaemolyticus* could only be isolated from enrichment culture and not from those grown in ABPW without sodium chloride. Our results support this recent findings and additionally suggest that a pre-enrichment of environmental samples with 1% NaCl helps the *V. parahaemolyticus* to survive for at least 7 days until they are enriched with alkaline peptone water in the laboratory for further growth.

In a previous study, we compared the survivability of the *V. cholerae* O1 organisms in artificial microcosms under different stressed conditions, such as salinity, pH and temperature, for different periods of time [34]. It was found that at 0% salinity, neutral pH and at 37˚C, *V. cholerae* O1 El-Tor Ogawa biotype undergoes to VBNC state 5 days after the incubation period and could not be grown on standard culture media. In this study, we also observed similar results. The Buriganga river sediment sample, without any pre-enrichment with NaCl, obtained an MPN value of <30 after 7 days (**Table 1**). Here the cells had probably undergone to the VBNC state and could not be recovered on culture plates. However, the same sample, when pre-enriched with 1% NaCl, showed an MPN value of 1500 after 7 days of incubation at room temperature. All these data strongly suggest that a 1% NaCl pre-enrichment of the environmental samples, both estuarine and fresh water, helps the *V. parahaemolyticus* to survive and can later be cultivated on standard culture media. In addition, it was found that river sediment sample contained the serotype O9:K41 of *V. parahaemolyticus* and the estuarine sample contained O3:K41 and O3:KUT. We are now investigating other environmental factors, if any, which help the *V. parahaemolyticus* to

Table 1. Quantitative and qualitative recoveries of *V. parahaemolyticus* from estuarine and fresh water sediment samples with or without pre-enrichment with sodium chloride.

Sample site	Pre-enrichment with NaCl	MPN/g sediment on day 7	Serotype
Buriganga river	+	1500	O9:K41
	-	<30	O9:K41
Karnaphuli estuary	+	930	O3:K41 O3:KUT
	-	<30	None

+done; -not done.

survive in stressed environmental conditions.

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