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Original Article

Role of Chitin for Harbouring of Toxigenic *Vibrio cholerae* O1 El Tor in Aquatic Environment

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***Vibrio cholerae*, the etiological agent of cholera is an autochthonous bacterium of aquatic environment, often found attached to crustacean zooplankton. Chitin is the main component of crustacean exoskeleton, an insoluble polysaccharide. *V. cholerae* can secrete chitinase enzymes, which can facilitate the bacterium's association with chitinous aquatic organisms to utilize it as a potential nutrient source. In the present study, we checked the role of chitin for long term survival of *V. cholerae*. Laboratory based microcosms were prepared with purified artificial chitin chips and a toxigenic strain of *V. cholerae* O1 El Tor. In the presence of chitin, *V. cholerae* was found in a higher cellular density for >61 days in culturable condition than control water. We observed that the older chitin chips gradually thinner which indicated that *V. cholerae* utilized chitin. PCR experiment confirmed the presence of the bacterium's cholera toxin coding gene (*ctxA*) and membrane associated virulent gene *ompU* remained unaltered. Finally, from randomly amplified polymorphic DNA (RAPD) analysis no significant genotypic variation was detected in the bacterium's genome after its long time association with chitin.**

Keywords: *Vibrio cholerae*, Chitin, Polymerase chain reaction (PCR), Microcosm

Introduction

Cholera is acute, potentially epidemic and life-threatening secretory diarrhoea characterized by voluminous watery stools. The causative agent of cholera is *Vibrio cholerae*, a Gram-negative motile bacterium, is a natural inhabitant of aquatic ecosystem[1]. Out of more than 200 known serogroups of the bacterium, only the O1 and O139 serogroups have been found to be associated with epidemic cholera. Cholera is endemic or epidemic in areas with poor sanitation, it occurs sporadically or as limited outbreaks in developed countries.

Chitin is an insoluble linear β-1, 4-linked polymer of *N*-acetyl D-glucosamine (GlcNAc) and is the second most abundant polymer after cellulose in nature. This polysaccharide is particularly an important nutrient source for maintaining the ecosystem in the aquatic environment[2]. Each year, 100 billion tons of discarded crustacean shell sink through the world's oceans – more than a billion tons of this have been moulted by copepods alone[3], the rest from shrimp, crabs and multitudes of other crustaceans and some diatoms, which are spread over wide geographic and spatial ranges in rivers, estuaries and oceans[4]. A major component of these shells is chitin. In aquatic environment *Vibrio cholerae* may persist in association with chitinous exoskeleton of aquatic crustacean copepods[5-8] as well as other aquatic crab[9], shrimp[10] and prawn[11]. Many *Vibrio* species are able to use chitin as a sole carbon and nitrogen source[1]. Marine bacteria including *V. cholerae* possess complex signal transduction systems for finding chitin, adhering to chitinaceous substrata, degrading it to oligosaccharides, transporting the oligosaccharides to the cytoplasm, and catabolizing the transport products to fructose-6-P, acetate and NH_3[3].

In the present investigation, we prepared microcosm with natural water, artificial chitin chips and inoculated *V. cholerae* to observe their growth on chitin chips. Toxigenic *V. cholerae* is extremely difficult to isolate from the aquatic sources and generally not found through out the year except cholera seasons. Moreover there is no quantitative estimation of *V. cholerae* survival on artificial chitin chips that can be used as bait to detect toxigenic *V. cholerae* in the environment. Recently, scientists have discovered that *ctx* is basically a part of genome of phase called *ctxö* and is a transmissible element in aquatic environment. Therefore, we also observed whether during their long term association with chitin *V. cholerae* do not loose its toxigenic gene *ctx* and whether there is any major change in their genome by molecular techniques.

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Materials & Methods

Collection of strain

A recently isolated strain of *Vibrio cholerae* O1 El Tor (1780) from culture collection of Environmental Microbiology Laboratory of ICDDR,B was used in this study. Identification of the strain was confirmed by a series of biochemical tests and serotyping[12]. After confirmation biochemically and serologically, remaining portion of the isolated colony was streaked onto a gelatin agar (GA) plate and grown overnight at 37°C to get a pure culture.

Sources and preparation of water

The natural fresh water for microcosm preparation was collected from Dhonagodha River (a branch of Meghna River) of Matlab, a cholera endemic area of Bangladesh. The river water was then filtered through 0.22 µm Millipore filter and used to the microcosm.

Preparation of microcosms

Laboratory microcosms were prepared consisting of a natural water [with pH 7.7, temperature 25°C, salinity 0.1 ppt, dissolved oxygen (DO) 6.96 mg/l, total dry solid (TDS) 55.5 mg/l and conductivity 116.8 µS/cm] in Erlenmeyer flasks. Erlenmeyer flasks (500 ml) were cleaned with acid hydrochloride and rinsed 10 times with double-distilled, deionized water. For each microcosm experiment the selective strain of *V. cholerae* O1 El Tor (1780) was added in two separate Erlenmeyer flasks, each of which contains 200 ml of stock water at room temperature. In the first flask of this setup 200 ml of the stock water was used as control water and in the second flask 12 pieces of autoclaved artificial chitin chips (each of which 1 g in weight) were added.

Preparation of inoculum

A loopful growth of fresh cultured *V. cholerae* O1 El Tor from the GA plate was re-suspended in LB brought and incubate at 37°C for about 6 h. The 90% transmittance of the suspension at 585 nm wave length was measured with spectrophotometer and the number of cells per ml had been assessed by using drop plate method as described by Hoben and Somasegoran[13] to ensure about 10^7 colony forming unit (CFU)/ml. Then a ten-fold dilution was prepared by using phosphate buffer saline (PBS) pH 8.4. Then 2 ml of diluted inoculum (PBS) of *V. cholerae* O1 El Tor was added by a pipette in each microcosm flask so that the final concentration of the strain would be 10^4 CFU/ml in each microcosm and then monitoring the number of *V. cholerae* O1 by bacteriological culture method.

Processing of samples

Samples processing were started within 5 min after added the inoculum and this was considered as 'zero hour' sampling or reading. Subsequently, sampling was carried out at different time intervals (0 h, 4^{th} h, 8^{th} h, 24^{th} h (1^{st} day), 2^{nd} day, 4^{th} day, 8^{th} day, 15^{th} day, 25^{th} day, 40^{th} day and 61^{st} day) until all the chitin chips were fully finished. Three types of samples were examined, water from the control flask (CW), chitin chips (CC) and surrounding water of chitin chips (MW). Water (100 ml) was taken from CW and MW in separate Eppendorf tube by separate micropipette and one chitin chip was taken in a Petri dish and homogenized with 2 ml PBS. A series of ten fold dilutions were prepared separately for each sample by PBS. The dilutions were properly mixed by a vortexer and 25 ml portion from each serial dilution were inoculated onto 2 to 3 TTGA plates in a duplicate fashion, using drop plate technique and incubated at 37°C for 18-24 h for counting of the colonies of *V. cholerae* O1.

Counting procedure for Vibrio cholerae O1

After incubation, one colony from the resulted growth in each plate was tested by serological methods[12] for confirmation of the serotype. Bacterial counts were derived from the counts of individual colony and were expressed as colony forming unit (CFU)/ml or g. The arithmetic mean was calculated from the colony counts of duplicate plates.

DNA isolation

A single colony from pure culture of each sample was taken in 300 ml of autoclaved deionized water containing Eppendorf tube and mixed vigorously. Then the samples were subsequently heated in boiling water for 10 min. The samples were then cooled in ice for 10 min and followed by centrifuged at 12,000 rpm for 1 min. The supernatants were used as template for the RAPD and PCR of *ctxA* and *ompU* genes.

PCR assay for ctxA gene

Selected primers and PCR conditions for *ctxA* gene according to Singh *et al.*[14] were forward 5'-CGGGCAGATTCTAGACCTCCTG-3', reverse 5'-CGATGATCTTGGAGCATTCCCAC-3'. PCR amplification of the target DNA was carried out in a thermal cycler with a reaction mixture volume of 25 µl. Each of the reaction mixtures contained 2.5 µl of 10x PCR amplification buffer (Invitrogen, USA), 1.0 µl of $MgCl_2$ (50 m*M*), 1 µl each of 10 m*M* dNTP (Invitrogen, USA), 1.5 µl each of the forward and reverse primers for *ctxA*, 0.25 µl of *Taq* DNA polymerase at 5 U/µl (Invitrogen, USA), and Milli-Q water to a final volume of 23.5 µl, and 1.5 µl of cell lysate (template DNA). PCR condition was as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of 94°C for 1 min (denaturation), 60°C for 1 min (primer annealing), and 72°C for 1 min (chain extension), and a final extension at 72°C for 10 min.

PCR assay for ompU gene

Selected primers and PCR conditions for *ompU* gene[14] were forward 5'-ACGCTGACGGAATCAACCAAAG-3', reverse 5'-GCGGAAGTTTGGCTTG AAGTAG-3'. PCR amplification of the target DNA was carried out in a thermal cycler with a reaction mixture volume of 25 µl. Each of the reaction mixtures contained 2.5 µl of 10x PCR amplification buffer (Invitrogen, USA), 1.5 µl of $MgCl_2$ (50 m*M*), 1 µl each of 10 m*M* dNTP (Invitrogen, USA), 1.0 µl each of the forward and reverse primers for *ompU*, 0.25 µl of *Taq* DNA polymerase at 5 U/µl (Invitrogen, USA), and Milli-Q water to a final volume of 24 µl, and 1.0 µl of cell lysate (template DNA).

PCR condition was as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles consisting of 94°C for 1 min 20 sec (denaturation), 60°C for 1 min 20 sec (primer annealing), and 72°C for 2 min (chain extension), and a final extension at 72°C for 10 min. Amplified gene detection by agarose gel electrophoresis were detected described elsewhere[14].

Randomly amplified polymorphic DNA (RAPD)

Primer 5'-AACGCGCAAC-3' was selected for RAPD analyses of the inoculated *V. cholerae* O1 strain at different time intervals of microcosms. PCR amplification of the target DNA was carried out in a thermal cycler with a reaction mixture volume of 25 µl. Each of the reaction mixtures contained 2.5 µl of 10x PCR amplification buffer (Invitrogen, USA), 1.5 µl of $MgCl_2$ (50 m*M*), 2.5 µl each of 10 m*M* dNTP (Invitrogen, USA), 2.0 µl of the universal random primer, 0.25 µl of *Taq* DNA polymerase at 5 U/µl (Invitrogen, USA), and Milli-Q water to a final volume of 24 µl, and 1.0 µl of cell lysate (template DNA). PCR was programmed as follows: an initial denaturation at 95°C for 5 min, followed by 45 cycles consisting of 94°C for 1 min 30 sec (denaturation), 36°C for 1 min 30 sec (primer annealing), and 72°C for 2 min 30 sec (chain extension), and a final extension at 72°C for 7 min. The amplification of the desired polymorphic DNA through RAPD was examined by resolving the RAPD product in 2% agarose gel. During gel electrophoresis a molecular weight marker 100 bp (*Hae*III digested Øx174 RF DNA, Gibco-BRL, USA) was used as standards to compare the amplicon size of the PCR products.

Results and Discussion

Cholera is a dreadful diarrhoeal disease from the time immemorial in Bangladesh and other countries of the world. *Vibrio cholerae* is considered as most frightening agent that causes cholera globally. This global impact occurs since *V. cholerae* has been regarded as a member of a group of organisms whose major habitats are aquatic ecosystem[15-17]. *V. cholerae* is free-living in surfaces of freshwater, oceanic and estuarine environment. In aquatic environment it may persist in associations with chitinous exoskeleton of aquatic crustacean copepods[5], crabs[9], prawns[11], shrimps[10] and lobsters. Through this aquatic environment *V. cholerae* take the opportunity to flash out all over the world and threatening the different human communities.

The results of the microcosm study were observed at different time intervals until the chitin chips were completely finished by the bacteria. Microcosms were protected from any kind of aerobic or other contaminations and then carefully observed the number of *V. cholerae* O1 by direct culturable count. The result of cultarable counts (Table 1) clearly showed that initially *V. cholerae* O1 could not attach chitin chips but after a few hours they begin to attach with chitin. In the 2nd and 4th day cellular density of *V. cholerae* O1 increased rapidly and their growth rates are comparatively higher than control water and the surrounding water of chitin chips. Initially the artificial chitin chips were hard, insoluble and floating on water but after few days (4th day) they begun to sink down under water and soften. Initially, culturabale counts of *V. cholerae* O1 were increased rapidly which may be due to the fact that some dissolved nutrients were present within the filtered river water of both microcosm but when the nutrient was finished then bacterial density of the water decreased slowly. On the 8th day the number of *V. cholerae* O1 attached to chitin reached the highest position (9.594×10^6/g) than other two water sources and on the 8th day the cultarable count of control water as well as surrounding water of chitin began to decrease. On the 15th, 25th and 40th day the observation showed that the result of cultarable counts of chitin were still highest in comparison to other two sources and it was also observed that the culturable counts of *V. cholerae* O1 from surrounding water of chitin (MW) were higher than control water (CW). A possible reason was that after 15th day the chitin chips were dissolved in the water (MW) because of bacterial enzymatic action and thus some chitinous nutrients were present in dissolved condition in the surrounding water of chitin (MW). On the 15th, 25th, 40th and 61st day a dense layer of bacterial biofilm was found in each piece of chitin chips which completely coated them. On the 61st day the last piece of the chitin chip, control water and the water surrounding of chitin chips were processed and the result showed that cultarable *V. cholerae* O1 were still present only within the chitin chip (CC) but absent in control water (CW) and the water surrounding of chitin chips (MW). It was also observed that the cultarable counts of *V. cholerae* O1 found from chitin chip on the 61st day were higher than the inoculums, which were added to the microcosms at the initial stage of the experiment (0-hour reading).

Table1. *Direct counts of culturable Vibrio cholerae O1 at different time intervals of microcosms*

Time interval	Count of *culturable Vibrio cholerae* O1		
	Control water (CFU/ml)	Microcosm water (CFU/ml)	Chitin chips (CFU/g)
0 h	2.93×10^3	2.877×10^3	Nil
4 h	1.274×10^4	4.466×10^3	2.95×10^2
8 h	1.122×10^4	1.096×10^4	2.39×10^2
24 h	3.953×10^5	5.357×10^5	2.999×10^2
2nd day	5.211×10^6	6.637×10^6	7.947×10^4
4th day	5.395×10^6	2.754×10^6	8.729×10^5
8th day	2.6×10^6	1.757×10^6	9.594×10^6
15th day	1.22×10^3	5.236×10^5	8.871×10^6
25th day	29.99	5.47×10^5	1.548×10^7
40th day	1.3	1.279×10^4	5.508×10^6
61st day	Nil	Nil	7.194×10^4

The result of polymerase chain reaction (PCR), targeting the *ctx A* and *ompU* genes were obtained from gel electrophoresis (Figure 1 and 2 respectively) the amplicon size for *ctxA* gene and *ompU* gene were 564 bp and 869 bp respectively. It was observed that toxigenic genes (*ctxA* and *ompU*) were present in the experimental strain of *V. cholerae* O1 El Tor and there were no significant changes occurred of these genes. The result of RAPD (Figure 3) showed that there were no significant genotypic changes occurred

within the polymorphic DNA sequences of the bacterium's genome, collected from different (nutritional condition and time intervals) microcosms. So from this result of cultarable counts of *V. cholerae* O1 of the microcosm experiment it is clear that chitin play a great role as a source of nutrient, which increase the survival potentiality *V. cholerae* O1 in normal aquatic condition without affecting their toxin producing gene as well as their genotypes and the present findings of microcosm experiment exhibits similarity with that of the previous respected studies[5-8].

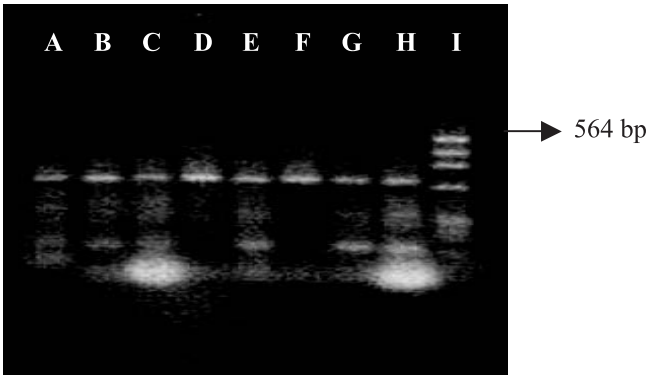

Figure 1. *Agarose gel electrophoresis of ctxA PCR product. CW = Control water; MW = Microcosm water; Lane A: CW-0-h, Lane B: CW 40th day, Lane C: MW-0-h, Lane D: MW 40th day, Lane E: CC 8th h, Lane F: 15th day, Lane G: CC 40th day, Lane H: 61st day, and Lane I = Marker DNA.*

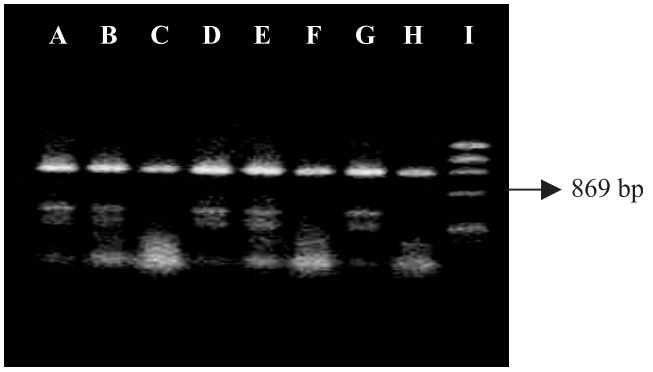

Figure 2. *Agarose gel electrophoresis of ompU PCR product. CW = Control water; MW = Microcosm water; Lane A: CW-0-h, Lane B: CW 40th day, Lane C: MW-0-h, Lane D: MW 40th day, Lane E: CC 8th h, Lane F: 15th day, Lane G: CC 40th day, Lane H: 61st day, and Lane I: Marker DNA.*

Several researchers thought that chitin is a principal component for reservoir of *V. cholerae* and hence they tried to prove that the relationships between *V. cholerae* and chitin by different artificial condition such as mutation on various chitinase gene[18], changing the pH[19], or temperature[20], and isolation, characterization, molecular cloning and sequencing[21-23] of different types of chitinase genes from several *Vibrio* spp. including *V. cholerae*. Most of the research was conducted by using liquid chitin but in

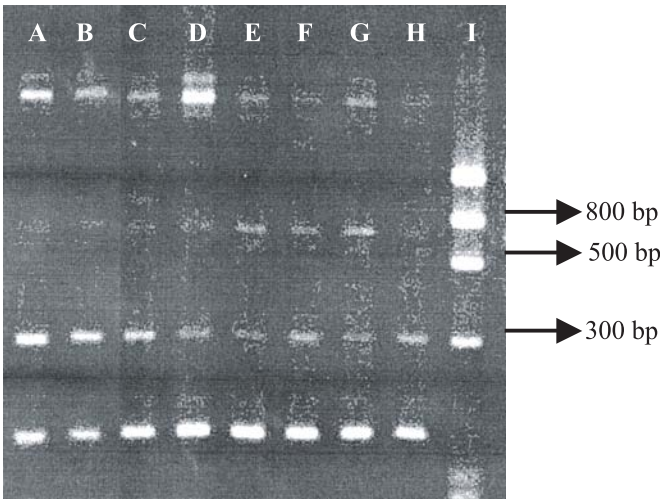

Figure 3. *Agarose gel electrophoresis of RAPD PCR product. CW = Control water; MW = Microcosm water; Lane A: CW-0-h, Lane B: CW 40th day, Lane C: MW-0-h, Lane D: MW 40th day, Lane E: CC 8th h, Lane F: 15th day, Lane G: CC 40th day, Lane H: 61st day, and Lane I: Marker DNA.*

the present study solid artificial chitin chips were used. The result of microcosm experiment in the present study showed that *V. cholerae* could utilize chitin chips as their potential nutrient in a simpler way in normal aquatic condition.

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