

Persistence of *Vibrio mimicus* During Inter-epidemic Period Associated with *Anabaena variabilis*

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ABSTRACT--- *Vibrio mimicus*, the causative agent of diarrhea, is one of the major public health issues in low-income countries like Bangladesh. Epidemiological studies of diarrhea in Bangladesh have demonstrated that surface-water sources can act as foci of infection. The present investigation was aimed to determine the role of blue green alga, *Anabaena variabilis* in the survival of *Vibrio mimicus* in laboratory microcosms. Survival of culturable *V. mimicus* in microcosms was monitored using drop plate method on TTGA plate. Viable but nonculturable (VBNC) *V. mimicus* were detected using fluorescent antibody (FA) and PCR techniques. *V. mimicus* was detected in association with *A. variabilis* for up to 10 days in a culturable state; whereas in algal water and control water the bacteria remained culturable state upto 5 days and 3 days respectively. The VBNC state of *V. mimicus* in association with *A. variabilis* was detected up to 60 days in microcosms. However, the bacteria were unable to detect after 25 days in algal water and after 12 days in control water. The VBNC state of *V. mimicus* was detected in the microcosms by PCR technique taken sample at different time interval. Both the fluorescent antibody technique and PCR results revealed that *V. mimicus* can survive longer period (as VBNC) in association with *A. variabilis* compared to control and algal water. The results suggested that, *A. variabilis* may act as a reservoir of *V. mimicus* in an aquatic environment. In the present study, pH was lower in case of algal water than control water. It may cause the hydrogen ion secretion by *A. variabilis*. The salinity of algal water was increased compare to control water in the present study. *A. variabilis* may excrete various salts during the association with *V. mimicus*. Further epidemiological study is required to trace its ecological niche.

Keywords--- *Vibrio mimicus*, inter epidemic period, persistence, *Anabaena variabilis*.

1. INTRODUCTION

Vibrio mimicus is an important member of vibrios which was first proposed to encompass biochemically atypical non O1 *V. cholerae* isolates [1]. *V. mimicus* has been implicated as the causative agent of diarrhea, gastroenteritis and various other infections [2]. *V. mimicus* is a variant of *Vibrio cholerae* or a separate species has been the subject of taxonomic controversy. Five to ten percent of all diarrhea cases can be linked to *V. mimicus* infection, which is two-thirds of all cases deaths occur in children younger than 5 years [3]. *V. mimicus* is most common in developing countries where there is poor hygiene and limited clean drinking water.

V. mimicus carries virulence associated genes namely cholera toxin (CT) as well as toxin co-regulated pilus (TCP) genes [4]. They share somatic antigens, that virulence related genes are associated with sporadic and epidemic diarrhea [5]. Recent horizontal transfer of CTX Φ and the VPI Φ between clinical strains of *V. mimicus* and *V. cholerae* has been reported [6]. *ctxA* and *tcpA* genes reside in the CTX Φ and VPI Φ respectively. This suggests that *V. mimicus* can serve as a reservoir of these bacteriophages from where these phages can transmit to other vibrios such as non O1 *V. cholerae*.

Diarrhea epidemics occur twice a year in Bangladesh, with a clear seasonal pattern [7]. During epidemics *Vibrio cholerae* O1 and O139 and *V. mimicus* have been isolated from patients as well as from surface water but disappear between inter epidemic period [8, 9]. So mechanism of maintenance of diarrhea seasonality and endemicity remains an enigma to aquatic flora and fauna may act as possible reservoirs of diarrhea in endemic areas [10]. From this evidence, it can be said that aquatic flora and fauna may act as possible reservoirs of *V. mimicus* in endemic areas.

Aquatic flora are involved in the transmission and reservoir mechanism of the *V. cholerae*[10] Vibrios were isolated from two marine algae, *Polysiphonialanosa* and *Ascophyllumnodosum*. A copious amount of extracellular products produced by marine algae, which contains peptides amides and free nitrogen[11, 12] that attracted vibrios on algal surfaces. Laycock[13] found vibrios associated with brown algae *Laminarialongicruris*. He observed that isolated vibrios could hydrolyse laminaran (an extracellular product of *Laminariaspllnza*; one red algae *Porphyrasuborbiculata*; and brown algae *Eiseniabicyclis*. Islam *et al.*[14] studied the survival and attachment of toxigenic *Vibrio cholerae* O1 to four marine algae, e.g. *Ulvalactuca*, *Enteromorpha intestinalis*, *Ceramium rubrum*, and *Polysiphonialanosa* in artificial aquatic ecosystems (Laboratory microcosm). Bacteria can provide the algae with CO₂, vitamins, growth factors, and buffering' capacity. Algae can supply bacteria with oxygen and extracellular products that can serve as organic substrates for growth. A similar relationship may exist between the blue-green algae named *Anabaena spp.* and *V. cholerae* O1 [15, 16] *V. cholerae* produces mucinase, an enzyme that degrades mucin and mucin-like substanc.). Shiba *et al.*[17] isolated vibrios from two green algae *Monostromanitidum* and *Enteromorpha* that are often encountered in the gut. Although clearly involved in intestinal colonization during the infection process, this enzyme can also degrade similar substances in plants and algae [18]. Mucinase is a soluble haemagglutinin protease, which may be important for the survival of *Vibrio cholerae* in association with mucilaginous blue-green algae (cyanobacteria) [19]. The association between blue green algae and bacterium during a bloom is also reported [20]

2. METHODS AND MATERIALS

In two sets of experiments, in the survival study were similar in design and include the addition of *V. mimicus* (10⁷ cells/ml) and 1 gm (wet weight) of *A. variabilis* in 100 ml of microcosm water for study flask and the addition of *V. mimicus* (10⁷ cells/ml) in 100 ml of microcosm water for control flask. The culturable count of *V. mimicus* on control water, algal water (water on which algae remain floating) and in association with algae were observed for a defined period of time and the technique .

Preparation of microcosm water

2 mg of NaCl was added to 2000 ml of distilled water to make 0.1% (or 1 ppt) salinity of this water which checked with digital salinity meter. Then the pH adjusted to 8 and autoclaved. After autoclaving and cooling, pH of the water adjusted for several times until it fixed to ~8. Then the water was used in experimental microcosm.

Cultivation of blue green algae

A. variabilis was grown in 250 ml conical flasks in a standard mineral medium, BG11[21] in the algal growth room of Dept. of Botany, University of Dhaka and maintained as pure culture. The temperature, humidity, and light intensity of the growth room were 27.5°C, 20%, 3670-6480 lux, respectively.

Preparation of bacterial inoculums

V. mimicus strains were grown on GA (Difco, USA) plates at 37°C for 18–24 h. A loopful of growth was suspended in 7 ml of 0.1% NaCl (pH 7.0) and the 90% transmittance of the suspension at 585 nm was measured with a Colman Junior spectrophotometer (The Perkin Elmer Corporation, Norwalk, Conn., USA). Concentration of the cell suspension was adjusted to 10⁷ cells/ml. Then the number of cells per milliliter was assessed by viable counts on GA plate using the drop-plate method [22]

Experimental condition

Here the experimental conditions were pH 8 in microcosm water and time intervals were 0 hr, 2hr, 4hr, 8hr, 12hr, 24hr and so on. The experiment was performed at room temperature (~25°C) and the plates were incubated at 37°C for 18 to 24 hr.

Preparation of experimental microcosm

1ml of inoculum was added in 100 ml of microcosm water (pH 8) in 4 flasks. Here, four microcosms were prepared and blue green algae were added in two duplicate flasks (study flask) and other two flasks were without blue green algae (control flask). Sampling were started within 30 min, after the addition of blue green algae. This was taken as 0hr

sampling. Subsequently sampling was carried out after interval of 2 hours from the starting time and to period of non-culturable stage. The flasks were stored at room temperature (25°C).

Processing of control water sample

After proper shaking 100 µl of water in an eppendorff tube by a micropipette from control microcosm (first flask) prepared for each set and tenfold serial dilution were prepared in normal saline (0.85% NaCl, pH 8). The dilution was properly mixed by a mistral mixer (Lab line) and 25 µl portions of these dilutions were inoculated by a micropipette onto TTGA and GA plates in duplicate by drop plate method. The plates were then incubated at 37°C for 18-24 hours for counting the colonies of bacteria.

Processing of water sample where blue green algae were floating

100 µl of water was taken by a micropipette. Four fold serial dilutions *A. variabilis* sample were made in normal saline. 25 µl portion from these dilutions were served on TTGA and GA plates in duplicate using drop plate technique and incubated at 37°C for 18-24 hours for counting the colonies of bacteria.

Processing of blue green algae from microcosm

1 ml algaewas taken from different microcosm water by a micropipette with the cut tips from the microcosm flasks. While *Anabaena* was washed in eppendorf tube using 0.1% normal saline and centrifuging at <5000 rpm and then crashed in homogenize tube. Then 100 µl of the processed sample was taken into an eppendorf tube, tenfold serial dilution were prepared using normal saline and 25 µl portions from the dilutions were served on TTGA and GA plates by drop plate technique. The plates were then incubated at 37°C for 18-24 hours for counting the colonies of bacteria.

Enumeration of *V. mimicus* by conventional culture technique

Survival of *V. mimicus* was assessed by culturable counts on tarurocholatelurite gelatin agar (TTGA) media. For algal sample, 4 fold dilutions of the homogenate were prepared and 25 µl from each dilution was inoculated on to duplicate plates of TTGA by the drop plate technique [22]The plates were then incubated at 37°C for 18-24 hours. The collected algal water sample was similarly diluted and spread on to duplicate plates of TTGA. The plates were incubated at 37°C for 18-24 hours. After incubation, the counts of *V. mimicus* were derived from the number of individual colonies and expressed as CFU/ml or gm. The counting of test suspension was discontinued after failure to recover *V. mimicus* after 10 days sampling following enrichment technique. Bacterial counts were derived from the counts of individual colonies and expressed as colony forming unit (CFU) per ml or gm. The arithmetic mean was calculated from the colony counts of duplicate plates

Detection of non-culturable state of *V. mimicus* in the microcosms

After some days, no *V. mimicus* colonies could be detected by culture method on GA and TTGA plates, even pellet from centrifuged 1 ml sample (taken in an eppendorff tube) did not produce any characteristic colony. When there was no growth of organism, it was considered that the cells have been transformed into non-culturable state. Then the existence of *V. mimicus* was examined by fluorescent microscopy.

Sample preparation for fluorescent antibody (FA) technique

When the cells of *V. mimicus* failed to grow on GA plates, then they were thought to enter into the non-culturable state. The viable but non culturable (VBNC) state of *V. mimicus* was assessed at different time intervals after inoculation. 1 ml water, each from study and control flasks was used for testing the viability of non culturable cells of *V. mimicus*. In case of algal sample, 50 µg of biomass was placed in 1 ml of 0.1% NaCl solution and homogenized manually in a glass homogenizer (Wheaton, USA), then centrifuged at 3000×g for 3 min in a micro centrifuge tube. The pellets were then resuspended in 25 µl of PBS with 0.025 % yeast extract and 0.002 % nalidixic acid [23].In this viability test, non culturable cells utilize yeast extract, start growing and elongate but cannot multiply due to the addition of nalidixic acid; therefore, viable cells appear greater than their normal size [23]. The tubes were incubated overnight in a dark place at room temperature. After incubation, 114 µl formaldehyde (40%) was added to each eppendorff tube and kept it for the fluorescent antibody (FA) test.

Slide preparation for fluorescent microscopic observation

A 5 µl portion of sample was placed on PTFE (polytetrafluoroethylene) coated well glass slide.The slide was air dried and flame fixed.The slides were air dried and flame fixed.1 drop (20 µl) of *V. mimicus* polyclonal antiserum (1:10) was added in each well. This procedure was done in a dark room.The slide was incubated at 37°C for 30 minutes in a moist chamber, keeping the slides in the dark.The slide was washed gently with phosphate buffered saline (PBS) for 3-5 minutes.The slides were air dried. This procedure was done in the dark place.Fluorescein isothiocyanate (FITC) conjugated anti-Rabbit globulin goat serum (20 µl diluted in PBS 1:80) was added on each well of the slide. This procedure was done in the dark room.The slide was again incubated at 37°C for 30 minutes in a moist chamber, keeping the slides in the dark. The slide was washed gently with phosphate buffered saline (PBS) for 3-5 minutes.The slides were air dried. This procedure was done in the dark place. Mounting oil was added to each well of the slide, and then placed the cover slip. The slide was examined at 1000X magnification by using an epifluorescence microscope, Olympus model

AH-2. Before adjusting the lens of the microscope at 1000X magnification, always a drop of the emulsion oil was put on the cover slip. Positive and negative controls were always inoculated on each PTFE coated slide, e.g. freshly cultured *V. mimicus* were used as positive control to compare the lengths of VBNC cells and normal cells.

PCR primers used to detect *V. mimicus* specific 16S rDNA gene

Primer	Sequence
Upstream primer of 16S rDNA	5'-TTAAGCSTTTTCRCTGAGAATGC-3'
Downstream primer of 16S rDNA	3'-AGTCACTTAACCATAACAACCCG-5'

3. RESULTS

Conformation of *V. mimicus* by molecular technique

A multiplex PCR was performed by using vibrio specific 16S rDNA primer, *V. mimicus* specific 16S rDNA primer and *ctxA* primer (Fig. 1).

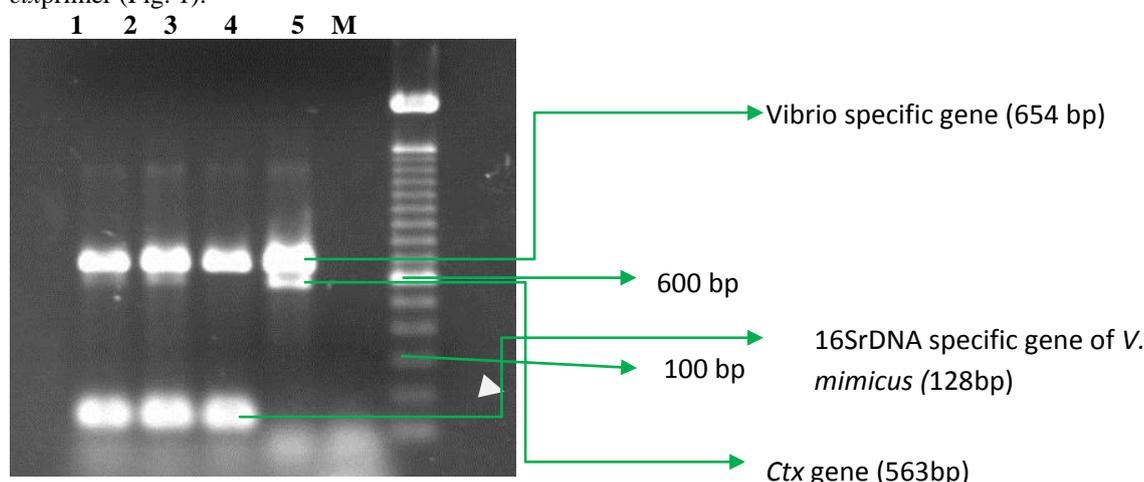


Fig. 2 Multiplex PCR using vibrio specific 16S rDNA template; *V. mimicus* specific 16S rDNA primer and *ctxA* primer. Here, 1-3 = different isolates of *V. mimicus*; 4 = (+Ve) control of *V. mimicus* and *ctxA*; 5 = (- Ve) control of *V. mimicus*; M=100 bp ladder.

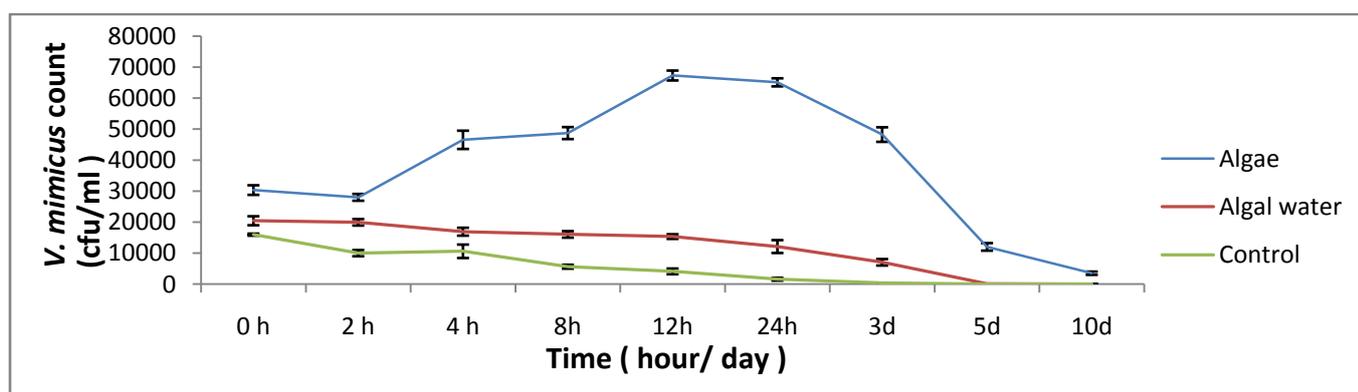


Fig. 3 Culturable counts of *V. mimicus* in various components of microcosm. Each line represents the viable *V. mimicus* count (cfu/g or ml) \pm SD of two experiments.

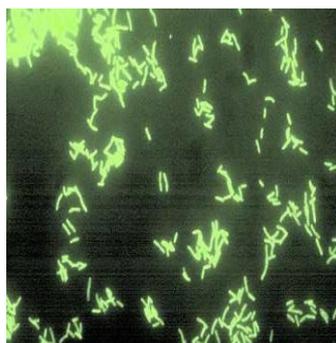


Fig. 4 Microscopic image of elongated *V. mimicus* cells on the DFA slide under epifluorescence microscopy at VBNC state.

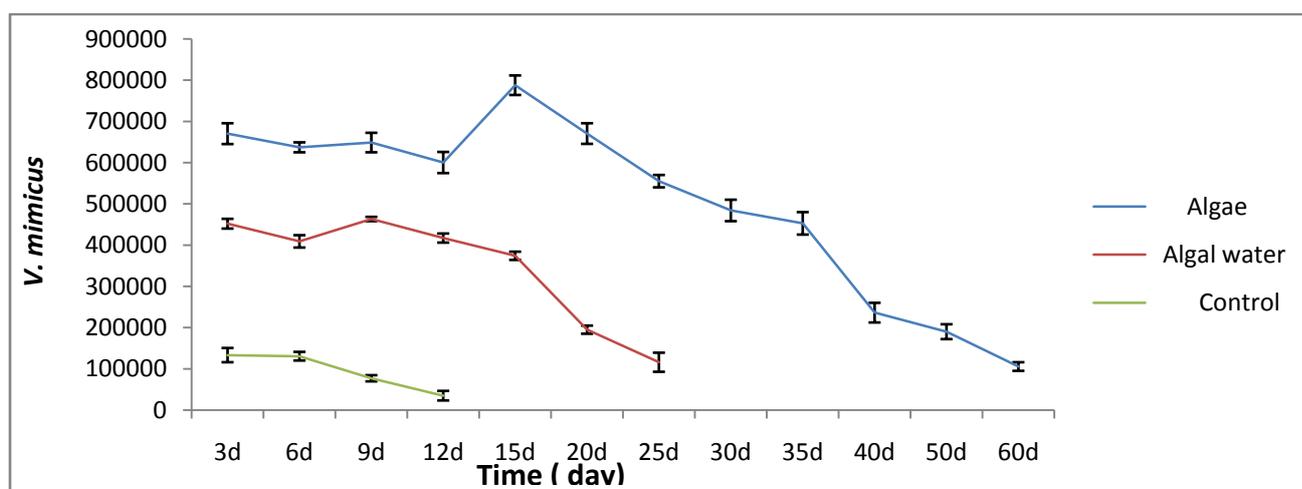


Fig. 5 Fluorescent antibody count of *V. mimicus* in various components of laboratory based microcosm. (cfu/g or ml) \pm SD of two experiments

3d 5d 8d 10d 15d 20d 25d 30d 35d 40d 45d 50d 60d (-)(+) Ladder

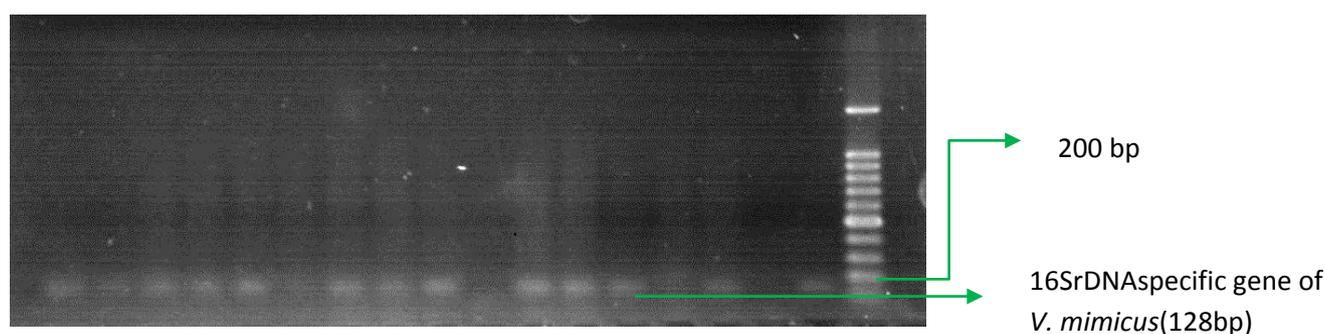


Fig. 6 16S rDNA gene of *V. mimicus* was detected from the study flask taken by different interval during VBNC state.

4. DISCUSSION

In the present study *V. mimicus* was detected with *A. variabilis* as viable but non-culturable state even after 60 days of inoculation in microcosm that supports Islam's hypothesis. The culturable count of *V. mimicus* in control water, algal water and in association with algae were enumerated at different time intervals. In case of control flask microcosm, *V. mimicus* remained in culturable form for up to 3 days. In study flask microcosm, water where *A. variabilis* was floating, the culturable count of *V. mimicus* was observed up to 5 days. In study flask microcosm, the culturable count of *V. mimicus* in association with *A. variabilis* was observed up to 10 days. When the cells of *V. mimicus*, no longer be

cultured using the conventional drop plate method, it was thought that they were enter into a viable but non culturable (VBNC) state. The VBNC state of *V. mimicus* was detected in microcosm using fluorescent antibody and PCR technique, when the cells could not be cultured using the conventional culture technique. It was observed that *V. mimicus* entered into a VBNC state after few days of survival in the culturable form. In the present study, VBNC state of *V. mimicus* was detected in control water up to 12 days and in algal water up to 25 days through fluorescent antibody technique. Whereas, the VBNC state of *V. mimicus* was detected with *A. variabilis* even after 60 days of inoculation in microcosm. The initial count of VBNC cells in association with *A. variabilis* was 670250 cfu/g, which increased to 787700 cfu/g on 15 days. There was a 117450 cells higher count on day 15 than the initial count. This implies that *V. mimicus* not only persist with *A. variabilis* but also multiply within it. Therefore, it can be inferred that *A. variabilis* can serve as a reservoir of *V. mimicus* in the aquatic environment. *V. mimicus* entered the mucilaginous sheath of *A. variabilis* became nonculturable but found dividing by binary fission and clustering around the heterocysts. When *V. mimicus* remain inside the mucilaginous sheath excrete mucinase enzyme to degrade the mucin of *A. variabilis*. *V. mimicus* use this mucin as their nutrient. Thus they survive and multiply up to 60 days which is longer than the cell without algal association. In developing country, like Bangladesh, transmission through contaminated drinking water can be an important mode of infection. The ability of *V. mimicus* to enter the VBNC state and persist for a long time with *A. variabilis* complicates the situation. As a mechanism of survival, as suggested by Roszak *et al.* (1984), the VBNC strategy perpetuates the organism after exposure to conditions less than optimal for cell growth and division.

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