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Original Article



# Metagenomic 16S rDNA Targeted PCR-DGGE in Determining Bacterial Diversity in Aquatic Ecosystem

Nur A. Hasan, W. Bari Chowdhury[1], Niaz Rahim[1], Marzia Sultana[1], S. Antara Shabnam[1], Volker Mai[2], Afsar Ali[2], Glen J. Morris[2], R. Bradley Sack[3], Anwar Huq[4], Rita R. Colwell[3,4,5], Hubert Ph. Endtz[1], Alejandro Cravioto[1], and Munirul Alam[1*]

*[1]International Center for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh; [2]Emerging Pathogen Research Institute, University of Florida, Gainesville, FL; [3]Johns Hopkins Bloomberg School of Public Health, Baltimore, MD; [4]Maryland Pathogen Research Institute, University of Maryland, College Park, MD; [5]University of Maryland Institute for Advanced Computer Studies, College Park, MD, USA.*

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**Bacterial numbers in surface water samples, collected randomly from six different water bodies, were estimated by acridine orange direct counting (AODC) and conventional culture-based heterotrophic plate counting (HPC). Bacterial genomic DNA was prepared from water samples by employing methods used for stool samples, including the population dynamics, were determined by primer extension of the 16S rDNA (V6/V8 region) using polymerase chain reaction (PCR), followed by denaturing gradient gel electrophoresis (DGGE), a metagenomic tool that is capable of separating unrelated DNAs based on the differences in their sequences and GC contents. The bacterial numbers in water samples ranged from $10^3$ – $10^6$ CFU/ mL for HPC and $10^4$ – $10^7$ cells/ mL for AODC, showing that a great majority of bacteria prevail as uncultivable which do not respond to culture methods that are used widely for tracking bacterial pathogens. The acridine orange-stained bacteria varied in sizes and shapes, and appeared either as planktonic (solitary) cells or as clusters of biofilms, showing the presence of diverse community under the epifluorescence microscope. The DGGE of the ca. 457 bp amplicons, as confirmed by agarose gel electrophoresis, produced bands that ranged in intensities and numbers from 18 to 31, with each band possibly indicating the presence of one or more closely related bacterial species. The enrichment of pathogenic bacteria in the aquatic ecosystem is known to precede the seasonal diarrhoeal outbreaks; therefore, bacterial community dynamics determined by Metagenomic 16S PCR-DGGE during pre-epidemic enrichment appears promising in predicting the upcoming diarrheal outbreaks.**

**Key words: Metagenomics, 16S rDNA, PCR, DGGE, bacterial community dynamics**

## Introduction

Metagenomics, the study of genomes, has been successful in elucidating the complex microbial communities present in the natural ecosystem[1,2]. The use of conventional culture-based methods is limited to estimation of bacterial population numbers from mixed microbial communities, and thus cannot represent much of the microbial world, as indicated by the inconsistency in "the great plate count anomaly"[3,4]. The culturable fraction of bacteria from aquatic environments has been found to be ten to a thousand times lower than those cultured from the soil[3]. However, over the last several decades, the development of ribosomal RNA (rRNA) phylotyping in identifying and enumerating microbes from the environment has shown that the variability within the highly conserved regions of rRNA sequences increases as the evolutionary distance increases between two organisms, thus providing the means to determine phylogenetic relationships between microorganisms[5-12]. Metagenomic rDNA-based techniques have employed the advanced molecular technique of polymerase chain reaction (PCR)[13], such as universal primer PCR targeted against 16S rDNA, followed by separation of amplicons using Denaturing Gradient Gel electrophoresis (DGGE) and Temporal Temperature Gradient Gel electrophoresis (TTGE) to analyze complex microbial communities present in environmental samples[19–22], including the soil[14–18], water, and the human distal gut microbiome[23-26].

In DGGE, denaturation of DNA strand depends on nucleotide sequence composition, therefore PCR products with different compositions but of same length will migrate different distances when exposed to a gradient of denaturing conditions, resulting in distinct fingerprints during separation[6].

In the present study, the conventional culture and Acridine Orange Direct Count (AODC) techniques were used to estimate the bacterial population size in water samples randomly collected from six sites, most being located at the Dhaka city. In addition, 16S rDNA PCR DGGE, which has been previously used

***Corresponding Author:***
*Munirul Alam, Enteric and Food Microbiology Laboratory, Lab Sciences Division, International Center for Diarrheal Disease Research, Bangladesh, Dhaka, Bangladesh, Tel.: +88-02-8860523 – 32 Fax: +88-02-8812529, 8821116, E-Mail. : munirul@icddrb.org*

successfully to compare microbial communities in a variety of ecosystems[6,21,27–29], was performed in order to determine the bacterial community dynamics in the surface water.

**Materials and Methods**

*Counting of bacteria by HPC and AODC*

Hundred micro-liters of each of the sample were plated onto Heterotrophic Plate Count (HPC) agar plate, which is a non-selective medium, for total culturable bacterial count. For HPC counting, samples were subjected to 10-fold dilution prior to spread plating. The plates were then incubated at 37ºC for 18 to 24 hours. Acridine Orange Direct Counting (AODC) was performed according to an earlier described method[30]. The samples were pre-incubated overnight in the dark with 0.025% yeast extract (Difco Laboratories, Detroit, Michigan) and 0.002% naldixic acid (Sigma), after which the cells in the samples were fixed with 4% formaldehyde and diluted 10-fold in a series. The bacterial cells were stained with Acridine Orange (Sigma, final concentration 0.1% w/v) for 2 minutes and filtered through black polycarbonate filters (Millipore, pore size 0.2 µm, diameter 25 mm) which were prestained with Irgalan black dye. The stained bacterial populations on the membrane filters were counted under UV light using an epifluorescent microscope (Carl Zeiss, Axioskop 40). The total bacterial counts were averaged from the bacterial numbers obtained from 20 microscopic fields and the images were captured using a digital camera (AxioCam MRc).

*DNA extraction from environmental samples*

The DNA from the environmental samples was isolated using method used for clinical samples. In brief, 100 ml of water samples were centrifuged at 13k rpm for 10 minutes and filtered through 0.22µm membrane filter. The pellets were retained and the membranes were washed off with 5ml of buffer A (50 mM Tris, 5 mM EDTA, pH 8.0) at least three times to recover maximum amount of retained materials. Centrifugation was performed at 13k rpm for 10 minutes and the pellet was retained, to which 467µl of lysis buffer (100 mM Tris, 100 mM EDTA, 1.5M NaCl, pH 8.0) was added and mixed by repeated inversion for 10 minutes. This was followed by addition of 30 µl of 10% SDS and mixing by inverting, and incubation at 65ºC for 15 mins or until a clear solution was obtained. Thirty micro-liters of proteinase K (20 mg/ml) was added to the mixture followed by incubation at 37ºC for 1 hr and addition of 100µl of 5M NaCl with thorough mixing. The next step involved addition of CTAB/NaCl solution (80µl); this was mixed thoroughly and then incubated at 65ºC for 10 minutes. Following incubation, the contents were cooled to room temperature and equal volumes of chloroform/isoamyl alcohol was added and mixed thoroughly by repeated inversion. Centrifugation at 12 rpm for 5 minutes produced a clear supernatant which was promptly transferred to a new eppendorf tube, without disturbing the interface. Equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) mixture were added to the supernatant and mixed by inverting, followed by centrifugation at 13 rpm for 5 minutes. The clear supernatant obtained was subjected to DNA precipitation by addition of 0.6 volume of isopropanol, accompanied with vigorous shaking of the tubes until stringy white DNA precipitates were visible.

The extracted community DNA was purified by adding 20 µl sodium acetate and 300µl absolute alcohol, after which they were left for one hour at -80ºC and centrifuged for 10 minutes at 13.2 rpm. The pellet was retained with the addition of 200µl of 70% ethanol and centrifugation at 13K rpm for 10 minutes. The saved pellet was dissolved with 200µl of 70% alcohol and centrifuged at 13K rpm for 10 minutes. Finally the pellet was air-dried.

DNA fragments for DGGE analyses were obtained from PCR with primers specific for conserved regions of the 16S rRNA gene. The primers with their respective sequences are provided in Table 1.

**Table 1:** *Primers used for 16S rDNA PCR-DGGE*

| Primer | Sequence |
|---|---|
| 6968 GC (V6/V8) | 5' – AA CGC GAA GAA CCT TAC – 3' |
| L1401 | 3' – GCG TGT GTA CAA GAC CC – 5' |

*PCR*

The oligonucleotide primers 6968 GC which amplify variable regions 6 and 8 (V6/V8) and L1401 were used to selectively amplify 16S rDNA genes of eubacteria. A 40 nucleotide GC-rich sequence was attached to the 5'- end of primer 6968 GC to improve the detection of sequence variation in amplified DNA fragments by subsequent DGGE. PCR amplification was performed in a thermal cycler (PTC-200 Peltier Thermal Cycler, MJ Research, Cetus, USA).

Each sample consisted of 50 ng of extracted DNA with 0.05µM of each of PCR primer, 200µM each of dNTP, 1.5 mM $MgCl_2$, 1.25U of Taq DNA polymerase in storage buffer A (Promega Co., USA), 5 µl of thermophilic 10X PCR buffer and sterile water added to make it upto 50 µl. The program consisted of 2 minutes of initial DNA denaturation and enzyme activation at 94ºC. This was followed by 34 cycles, with each cycle consisting of 30 seconds of denaturation at 94ºC, 30 seconds of annealing at 56ºC, 1 minute of elongation at 68ºC, and a final elongation for 7 minutes at 68ºC.

The PCR amplified products were separated in a 1% agarose gel by using Horizon 11-14 (Life Technologies, USA) electrophoresis apparatus. The gel was stained with ethidium bromide (10 mg/ml) and visualized under a UV trans-illuminator.

*Denaturing Gradient Gel Electrophoresis (DGGE)*

DGGE of the 16S rDNA amplicons (n=6), which had an approximate size 457 bp, was performed by following the methods, as described elsewhere[6,21,26]. The characteristic profiles of the community obtained by DGGE separation of the PCR amplicons were analyzed.

The DCode® Universal Mutation Detection System with gradient former model 475 was used according to the manufacturer's instructions (Bio-Rad). The acrylamide concentration in the gel was 6% and the denaturing gradient was between 40 and 50%. Both the 100% and 0% denaturant solution contained 7M urea with 40% (v/v) formamide, 6% acrylamide/bis-acrylamide (37.5:1) and 0.5X Tris-acetate EDTA (TAE) buffer at pH 8.0 in ultra pure water. Fifteen microliters of purified PCR amplicons were mixed with equal volumes of loading buffer and loaded to gel wells. The gels were run in 0.5X TAE buffer at $60^0$C for 16 hours at 65V as per the previously established method[31], followed by 30 minute incubation in the dark, staining with 1µl of SYBR™ Gold and visualizing under UV light with the Gel Doc 2000 System (Bio-Rad).

**Results and Discussion**

*Estimation of bacterial counts using HPC and AODC*

The number of total culturable bacteria estimated by heterotrophic plate counts (HPC) varied from $10^3$–$10^6$ CFU/ml (Fig. 1). The total number of bacteria, as determined by Acridine Orange Direct Counting (AODC), varied from$10^4$–$10^7$cells/ml, as displayed in Fig. 1. The comparative data revealed that the actual bacterial number determined by AODC was higher by 1 to 2 logs than that for HPC (Fig. 1), and this seemed to be in accordance to an earlier report that more than 99% of the bacteria do not respond to conventional (routine) culture, and are thus nonculturable[4].

The micrographs of the Acridine Orange-stained samples showed the presence of bacterial cells that appear in various sizes and shapes (rods, spheres, etc.), either free-living, or in clusters of biofilms, as depicted in Figs. 2. These images provide an in situ evidence of how bacterial communities modulate their survival in the natural aquatic ecosystem that serves as niche for many bacterial enteric pathogens. It has already been established that in non culturable states, bacteria survive without responding to conventional culture techniques and mostly remain undetected[32,34]. They undergo morphological changes, whereby the rod shapes of bacteria turn coccoid by reducing in size, including secretion of polysaccharides or extracellular matrices (biofilms) for protection against adverse environmental conditions[34]. Similarly, the nonculturable states as a means to overcome stresses has been well documented in several enteric pathogens, including *Vibrio cholerae*, the cause of cholera[32,34]. Although little is known about the mechanism of how nonculturable bacteria regain culturability to multiply and increase their number to be able to initiate seasonal diarrheal epidemics, the characteristic seasonality and the isolation of culturable *V. cholerae* prior to cholera epidemics in Bangladesh indicate a seasonal shift in the bacterial flora in the aquatic environments that serve as the reservoir for them[34]. Evidences in the past suggested that the nonculturable *V. cholerae* cells retain the metabolic activity within biofilms and can revert to culturability when favorable conditions recur, and can pose a major health risks for human[32-34]. Thus, the nonculturable bacterial communities that prevail in the aquatic ecosystem requires continuous monitoring to be able to track the floral shifts resulted by competition and proliferation of a particular population of major public health concern[34]. As evident from results of the current study, the major limitations of the HPC and AODC may be that none of these methods precisely and reliably reflect the seasonal changes that bring about changes in bacterial community dynamics in the aquatic ecosystem.

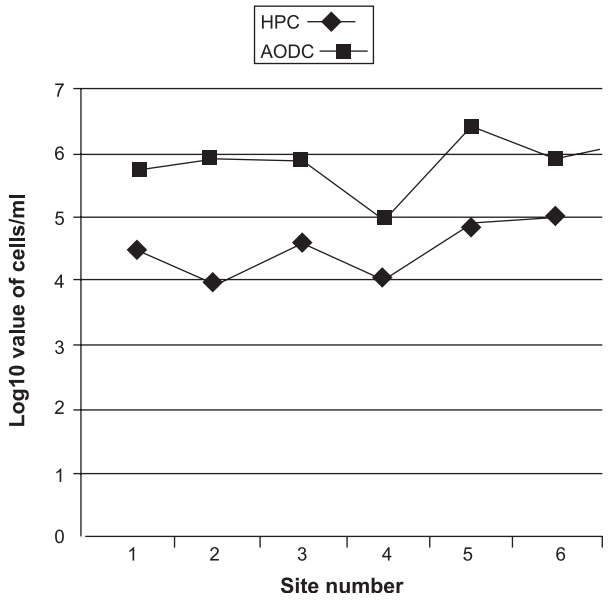


**Fig 1:** *Logarithmic graph showing line diagram of AODC and HPC counts of bacteria occurring in natural surface water samples.*

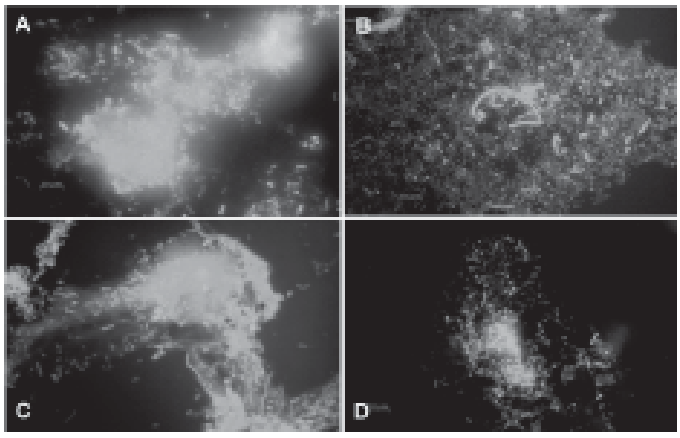


**Fig 2:** *Epifluorescence micrographs of microbial communities showing bacterial cells stained with Acridine Orange dye, found in the surface water samples. Images were captured digitally (Carl Zeiss, Axioskop 40).*

*Agarose gel electrophoresis of 16S rDNA amplified by PCR*

The PCR using primers that successfully amplified the V6/V8 regions of 16S rDNA produced amplicons of an approximate size 457 bp in all water samples tested. The six samples that produced the PCR amplicons were further analyzed by DGGE for the determination of bacterial community dynamics.

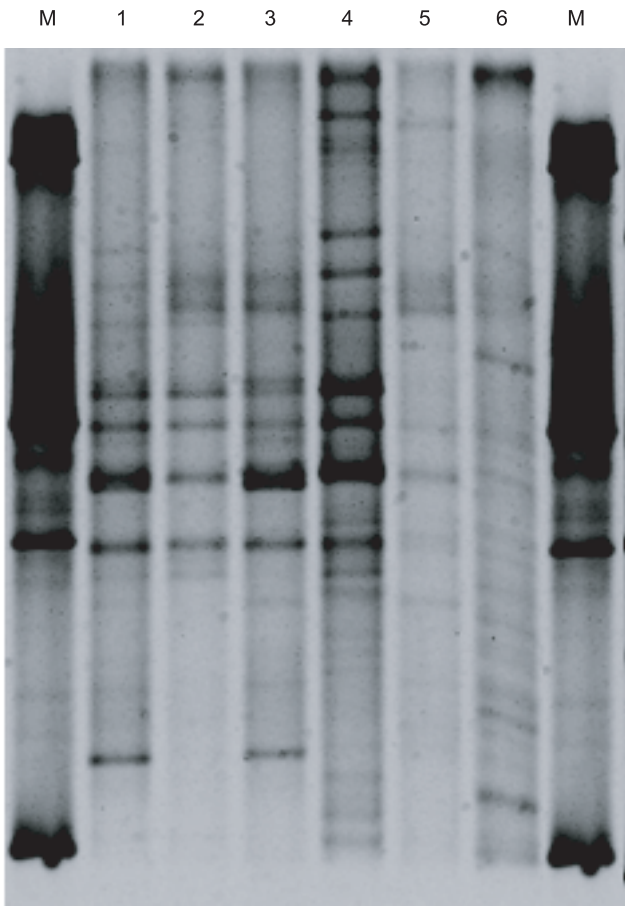


**Fig 3:** *The 16s rDNA amplified from natural surface water samples by PCR were analyzed by denaturing gradient gel (40% -50%) electrophoresis (DGGE).*

Lanes M: Marker; Lane 1: Jahurul Haq Hall, Dhaka University (DU); Lane 2: Fazlul Huque Hall, DU; Lane 3: Jagannath Hall, DU; Lane 4: Mathbaria Pond; Lane 5: Rampura Lake; Lane 6: River Buriganga.

*DGGE*

The DGGE band patterns varied between the water bodies; the band numbers ranged from 18 - 31 (Fig. 3).

The maximum number of bands was observed for water sample collected from the Buriganga River, labeled as Site 6, from which 31 bands were obtained. Minimum number of bands (n=18) were found from Site 5, a lake located in Rampura which was also connected to the sewage system. The variation of band patterns within DGGE profiles, which is dependent on the bacterial population dynamics, may have been influenced by the physical and chemical quality of water, such as the availability of nutrients, since connections of sewage systems with the pond and river waters allow fecal and other domestic and industrial effluents to be washed into the waters. For instance, the maximum number of bands within the DGGE profiles for the river Buriganga can possibly be attributed to the freely flowing, efficiently aerated waters that support greater diversity and enrichment of bacterial communities resulting from the discharge of approximately 15000 $m^3$ of untreated chemical wastes into the water[36], whereas the minimum number of bands corresponding to the sample collected from the Rampura lake may be highly eutrophic since the stagnant waters are not properly aerated and may not be supportive for the complex microbial communities that usually prevail in surface waters. Based on the DGGE profile, especially the numbers and intensity of the bands in water samples, it may be suggested that the DNA isolation methods used for stool samples was efficient in elucidating the bacterial diversity in the aquatic ecosystem. The recurrent diarrheal diseases that occur endemically in Bangladesh are climate driven, that is, seasonal[34]. Under the changing climate, which is presumed to contribute to infectious diseases, the determination of the bacterial community dynamics in aquatic ecosystem is crucial for understanding the floral shifts that are believed to contribute to the proliferation of a particular population of diarrheal implication[34–35]. Whereas the culture methods fail to detect most bacteria and direct microscopic observation only indicate the physical presence of bacteria, the diverse 16SrDNA bands, some of which appeared as intense bands, represent one or more closely related species in the aquatic ecosystem. This study was limited in that the samples were not tested in length to elucidate the yearly seasonal patterns; nonetheless, the DGGE band patterns will show variations due to seasonal climate change, and the resultant floral shift in the aquatic ecosystem. Therefore, the characteristic DGGE patterns that are likely to recur during the seasonal change can be translated into a prediction model for upcoming diarrhea. The DNA band(s) in the DGGE profiles can be sequenced to provide comprehensive identification of bacteria up to the species level. Nonetheless, the relative positioning and the intensity of bands with reference to DGGE markers can be reliable in predicting the emergence of a bacterial species of epidemic implication. Further work is in progress in our laboratory.

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