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# Molecular Identification of Bacterial species in Gundaru River Basin of Thirumangalam, Madurai District, South India

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**In the study area of Gundaru river basin, more than 60% of the population is dependent on underground water for drinking purposes. Water sample was collected from the river basin and bacterial species were isolated, characterized and identified using 16S rRNA sequence analysis. Five species observed in the study were *Salmonella* species, *Enterobacter* species, *Alcaligenes* species and sequences were deposited in Genbank database. Results of analysis of surface water show it is highly polluted and unfit for consumption.**

**Key words:** Microbial diversity, 16S rRNA sequencing, Phylogenetic tree.

Microbial diversity, being an integral part of biodiversity includes bacteria, archea, fungi, algae, protozoans and protists and seems to be an unnoticed normal resource that deserves greater attention. Microbial biodiversity is the variety that exists among microorganisms and their environments. The long evolutionary history has likely been the major factor in determining the vast diversity of microbial life (Woese, 1998). In particular bacteria are remarkable in the abilities to live in soil environments that are hospitable for life and the greatest among energy sources. Where there is life on earth, there is microbial life

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Microorganisms are found in all ecosystems (Stanley *et al.,* 2002). The environmental conditions to which flood plain soils have exposed may affect soil microbial biomass, function, communities and composition. To examine and to quantify these impacts soil microbial carbon (C mic) basal respiration (BR) metabolic quotient ($qCo_2$) and PLFA were determined in three selected flood plan soils at the Elbe river (Germany) to characterize microbial diversity in three flood plan soils and to discriminate the soils in the microbial parameters (Rinklebe, 2004). The functional groups and bacteria tested for by PCR were sulfate reducing bacteria, denitrifiers, nitrifiers, ammonia oxidizers and *Cyanobacteria.* Sulfate, nitrate, ammonia, dissolved organic carbon and dissolved oxygen were measured to monitor the biogeochemical process of the systems and the functional groups for which molecular presence were being tested (Levin *et al.,* 1996). The overall burden of infectious disease remains high. In 2001,

infectious diseases accounted for an estimated 26% of deaths worldwide (Kindhauser, 2003). Emerging pathogens are those that have appeared in a human population for the first time, or have occurred previously but are increasing in incidence or expanding into areas where they have not previously been reported, usually over the last 20 years (WHO, 1997). Re-emerging pathogens are those whose incidence is increasing as a result of long-term changes in their underlying epidemiology (Woolhouse, 2002).

The most commonly used procedure to assess microbial diversity is originated in Pace's laboratory (Pace *et al.,* 1986). The DNA is extracted from community samples. This is followed by polymerase chain reaction PCR (Mullis *et al.,* 1987) has begun to be applied to environmental detections of microorganism. Only a few reports investigated soil respiration (Megonigal *et al.,* 1996) or the impact of inundation dynamics and different management systems on soil respiration and diverse enzyme activities (Emmerling, 1993) in floodplain soils. However, the microbial diversity in floodplain soils is poorly understood. Gundaru River flows in the Southern part of Tamil Nadu. It originates at an atitude of 500 m near Kottamalai of Saptur reserve forests belonging to Varushanad hills after traversing about 150 Kms, it falls into Gulf of Mannar at about 6 Kms southeast of Sayalkudi. Gundaru basin lies in between 9° 05' N - 10° 03' N latitude and 77° 35' E longitude covering an extent of 5,647 sq. kms.

The objective of the present investigation is to study the microbiological status of Gundaru River. The use of specific 16S rRNA probes has enabled researchers to amplify specific 16S rRNA sequences from mixed cultures and therefore allows phylogenetic analysis, estimation of bacterial diversity and identification of isolates directly from clinical or environmental sites (Martinko *et al.,* 1997). Hence molecular technique has been adopted for this study to confirm the bacterial species present in the water sample.

## MATERIAL AND METHODS

### Sample collection

For collection, heat – sterilized bottles were used. Collection bottle was opened at a depth of about 30cm with its mouth facing the water current to ensure that water entering the bottle had not been in contact with the hand. The bottle was stoppered and labelled with full details of the source of the water and time and date of collection, and taken to the laboratory as quickly as possible, at least within 6 hours, and kept in a cool container protected from light.

### Enrichment and Isolation of bacterial species

The sample bottle was shaken vigorously about 25 times. Serial decimal dilutions were made using 10% peptone water, enriched and plated on sterile media plates. Total number of bacterial colonies present in the water sample was enumerated using spread plating technique. Five of the colonies observed on the plates were taken and sub-cultured in nutrient medium for further analysis. The sub-cultured isolates were plated on Salmonella-Shigella agar, Bismuth Sulfite agar, Hektoen Enteric agar, Deoxycholate Citrate agar and *Alcaligenes* medium.

### Morphological and Biochemical characterization

The isolates designated as G1, G2, G3, G4 and 5 were analyzed for their morphological and biochemical characters as given by Bergey's manual of Determinative Bacteriology.

### Genomic DNA isolation, PCR amplification and Sequencing of 16S rRNA genes

Bacterial culture (1.5 ml) was taken and centrifuged at 10000 rpm for 10 minutes at 4°C. To the pellet, 500 µl of TE buffer suspended with 300 µl of lysozyme was added and incubated at 37°C for 30 minutes. To the above mixture, 3 ìl of proteinaseK was added along with 60 µl of SDS and incubated at 55°C for 2 hours. Then equal volume of phenol was added and centrifuged for 10 minutes at 10000 rpm. The aqueous (top) layer was transferred to a fresh tube and equal volume of PCI (phenyl chloroform isoamylalcohol) was added and centrifuged for 10 minutes at 10000 rpm. The extraction steps were repeated until no protein precipitate was observed. The DNA in top phase was precipitated with 5% ammonium sulphate and 100% ethanol at 20°C. The precipitate was collected by centrifugation and the pellet was dissolved in 70% ethanol decant ethanol rinsed and the pellet was air dried. DNA in TE buffer was resuspended.

Polymerase chain reaction (PCR) was performed with a final volume of 50 µl in 0.2 ml thin walled tubes. The primers (Teske *et al*., 2002) used for PCR amplification of 16S rRNA gene are 8F 5'-AGA GTT TGA TCC TGG CTC AG - 3' and 1492R 5' -GGT TAC CTT GTT ACG ACT T-3' (Sigma genosys). Each reaction mixture contained 2 µl of template DNA (100 ng), 0.5 µM of two primers, and 25 µl of Enzyme Master Mix (Bioron). The PCR program consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of DNA denaturation at 92°C for 30 sec, primer annealing at 50°C for 1 min, and primer extension at 72ºC for 2 min carried out in Thermal Cycler (Thermo Hybaid). After the last cycle, a final extension at 72ºC for 20 min was added. The PCR products were purified by QIAquick PCR purification kit as described by the manufacturer and cloned using QIAGEN PCR cloning plus kit as described by the manufacturer. Clones were selected and isolated plasmids with insert were sequenced with M13 Sequencing Primers using ABI Biosystems automated sequencer.

**Phylogenetic analysis of 16S rRNA sequences**

The 16S rRNA sequences were aligned using Multiple sequence alignment program CLUSTALW (Higgins *et al*., 1994) and phylogenetic tree was plotted using NJPLOT (Saitou and Nei, 1987) and PHYLODRAW (Choi *et al*., 2000) programs. A bootstrap analysis was performed to validate the reproducibility of the branching pattern.

**Table 1.** Biochemical Characters

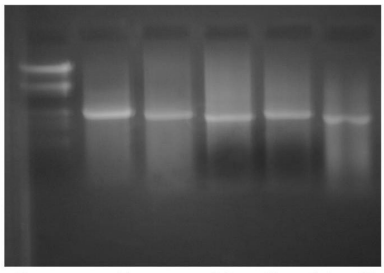
| S.No. | Test | Isolate G1 | Isolate G2 | Isolate G3 | IsolateG4 | Isolate G5 |
|---|---|---|---|---|---|---|
| 1. | Gram reaction | Gram Negative | Gram Negative | Gram Negative | Gram Negative | Gram Negative |
| 2. | Shape | Rods | Rods | Rods | Rods | Rods |
| 3. | Motility | + | + | + | + | + |
| 4. | Indole test | - | - | - | - | - |
| 5. | Methyl red test | - | + | + | + | + |
| 6. | Voges Proskaur test | - | + | - | - | + |
| 7. | Citrate Utilization test | + | - | - | - | - |
| 8. | Carbohydrate fermentation of | - | + | - | - | + |
| | a. Acid production | - | - | | | - |
| | b. Gas production | | | | | |
| 9. | Starch hydrolysis test | - | + | - | - | + |
| 10. | Catalase test | + | + | + | + | + |
| 11. | DNA digestion test | - | - | - | - | - |

**Table 2.** Identified species

| S. No. | Isolates | % Similarity with database sequences | Name of the Microorganism | GenBank Accession number |
|---|---|---|---|---|
| 1. | G1 | 99 | *Alcaligens sp.* | EF195165 |
| 2. | G2 | 99 | *Enterobacter aerogenes* | EF195172 |
| 3. | G3 | 99 | *Salmonella typhi* | EF195174 |
| 4. | G4 | 98 | *Salmonella enterica* | EF579646 |
| 5. | G5 | 99 | *Enterobacter sp.* | EF579647 |

## RESULTS AND DISCUSSION

Bacterial enumeration in the water sample was $262\times10^{-6}$CFU/ml. Five colonies were selected and their morphological and biochemical characters were analyzed (Table1). The pathogen *Salmonella* presence was confirmed by aseptic inoculation of sample in SS agar and Selenite broth. *Salmonella* colonies appeared as black color colonies in *Salmonella – Shigella* agar and *Salmonella* appeared as black centre with light edges colonies in BSA agar. In Hektoen Enteric agar *Salmonella* colonies appeared as Blue green with a black centre. *Enterobacter* presence was confirmed by pink colour colonies of *E. coli* in Deoxycholate Citrate agar. *Enterobacter* appeared as red colonies in Macconkey agar, red with a permanent metallic sheen in endo agar. The presence of *Alcaligenes* sp. was confirmed by the presence of thin white spread colonies on *Alcaligenes* medium.16S rRNA genes (Fig. 1) were PCR amplified, cloned and sequenced. Sequences obtained were analyzed and BLAST analysis show the presence of *Salmonella* species, *Enterobacter* sp. and *Alcaligenes* sp. having nearly 99% similarity with the database sequences and sequences were submitted in Genbank database (Table2). Phylogenetic tree of the 16S rRNA sequences (Fig. 2) reveal the genetic relationship between the sequences.



**M G1 G2 G3 G4 G5**

**M– Marker (Lambda Hind III)**
**G1-G5- PCR amplified products (1.5 Kb) of the isolates**

**Fig. 1.** Electrophoreto graph of PCR amplified 16S rRNA genes

Pathogenic microbes, though constitute a very small proportion of the microbial species, are nevertheless characterized by high genetic diversity. Genotypic variation in pathogen populations poses a major barrier to disease control. As of today, amongst the pathogenic microorganisms, diversity of bacterial pathogens infecting human or animal hosts is the most studied. Epidemiological data suggest that waterborne transmission of *Salmonella typhi* usually involves small inocula, whereas food borne transmission is associated with large inocula and high attack rates over short periods. The inoculum size and the type of vehicle in which the organisms are ingested greatly influence both the attack rate and the incubation period (WHO, 2003).

The diversity and richness of soil bacterial communities differed by ecosystem type, and these differences could largely be explained by soil pH. Bacterial diversity was the highest in neutral soils and lower in acidic soils, with soils from the


**Fig. 2.** Phylogenetic tree

Peruvian Amazon. Microbial biogeography is controlled primarily by edaphic variables and differs fundamentally from the biogeography of "macro" organisms (Noah and Jackson 2005). The active communities (16s rRNA) were further discriminated by farm location and, to some degree, by land-use practices. The results of this investigation indicated that soil type was the key factor determining bacterial community composition in these arable soils (Girvan *et al.,* 2003). Molecular and biochemical techniques of estimating abundance and number of each species are means of approaching an understanding of the community composition and diversity of soil bacterial communities at the genetic level. Furthermore, information about microbial community structure and diversity is important for understanding the relationship between environmental factors and ecosystem functions (Torsvik *et al.,* 1998). The benefit of a high genetic diversity is currently under debate because it is not always correlated to functional diversity (Griffiths *et al.,* 2000). Furthermore, the relationship between soil health and biodiversity is not completely understood, although a medium to high diversity is generally considered to indicate a good soil health. Microbial diversity measurements have thus been recommended in soil health monitoring programmes (Nielsen and Winding, 2002; Turco *et al.,* 1994).

The present study shows the microbial diversity in one region of Gundaru River and the contamination may be due to the disposal of domestic sewage and industrial untreated effluents into river basin.

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