RESEARCH ARTICLE

MICROBIAL DIVERSITY ANALYSIS, SEQUENCE ISOLATION AND PHYLOGENETIC ANALYSIS OF SOUTH INDIAN GUNDARU RIVER BASIN

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ABSTRACT

River water sample was collected at five different locations in Gundaru river, Tirumangalam. Then these samples were tested for pH factor. It showed neutral pH and electrical conductivity. The water sample was also analysed for color, dissolved oxygen and BOD. The examined river water contained chloride, calcium, phosphorus, sulfate, potassium, total solids and nitrogen. The soil samples were collected from five different places near Gundaru river basin. The colony forming units were countable and maximum in first three sites and minimum in the next two sites. The first three sites namely Arukannu bridge, Santhapettai and Near Girls school were very much contaminated with sewage water and the other two sites namely Sengapadai and Sonnaisamy bridge were not so much contaminated with sewage water. In MPN method, it was found that the sewage enteric organisms are present in Gundaru River. Molecular techniques were used in estimating the phylogenetic relatedness of 15 bacterial species isolated.

INTRODUCTION

Microorganisms are found throughout the environment: in the air, water and soil; on the surface of any object such as clothes, walls, furniture; in soil and dust; and on and in our own bodies (skin and mucous membranes). Microbes are even found in extreme, seemingly inhospitable environments: living and thriving microbes are common in hot springs with temperatures in excess of 95°C, in Antarctic seawater below 0°C, and in acid mine waters whose pH is below 4. Ordinarily these ubiquitous microorganisms are of no threat to healthy humans, and quite a few are actually beneficial. Microorganisms are found in all ecosystems and microbes present in rivers and river banks are much concern in the rural people's health (Stanley *et al.,* 2002). In particular bacteria are remarkable in the abilities to live in soil environments that are hospitable for life and the greatest among energy sources. The long evolutionary history has likely been the major factor in determining the vast diversity of microbial life (Woese 1998). In the environment, many organisms in soil play important geochemical roles in processing vital elements such as phosphorus, nitrogen and making them available to other living things. Biodiversity, which is defined as the variety and variability among living organisms and the ecological complexes in which they occur, is measured at three levels – the gene, the species, and the ecosystem. Microorganisms in river water and soil play an important role both in natural and managed agricultural soils in processes such as maintenance of soil structure [Wright 1982; Dodd et al., 2000] decomposition of organic matter, toxin removal, the cycling of carbon, nitrogen, phosphorus and sulphur [Molin and Molin, 1997;

Trevors 1998b; Wall and Virginia 1999]. Here we perform the ecological flow of microbial species from one of the river water and soil surroundings in South India. Gundaru River flows in the southern part of Tamilnadu and it takes its origin from Varushanad hills of the Western Ghats and flows through Madurai, Virudhunagar, Sivagangai, Ramnad and Tuticorin districts. It irrigates the agricultural lands of these areas and helps in raising the level of water table and meets the drinking water need of the people to a certain extent. The purpose of the present study is to analyze about microbes present in the river water and the microbes present in the river banks. Water borne diseases are essentially biological caused by bacteria, viruses and parasites. There are various forms of illness due to infection of waterborne pathogens and most important among them is diarrhea. The source of water is extremely important to denote its quality in many locations. The contamination of surface water by domestic and industrial wastes causes environmental problems. The objective of the present investigation is to study the ecological and environmental status of Gundaru River with a special reference to the catchment area. For understanding the microbes present in this river and river banks this research work has been carried out with experimental techniques, the sequence information and relation of each species were analyzed.

MATERIALS AND METHODS

Sample collection and location description

Gundaru River flows in the southern part of India (Tamil Nadu). Gundaru river basin lies in between 9°05' N – 10°03' N latitude and 77°35' E longitude covering an extent of 5,647 sq. km. It originates at an attitude of 500 m near Kottamalai of

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Saptur reserve forests belonging to Varushanad hills and after traversing about 150 kms. In the present study, we concentrate on five different sites of location, particularly Gundaru river flows. The details of locations of samples have been included in supplementary information. The samples of soil and water were collected from five different locations using sterile containers.

Analysis of Physico chemical properties

The obtained samples were maintained under aseptic condition to avoid unwanted contaminations and their physico-chemical properties. The properties of the samples such as pH, electrical conductivity [Jackson 1998], calcium phosphorus [Allen *et al.,* 1980] magnesium, chloride, nitrogen, sulfate, potassium, Total Dissolved Solids (TDS), biological oxygen demand (BOD) (Frank *et al.,* 2000) were analyzed using standard procedures.

Microbial analysis of soil samples using Winogradsky column

Winogradsky column (**Tortoso *et al.,* 1990**) named after the Russian Microbiologist Sergei Winogradsky is a model ecosystem that is used for the study of aquatic and sediment microorganisms. This helps to develop a gradient of redox potentials and pigmented microorganisms which show up clearly against the light coloured sand. This method classifies the soil microorganisms. From the Winogradsky column the microorganisms were isolated and plated through serial dilution (Jensen 1968). The soil samples were collected from Gundaru river basin at five different places namely Arukannu Bridge, Santhapettai, P. K. N Girls School, Sengapadai village and Sonnaisamy Bridge. The colony forming units were countable that was maximum in first three sites and minimum in next two sites. The first three sites namely Arukannu Bridge, Santhapettai, P. K. N Girls School was well contaminated with sewage water and the other two sites namely Sengapadai and Sonnaisamy Bridge were not so contaminated comparatively. Winogradsky column was prepared with a tall cylindrical container (Tortoso *et al.,* 1990). Each column was prepared by adding cellulose and sulfur. Paper towels were cut into small strips and used as source of cellulose. Two grams of paper per 500-milliliter container was mixed with the mud. One gram of calcium sulfate was added to the mud as the sulfur source. The containers were sealed using plastic wrap secured with rubber bands and placed in front of a light source. After a period of several weeks, patches of red green and purple colors appeared at different depths of the column indicating the enriched media of cellulose and sulfur and it provides a suitable environment for colonization of aerobic and anaerobic bacteria.

Isolation

Samples collected from the Winograsky column solution were serially diluted and inoculated into nutrient agar (bacteriological medium) and incubated at room temperature. After 24 hours, the viable colony forming unit per gram of soil sample was counted (Jensen 1968).

Molecular identification of bacterial isolates from Gundaru river basin

Isolation of bacterial Genomic DNA

Genomic DNA was isolated with Lysozyme/SDS/Phenol/Chloroform method described by Wawer and Muyzer. Bacterial culture (1.5 ml) was taken and centrifuged at 10,000 rpm for 10 minutes at 4°C. To the pellet obtained, 500 µl of TE buffer was added along with 300 µl of lysozyme and incubated at 37°C for 30 minutes. To the above mixture, 3 µl of proteinase K 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 at 10,000 rpm for 10 minutes. The aqueous (top) layer was transferred to a fresh tube and equal volume of PCI (phenyl chloroform isoamylalcohol) was added and centrifuged at 10,000 rpm for 10 minutes. 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. Ethanol was decanted and the pellet was air dried. DNA was resuspended in TE buffer.

PCR amplification, cloning and sequencing of 16S rRNA genes

Polymerase chain reaction (PCR) was performed with a final volume of 50 µl DNA in 0.2 ml thin walled tubes. The primers (Teske *et al.,* 2007) 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 (Genei, Bangalore). The PCR program consisted of initial denaturation at 94 °C for 5 mins , followed by 30 cycles of DNA denaturation at 92 °C for 30 secs , primer annealing at 50 °C for 1 min , and primer extension at 72 °C for 2 mins carried out in Thermal Cycler (Thermo Hybaid). After the last cycle, a final extension was performed at 72 ºC for 20 mins. The PCR products were purified by PCR purification kit (MBI Fermentas) and cloned using InsTA PCR cloning kit as described by the manufacturer. Clones were selected, isolated and plasmids with insert were sequenced with M13 Sequencing Primers using ABI Biosystems automated sequencer.

Phylogeny tree construction

Phylogenetic and molecular evolutionary analysis was conducted using clustal X 2.0. The multiple alignments of sequences of bacterial isolates from Gundaru river basin was used to create phylogenetic trees. The bootstrapping of the 'alignment dataset' was restricted to 1000 times owing to the large number of sequences in the alignment. Protein distances were calculated employing Clustal x2.0 package (Eigel and Whitney 1984; Julie and Thompson Toby 1997). The trees were calculated using the rooted Neighbor-Joining (NJ) method (Saitou and Nei 1987) on distance matrices employing NEIGHBOR from the Clustalx package (Larkin *et al.,* 2007). The Default p- distance method was used for distance analysis. These trees were analyzed employing of Clustalx package to derive a consensus tree. A rooted tree was plotted using NJ Plot software package (Saitou and Nei 1987). Sequences with more than 50% bootstrap support values were confirmed and grouped

RESULTS AND DISCUSSION

Analysis of Physico – chemical Properties

The Physico - chemical parameters useful for water quality assessment are determined by the presence of both organic and inorganic compounds that are either suspended or dissolved in soil. While some of these compounds are toxic to the ecosystem, some constitutes nutrients to aquatic organisms and

others are responsible for the aesthetics of the water body.The physio-chemical characteristics of the soil such as pH, EC, soil phosphorus, organic carbon, magnesium, nitrogen, sulphate and potassium were analyzed and presented in Tables 1 and 2 and workflow of this work was represented in Fig 1. The pH of water determines the solubility and bioavailability of chemical constituents such as nutrients and heavy metals.
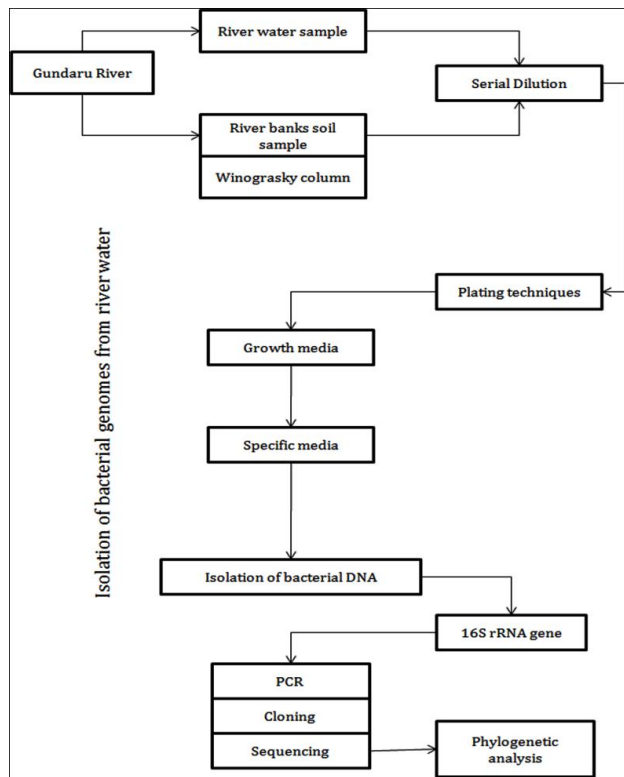
Fig.1. Workflow for isolation, purification and sequence analysis of bacteria isolates from river water sample.

Though an increase in pH levels may have no direct impact with aquatic life, they greatly influence the availability and solubility of all chemical forms in the water and this might aggravate nutrient problems leading to a destabilization of the ecosystem. The pH of the soil samples collected from five different sites ranged from 7.9 to 8.4. These analytical steps in the present study are in concurrence with reported readings of Mohan 1997, who had analyzed the Vellar River, Tamilnadu,

India and Shlyakhov *et al.,* 1998 who had analyzed physiochemical properties of river basin soil in Russia and the pH readings of soil are in accord with reported readings of Stanley *et al.,* 2002 who had analyzed Wisconsin River basin in USA. The wide range of pH variation in the profiles of meadow-swampy maritime soil is conditioned by the differences in composition of the upper (organic) and lower (mineral) parts of these soils. If peat layers are taken separately, the range of pH variation in them is much narrower (from 4.8 to 5.8) (Shlyakhov and Kostenkov 1998). The introduction of waste water, high in organic matter and essential nutrients brings about changes in microbial flora (Rheinheimer 1999 ; Ekhaise and Anyasi 2005). The pH, EC readings in present study are in concomitantly with that of Dennis *et al.,* 1999 who had determined pollution levels in tropical waters of Kampale, Uganda. Higher EC values indicate the presence of higher content of dissolved salts in river water (Abdullah *et al.,* 1999).

In our study EC values are ranged between 2.5 – 3.3 mmhos which is considered as dangerous. These BOD, total solids and chloride readings in the present study are in agreement with reported readings of Janakarajan, 2002 who had analyzed the physiochemical characteristics of Palar River, Tamilnadu, India. The nutrients nitrogen and phosphorus commonly occur naturally in ground water but elevated concentrations may be from influence of human activity (Nerbraska 2004). The readings of phosphorus, sulphate, chloride in the present study are in parallel with reported findings of Nebnaska Natural Resources Commission, (2004). They examined the quality of river water on Platte river valley. In the present study the readings of pH, EC, total solids, BOD, nitrate, calcium are analogous with reported readings of Ekhaise and Anyasi 2005, who had analysed physiochemical properties of Ikpoba River, Nigeria and the pH, EC, BOD, total solids readings of the present study are similar to the reported readings of Muhammad Ali *et al.,* 2005 who had examined physiochemical parameters of brackish water pond. The pH, potassium, phosphorus readings of the present study are in consensus with reported readings of Iversen *et al.,* 2006 who had analyzed river basin soil in Denmark. The readings of pH, BOD are in concurrence with reported readings of Jonathan *et al.,* 2007 who had analyzed physiochemical properties of Uppanar river water. In the present study, pH, EC, potassium, calcium, sulphate readings are correlated with the reported readings of Mackay *et al.,* 2007 who had analyzed Leichhardt River's physiochemical properties in Queensland.

Table 1. Physico – Chemical properties of samples

S.No	Parameter	Site - I	Site - II	Site - III	Site - IV	Site - V
1.	pH	7.8±0.02	8±0.02	8.2±0.02	7.8±0.02	7.6±0.02
2.	Electrical conductivity (m ohms/cm)	3.4±0.02	3.2±0.02	3.3±0.02	3.0±0.02	3.0±0.02
3.	Phosphorus (mg/l)	0.3±0.02	0.2±0.02	0.5±0.02	0.05±0.02	0.002±0.02
4.	Organic carbon (%)	0.78±0.02	0.92±0.02	0.98±0.02	0.9±0.02	0.08±0.02
5.	Magnesium (mg/l)	1.4±0.02	1.25±0.02	0.9±0.02	0.5±0.02	0.4±0.02
6.	Nitrogen (mg/l)	0.8±0.02	0.9±0.02	0.10±0.02	0.2±0.02	0.02±0.02
7.	Sulphate (mg/l)	0.10±0.02	0.12±0.02	0.08±0.02	0.05±0.02	0.07±0.02
8.	Potassium(mg/l)	0.17±0.02	0.19±0.02	0.13±0.02	0.10±0.02	0.20±0.02

Table 2. Physico – Chemical properties of Gundaru River water

S.No	Parameter	Site - I	Site - II	Site - III	Site - IV	Site - V
1.	pH	8.1±0.02	8.0±0.02	8.9±0.02	8.0±0.02	8.4±0.02
2.	Electrical conductivity (m ohms/cm)	3.0±0.02	3.3±0.02	3.1±0.02	2.5±0.02	2.7±0.02
3.	Phosphorus (mg/l)	0.002±0.02	0.01±0.02	0.003±0.02	0.1±0.02	0.001±0.02
4.	Calcium (mg/l)	30.6±0.02	51.3±0.02	62.7±0.02	58.7±0.02	59.5±0.02
5.	Magnesium (mg/l)	1.4±0.02	1.25±0.02	0.9±0.02	0.5±0.02	0.4±0.02
6.	Chloride (mg/l)	150.6±0.02	260.7±0.02	175.6±0.02	190.6±0.02	220.8±0.02
7.	Sulphate (mg/l)	96±0.02	95±0.02	92±0.02	90±0.02	89±0.02
8.	Nitrogen (mg/l)	0.5±0.02	0.4±0.02	0.2±0.02	0.1±0.02	0.01±0.02
9.	Potassium (mg/l)	0.03±0.02	0.10±0.02	0.09±0.02	0.07±0.02	0.09±0.02
10.	Total solids	390±0.02	682±0.02	490±0.02	350±0.02	560±0.02
11.	BOD	370	366	325	287	280

Table 3. Genbank accession numbers of Bacterial isolates from Gundaru River water

| S.No.	GenBank Accession number	Bacterial isolate strain source	Name of the Microorganism
1.	EF195165	River water (Site I)	*Alcaligens sp.*
2.	EF195166	River water (Site I)	*Alcaligens sp.*
3.	EF195167	River water (Site I)	*Alcaligens sp.*
4.	EF195168	River water (Site I)	*Alcaligens sp.*
5.	EF195169	River water (Site IV)	*Bacillus cereus*
6.	EF195170	River water (Site III)	*Alcaligens faecalis*
7.	EF195171	River water (Site I)	*Achrobacterium*
8.	EF195172	River water (Site I, II, III)	*Enterobacter aerogens*
9.	EF195173	River water (Site I)	*Brevundimonas sp.*
10.	EF195174	River water (Site I, II, III)	*Salmonella typhi*
11.	EF579646	River water (Site I, II, III)	*Salmonella enterica*
12.	EF579647	River water (Site I, II, III)	*Enterobacter sp.*
13.	EF579648	River water (Site I, II, III)	*Salmonella typhimurium*
14.	EF579649	River water (Site I, II, III)	*Salmonella sp.*
15.	EF579650	River water (Site I, II, III)	*Salmonella enterica*
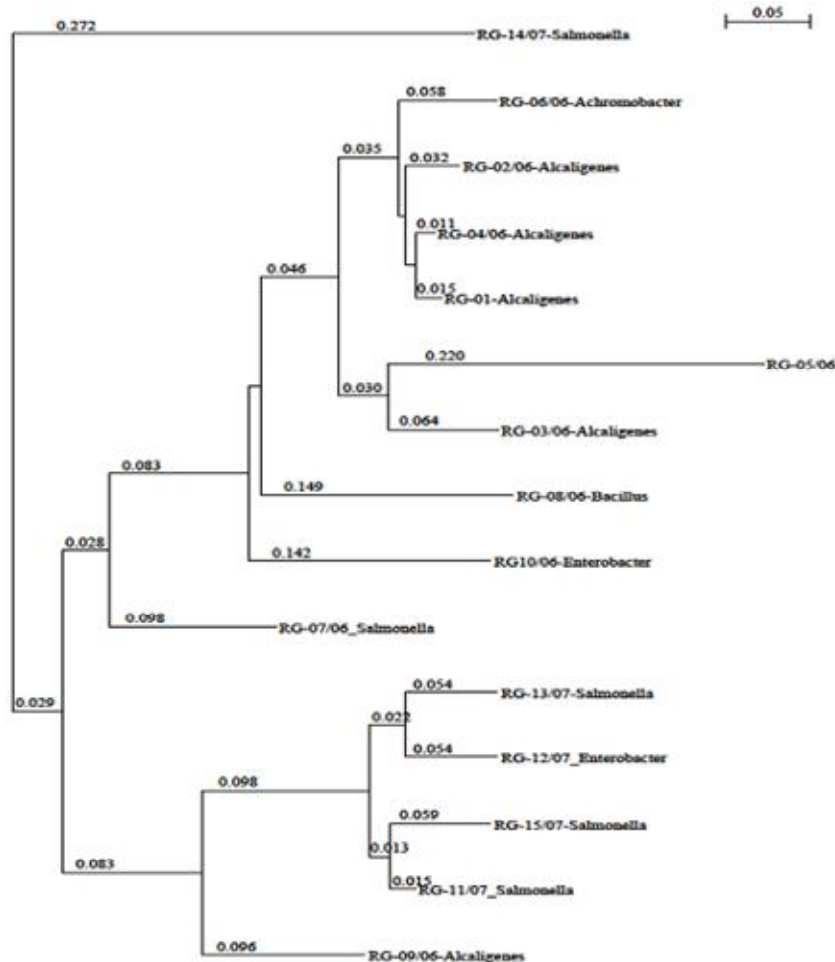
Fig 2. Phylogenetic tree for 16S rRNA sequence of bacterial isolates from Gundaru river basin

16S rRNA gene analysis provides accurate identification at the species level and can clarify their clinical importance. Comparison between a variety of identification systems including cellular fatty acid profiles, carbon source utilization and conventional biochemical identification with the 16S rRNA gene sequence to elevate both unusual aerobic gram negative bacilli and *coryneform* organisms isolated from clinical specimens (Jensen, 1985; Jones *et al.,* 1983). They found that 16S rRNA gene sequence provide more rapid, unambiguous identification could translate improved clinical outcomes. In the present study, phylogenetic and phonetic approaches to bacterial taxonomy were described and compared. Although a variety of molecular techniques were used in estimating the phylogenetic relatedness of bacteria, the comparison of 16S rRNA in bacterial isolates was submitted to the Genbank. Bacterial isolates from Gundaru river basin and their Genbank accession numbers are reported in Table 3.

Phylogenetic analysis

We generated a cladograme protein directed tree (Penny *et al.,* 1992) of 16S rRNA sequence of bacterial isolates from Gundaru river basin. The phylogenetic tree of these sequences shows RG-03 *Alcaligenes* & RG-05 *Brevundimonas* and RG-12 *Enterobacter &* RG-13 *Salmonella typhimurium* shows very much similarity between them. Comparison of 16S rRNAs have proven to be extremely useful for determining phylogenetic relationships among organisms from the level of domains to the level of closely related species. Phylogenetic tree generated using 16S rRNA sequence of bacterial isolates from Gundaru river basin is shown in the Fig 2.

Conclusions

In the present study on the microbial biodiversity of Gundaru river basin, it was found that, out of the five sites, Site I, II and Site III are highly polluted due to the dumping of solid waste by Tirumangalam municipality and mixing of sewage water into the river. It is disheartening to note that Salmonella pathogen is persistent in the soil as a result of this pollution. This could be avoided by establishing a sewage water treatment plant by the municipality. Moreover, the solid wastes could be dumped somewhere else keeping in mind of the pollution it causes to the river water and the health hazards it creates in general.

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