Isolation, characterization and molecular phylogeny of multiple metal tolerant and antibiotics resistant bacterial isolates from river Ganga, Varanasi, India

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Abstract: The present study was focused on the isolation of multiple metal tolerant and antibiotics resistant bacterial strains from water samples of five different Ghats of river Ganga, Varanasi, India. These strains were biochemically characterized and their phylogenetic relatedness was assumed using amplified ribosomal DNA restriction analysis fingerprinting and 16S ribosomal gene sequencing. The presence of heterogeneous groups of bacteria belonging to alpha, beta, gamma proteobacteria, and bacilli was noticed. Some of the bacterial strains like *Pseudomonas*, *Serratia*, *Enterobacter*, and *Proteus vulgaris* were mainly found at the Dashashwamedh Ghat and the Assi Ghat showing minimum inhibitory concentration 200–300 mg/L for copper, nickel, lead, and chromium. *Comamonas* sp. mainly isolated from the Samne Ghat and the Rajendra Prasad Ghat was able to grow at very high concentration of lead viz. 400 mg/L. Some of the strains showed multidrug resistant property against 10 different antibiotics which are of most serious concern because these drugs are frequently used against various bacterial infections.

Subjects: Environmental & Agriculture; Bioscience; Environmental Studies & Management

Keywords: bacterial isolates; multiple metal tolerant; antibiotics resistant; amplified ribosomal DNA restriction analysis; phylogeny

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PUBLIC INTEREST STATEMENT

Mixing of sewage along with industrial effluents into the Ganga river perturb water quality. Polluted water became a habitat of various pathogenic bacteria having dual properties in rendering multiple heavy metals tolerant capacity as well as antibiotics resistance. The present study was focused on the isolation of multiple metal tolerant and antibiotics resistant bacterial strains from water samples of five different Ghats of river Ganga, Varanasi, India. These strains were biochemically characterized and their phylogenetic relatedness was assumed using amplified ribosomal DNA restriction analysis fingerprinting and 16S ribosomal gene sequencing. Collaboration of physiological, biochemical, and molecular techniques could provide complete information about bacterial identification and characterization. Presence of harmful bacteria in the river Ganga invites serious attention from human health perspective since Ganga water is also used for drinking, holy bathing and irrigation purposes.
1. Introduction
Varanasi, India (25°20′ N, 83°00′ E) lies 8.77 m above sea level in the eastern Gangetic plains of India has at least 84 Ghats. Ghats are riverfront steps leading to the banks of river Ganga. The holy river Ganges and its Ghats are an abode for thousands of natives besides being a place visited by 300 people every hour in the mornings, and on festival days 2,500 people arrive per hour. According to World Health Organization (WHO) reports, in developing countries like India, almost 80% of the total water is being polluted by discharge of domestic sewage and untreated industrial effluents (Sangu & Sharma, 1987). The WHO reported (World Health Organization [WHO], 2002) that infectious disease like diarrhea, caused due to the intake of unsafe and extremely polluted water, is responsible for about 1.7 million deaths a year worldwide. Recently Times of India New Delhi September 2014 reported that the quality of Ganga water of two holy places Varanasi and Allahabad where millions take a dip everyday are most unfit for bathing purpose.

Water of river Ganga at Varanasi region is mainly polluted by industrial effluents, domestic sewage, and disposal of dead bodies (Mishra & Tripathi, 2007; Pandey, Shubhashish, & Pandey, 2010). About 1,503 industries (Chemical industries, metal processing industries, leather, and textile industries) are located in and around Varanasi (Anonymous, 2006; Industrial Directory, 1981; Rai & Tripathi, 2008). Escalating anthropogenic activities, indiscriminate urbanization, and accelerated industrialization gave birth to the river water pollution particularly heavy metals and microbial pollution (Rai, Mishra, & Tripathi, 2010). Either directly or indirectly metals have been known to play a major role in almost all metabolic processes, growth, and development of the microorganisms (Bergey & Breed, 1994; Beveridge & Doyle, 1989). However, these organisms are forced to adapt the increasing concentrations of metals beyond their normal tolerance level. Therefore, microbes have developed various biological mechanisms like complexation, metal efflux systems, utilization or reduction of the metal during anaerobic respiration in order to tolerate heavy metal stress (Ehrlich & Brierley, 1990). Metal resistant bacteria have the ability to grow under high concentrations of metals and can play an incredible role in the bioremediation of those metals (Bolan et al., 2014).

Introduction of antimicrobial agents such as antibiotics into the river and streams from industrial pollution as metals, emerged as an ever increasing health hazard due to spread of antibiotic resistant organism in water environment (Baquero, Martínez, & Cantón, 2008; Levy, 1997). Ability of different species of bacteria in acquiring multidrug resistance (MDR) has led to severe complication for human beings (Tenover & McGowan, 1996). A large proportion of MDR was found to contain plasmid borne mobile resistance genes that can transmit through bacterial populations (Kumarasamy et al., 2010).

Molecular techniques have initiated a new era of microbiology and may prove to be helpful in determining the inhabitants of freshwater microbial communities (Mukherjee, Kumar, Kumar, & Chakraborty, 2013). Sequences based on 16S ribosomal gene reveal numerous cultured as well as uncultured microorganisms in diverse environments as 16S ribosomal RNA (16S rRNA) molecules contain both highly conserved as well as variable regions (Woese, 1987). In the recent few years, amplified ribosomal DNA restriction analysis (ARDRA) has become a useful tool for assessment of microbial community structure changes as it is a simple, fast and reliable method for evaluation of bacterial diversity in different environments (Martínez-Murcia, Acinas, & Rodríguez-Valera, 1995).

In the present communication, the water quality of river Ganga has been analyzed using physicochemical properties. Isolation and biochemical characterization of multiple metal tolerant and antibiotics resistant bacteria have been investigated. Molecular characterization of isolates based on 16S rDNA gene sequences and ARDRA fingerprints were performed. The significance of the proposed study has been ascertained by applying statistical parameters such as pearson correlation/regression and principal component analysis (PCA) analysis.
2. Materials and methods

2.1. Sample collection and physicochemical analysis

The areas selected for the present study are based on the human use and pollution. Water samples were collected from the bank of five Ghats (5 km area) viz. Dhashashwamedh ghat, Harishchandra ghat, Samne ghat, Rajendra Prasad Ghat and Assi Ghat. Water samples (1 L) were collected monthly during the first week of each month from January 2014 to December 2014 from 15 cm depth at 8.00–10.00 am near bank of each Ghat in three replicate in 1 L acid washed glass bottles. Temperature, pH, and conductivity were measured on the spot using a thermometer, a pH-meter and a conductivity meter. Samples were then transported to the laboratory under sterile condition for isolation of bacteria and analysis of dissolved oxygen (DO), biochemical oxygen demand (BOD), total hardness (TH), total alkalinity (TA), calcium (Ca²⁺), magnesium (Mg²⁺), sulfate (SO₄²⁻), total dissolved solids (TDS), total nitrogen (TN) and total phosphorus (TP), the latter estimated using the American Public Health Association [APHA] (1998) method. Estimation of TN content was performed by following the micro Kjeldahl technique using a Gerhardt automatic analyser (model KB8S Kjeldatherm, Germany). Titrimetric methods were for testing DO, BOD and TA whereas a gravimetric method was used for TDS (Vogel, 1978). Turbidometric method was used for estimation of SO₄²⁻ (Vogel, 1978). An ethylenediaminetetraacetic acid titrimetric method was used to estimate Ca²⁺, Mg²⁺, and TH (Vogel, 1978). An atomic absorption spectrophotometer (Model 2380, Perkin Elmer, Inc. Norwalk, CT, USA), was used for determination of Cr, Cd, Ni, Pb, Zn, and Cu. A Pearson correlation matrix was constructed by using SPSS software version 16.0 in order to study correlation among physicochemical parameters at five Ghats of river Ganga Varanasi, India.

2.2. Isolation and maintenance of bacterial isolates

In order to get all types of bacterial strains, water samples from each Ghats were concentrated through centrifugation at 10,000 rpm for 15 min. Pellet was dissolved in 100 μL sterilized distilled water and spread on nutrient agar (NA) media containing 0.5% peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% NaCl, and incubated at 37°C. Numerous colonies were obtained on NA plates. The single bacterial colonies showing unique morphology were isolated and subcultured at least five times to ascertain purity of the isolates. The purity of the isolates was monitored routinely by microscopic analysis. Purified bacterial isolates were subjected to the biochemical and molecular identification tests.

2.3. Metal tolerance assays

2.3.1. Minimum inhibitory concentration of metals

The isolated pure bacterial cultures were checked for their respective minimum inhibitory concentrations (MICs) towards all the metal ions. Different concentrations of metals ions such as Zn, Cd, Cu, Ni, Pb, and Cr (0, 25, 50, and 100 mg/L) were added separately against each bacterial isolates incubated in solid NA medium and then kept for 24–48 h at 37°C in incubator. Those bacterial isolates, in which growth was visible up to 100 mg/L were further grown at higher metal concentration ranging from 150, 200, 250, 300, 350 to 400 mg/L. At that metal concentration where bacterial isolates failed to grow on plates even after 7 days of incubation were considered as the MIC for that particular bacterial strain (Narasimhulu, Rao, & Venu Vinod, 2010).

2.4. Antibiotics resistance assay

Bacterial strains were checked for antibiotics resistance pattern against ampicillin (Amp), chloramphenicol (Chl), nalidixic acid (Nal), tetracyclin (Tet), vancomycin (Van), ciprofloxacin (Cip), imipenem (Imi), erythromycin (Ery), streptomycin (Str), and polymixin (Pol) (Himedia, India) according to Kirby-Bauer disk diffusion method (Bauer, Kirby, Sherris, & Turck, 1966). A volume of 5 mL of nutrient broth was prepared for inoculating bacterial strains which were incubated overnight at 37°C. The pure broth cultures were then swabbed over the sterilized 20 mL Mueller Hinton pre-made agar plates. Disks of 6 mm diameter were prepared from sterilized Whatman filter paper. The 10 antibiotics at concentrations of 30 mg/L were delivered in 10 disks which were then dispensed on each of the
swabbed plates at appropriate distances from each other. Plates were then incubated at 37°C for 24 h. Zones of inhibition were measured in diameter across the centre of each zone in millimeters.

2.5. **Physiological characterization of isolated bacteria**
Gram staining was performed for differentiating Gram-positive and Gram-negative bacteria based on the differences in bacterial cell wall peptidoglycan layer (thick layer present in gram positive).

2.6. **Biochemical assay**

2.6.1. **Citrate test**
A volume of 100 mL Simmon citrate agar medium (15 g/L agar, 0.2 g/L ammonium dihydrogen phosphate, 0.08 g/L bromothymol blue, 0.8 g/L disodium ammonium phosphate, 0.2 g/L magnesium sulfate heptahydrate, 5 g/L sodium chloride, 2 g/L trisodium citrate and 15 g/L agar) pH 7.0 was prepared and then autoclaved. The sterilized Simmon citrate agar medium was poured in different glass tubes and placed obliquely to prepare slants. After that, the slants were streaked with different bacterial isolates and kept for 48 h in the incubator. The change in color from dark-green to blue was noticed after 48 h (Claus, 1989; Jawetz, 1989).

2.6.2. **Catalase test**
A small inoculum of bacterial isolates was transferred to a clean dry glass slide and few drops of hydrogen peroxide solution (3%) were added. Rapid evolvement of oxygen bubbles gave the positive result for catalase (Facklam & Elliott, 1995).

2.6.3. **Oxidase test**
One to two drops of Kovács oxidase reagent (1% tetra-methyl-p-phenylenediamine dihydrochloride, in water) (Sigma-Aldrich, USA) were placed on freshly grown bacteria on Nutrient agar media (Kovacs, 1956). Color change to dark purple gave positive test for oxidase.

2.6.4. **Indole test**
About 100 mL of tryptone broth was prepared and autoclaved. 7 mL of the medium was added in 20 test tubes. The bacterial colonies were inoculated in the test tubes exclusive of blank. These test tubes were kept at room temperature for 48 h. After 48 h 10 drops of Kovac’s reagent was added and kept for the color change (Isenberg & Sundheim, 1958).

2.6.5. **Urease test**
The bacterial isolates were inoculated in the urea agar media containing pH indicator phenol red and kept under 37°C under incubator for 24–48 h. Color change to deep pink color gave the positive reaction for the presence of urease (Seeliger, 1955).

2.7. **Molecular characterizations**

2.7.1. **Isolation of genomic DNA**
DNA was extracted from the cultures growing on nutrient agar media from 24 h, 37°C using Himedia Bacterial DNA purification kit (MB505). 16S ribosomal gene was amplified using 16S rDNA bacteria specific primers 27F forward (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′). The PCR amplification of 16S rDNA was performed in 25 μL aliquots containing 20-50 μg DNA template, 0.4 μmol/L of each primers, 1.5 mmol MgCl₂, 200 mmol dNTPs and 1U/μL Taq Polymerase. The PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide in 1X TBE buffer and visualized under gel documentation system.

2.7.2. **Cloning and sequencing**
The amplified 16S ribosomal RNA was purified from agarose (1.2% w/v) using QIA quick Gel extraction Kit according to manufacturer’s instruction. The purified PCR products were cloned in pGEM-T vector and transformed into competent E. coli DH5α cells using standard protocols. The plasmid
insert were amplified using vector specific primer SP6 (5'-GATTTAGGTGACACTATA-3') and T7 (5'-TAATACGACTCACTATAG-3'). PCR was performed in 25 μl aliquots containing 20–50 μg DNA templates, 0.4 μM of each primers, 1.5 μM MgCl₂, 200 μM dNTPs and 1U/μl Taq Polymerase. The PCR conditions comprised an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 50 s with final extension at 72°C for 20 min. The PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide in 1X TBE buffer and visualized under gel documentation system. The bands were cut and eluted using Qiagen quick Gel Extraction kit and eluted amplified products sent directly for sequencing (Sci Genome Cochin, Kerala, India).

2.7.3. ARDRA
ARDRA was performed prior to sequencing in order to determine number of genotypes and genetic diversity among bacterial strains. Generated amplified fragments of 16S ribosomal RNA were digested with restriction endonuclease AluI, HaeIII (Arya, Joshi, Gupta, Kumar, & Raturi, 2015) and AfaI/MspI (Zeng, Liu, Li, Yu, & Chen, 2007) in accordance with manufacturer’s instruction. The restriction products were resolved in 2% agarose (w/v solution of agarose in TBE buffer containing 1 μg/ mL of ethidium bromide). Gels were visualized using Bio Rad Gel Documentation system. All reactions were repeated at least three times for each restriction enzyme to ascertain the reproducibility of banding pattern.

2.7.4. Phylogenetic analysis of ARDRA fingerprints
The presence and absence of distinct and reproducible bands in each of the individual DNA fingerprinting pattern generated by AluI, HaeIII, and AfaI/MspI profiles were converted into binary data, and the pooled binary data were used to construct composite dendrogram respectively. The Biodiversity Pro software (Version 2) was used to perform the phylogenetic analysis using Bray-Curtis Cluster analysis.

2.7.5. Principal component analysis
DNA fingerprints obtained using all the 4 restriction endonucleases viz. AluI, HaeIII, and AfaI/MspI were utilized for the PCA. The software Sigma plot 11 was used to generate the graphical representation of the values generated by Biodiversity Pro software version 2.0.

2.7.6. Sequence analysis and construction of phylogenetic tree
Sequences of all the 20 isolates were validated by comparing them with sequences available in a public database (Ribosomal Database Project release 9.43 [RDP]) (Cole et al., 2005). The 16S rRNA sequences obtained were then subjected to nucleotide basic algorithm search tool (Blastn) [http://blast.ncbi.nlm.nih.gov/Blast.cgi] and aligned with the most similar bacterial species and then submitted to the NCBI database using the submission tool Sequin. Sequences exhibiting close relatedness in a BLAST search were used for construction of phylogenetic tree by neighbor joining algorithm method with the aim to study their evolutionary history (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) showed above the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method (Jukes & Cantor, 1969) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 120 positions in the final data-set. Evolutionary analyses were conducted by using MEGA 6 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

3. Results and discussion
In the present study, attempts have been made to isolate different metal tolerant as well as antibiotic resistant bacterial strains from the banks of Ghats of river Ganga and characterize them using various biochemical and modern molecular techniques. In order to analyze water quality, physicochemical characteristics of Ganga water near Ghats was performed and found to be higher than maximum permissible limit set by Indian Standard Specification for Drinking Water (IS: 10500)
Throughout the year (Table 1). Out of five selected Ghats, Assi ghat and Dashashwamedh ghat were found to be most polluted Ghats. Water quality of the Assi ghat has drastically deteriorated in the recent past as Assi Nala (Sewage) is directly connected to the Assi Ghat which is the mixing point of untreated waste water, domestic water and industrial pollutants with the river water. But in Dashashwamedh ghat, extreme level of human interference is possibly the major reason of increased water pollution. Level of nutrients viz. total nitrogen and total phosphorus were found to be higher in the Harishchandra ghat which is mainly due to the combustion of human bodies on this particular ghat. Other factors like total alkalinity and total dissolved solids were also high in the same ghat because washing of clothes is also very frequent. The level of heavy metals was found much beyond the safe limit at all the Ghats of river Ganga. According to Indian Standard Specifications for Drinking Water IS: 10500 safe limit (Indian Standard Specifications For Drinking Water IS: 10500, 2012), Cd concentrations beyond 0.01 mg/L becomes toxic, Cr above limit of 0.05 becomes carcinogenic and Pb beyond 0.1 could cause blood cancer in human. Atmospheric deposition of metals emitted from vehicles, direct anthropogenic activities and discharge of untreated industrial effluents as well as domestic sewages into the river could be the possible reason of metal load in river Ganga (Pandey et al., 2010). The higher level of Cu in water might be due to corrosion of pipes, fittings, and utensils.

A marked correlation between physico-chemical properties of water collected from different Ghats was observed after performing the statistical analysis using Pearson’s formula of correlation and regression (Table 2). Metals like Zn, Fe, and Ni showed significant correlation with different abiotic factors. At higher concentrations, metals exert toxic effects by forming complexes with organic compounds and toxic effects of metals can change by changing metals structure (Das et al., 2013).

| Parameters | Site 1 | Site 2 | Site 3 | Site 4 | Site 5 |
|------------|--------|--------|--------|--------|--------|
| pH         | 7.6 ± 1 | 7.6 ± 0.5 | 7.7 ± 0.5 | 7.4 ± 0.5 | 7.7 ± 1 |
| Temperature (°C) | 27.9 ± 15 | 27.8 ± 16 | 27.8 ± 15 | 27.6 ± 16 | 27.9 ± 15 |
| EC (μmho/cm) | 297 ± 1 | 296 ± 5 | 289 ± 3 | 290 ± 3 | 299 ± 1 |
| BOD | 11.7 ± 2 | 9.8 ± 1 | 9.9 ± 1 | 10.7 ± 1 | 12.3 ± 2 |
| DO | 5.9 ± 0.1 | 5.6 ± 0.01 | 5.2 ± 0.1 | 5.8 ± 0.1 | 6.1 ± 0.1 |
| TN (mg/L) | 3.28 ± 0.1 | 4.21 ± 0.1 | 4.34 ± 0.1 | 3.39 ± 0.1 | 3.39 ± 0.1 |
| TP (mg/L) | 0.15 ± 0.1 | 0.14 ± 0.01 | 0.16 ± 0.1 | 0.14 ± 0.01 | 0.17 ± 0.1 |
| TA (mg/L) | 236 ± 5 | 223 ± 4 | 221 ± 5 | 229 ± 3 | 245 ± 2 |
| TDS (mg/L) | 456 ± 5 | 439 ± 5 | 459 ± 5 | 439 ± 6 | 457 ± 4 |
| TH (as CaCO3) (mg/L) | 235 ± 1 | 230 ± 1 | 239 ± 2 | 229 ± 1 | 238 ± 2 |
| Sulfate (mg/L) | 9.1 ± 0.1 | 7.6 ± 0.1 | 6.1 ± 0.1 | 6.7 ± 0.1 | 9.8 ± 0.1 |
| Magnesium (mg/L) | 1.25 ± 0.1 | 1.11 ± 0.01 | 1.18 ± 0.01 | 1.28 ± 0.1 | 1.38 ± 0.1 |
| Calcium (mg/L) | 37.2 ± 0.1 | 29.6 ± 0.1 | 28.1 ± 0.1 | 33.5 ± 0.1 | 38.5 ± 0.1 |
| Cadmium (mg/L) | 0.08 ± 0.01 | 0.02 ± 0.01 | 0.02 ± 0.01 | 0.05 ± 0.01 | 0.15 ± 0.01 |
| Zinc (mg/L) | 0.25 ± 0.01 | 0.21 ± 0.02 | 0.20 ± 0.1 | 0.25 ± 0.1 | 0.27 ± 0.1 |
| Iron (mg/L) | 0.59 ± 0.3 | 0.64 ± 0.05 | 0.68 ± 0.4 | 0.59 ± 0.3 | 0.61 ± 0.2 |
| Copper (mg/L) | 0.05±0.03 | 0.02 ± 0.02 | 0.03 ± 0.02 | 0.03 ± 0.02 | 0.08 ± 0.01 |
| Lead (mg/L) | 0.09 ± 0.01 | 0.06 ± 0.01 | 0.17 ± 0.01 | 0.19 ± 0.01 | 0.09 ± 0.02 |
| Nickel (mg/L) | 0.31 ± 0.1 | 0.27 ± 0.1 | 0.22 ± 0.1 | 0.31 ± 0.5 | 0.35 ± 0.5 |
| Chromium (mg/L) | 0.06 ± 0.01 | 0.03 ± 0.01 | 0.08 ± 0.02 | 0.05 ± 0.02 | 0.09 ± 0.01 |

Notes: ± Represents mean standard deviation with three replicates; note that the values are minimum and maximum; Site 1-Dashashwamedh Ghat, Site 2-Harishchandra Ghat, Site 3-Samne Ghat, Site 4-Rajendra Prasad Ghat, Site 5-Assi Ghat; EC-Electrical conductivity, BOD-Biological oxygen demand, DO-Dissolved oxygen, TN-Total nitrogen, TP-Total phosphorus, TA-Total alkalinity, TDS-Total dissolved solid, TH-Total hardness.
### Table 2. Pearson correlation coefficient between physicochemical properties of 5 Ghats of river Ganga Varanasi, India

|       | Temp. | pH    | EC    | BOD   | DO    | TN    | TP    | TA    | TDS   | TH    | SO$_4^{2-}$ | Cd    | Zn    | Fe    | Cu    | Pb    | Ni    | Ca    | Mg    | Cr    |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Temp. | 1.000 | 0.349 | 0.298 | 0.315 | 0.497 | 0.002 | 0.166 | 0.576 | 0.285 | 0.329 | 0.203       | 0.617 | 0.435 | 0.919(*)| 0.639 | 0.617 | 0.451 | 0.628 | −0.569| 0.184 |
| pH    | 1.000 | 0.901 | 0.492 | 0.535 | 0.239 | 0.179 | 0.793 | 0.525 | 0.815 | 0.219 | 0.408       | 0.468 | 0.593 | 0.531 | 0.408 | 0.933 | 0.156 | −0.357| 0.791 |
| EC    | 1.000 | 0.659 | 0.768 | 0.612 | 0.518 | 0.931 | 0.494 | 0.917(*)| 0.466 | 0.665 | 0.633       | 0.491 | 0.660 | 0.665 | 0.853 | 0.055 | 0.032 | 0.859 |
| BOD   | 1.000 | 0.921(*) | 0.356 | 0.880(*)| 0.746(*)| −0.282 | 0.894(*)| 0.954(**)| 0.452 | 0.066 | 0.195       | 0.185 | 0.452 | 0.725 | 0.606 | 0.201 | 0.195 |
| DO    | 1.000 | 0.602 | 0.892(*)| 0.904(*)| 0.028 | 0.888(*)| 0.869(*)| 0.764 | 0.429 | 0.423 | 0.535       | 0.764 | 0.691 | 0.460 | 0.218 | 0.423 |
| TN    | 1.000 | 0.640 | 0.612 | 0.421 | 0.459 | 0.393 | 0.788 | 0.751 | 0.090 | 0.653 | 0.788       | 0.375 | 0.691 | 0.460 | 0.218 | 0.423 |
| TP    | 1.000 | 0.640 | −0.283| 0.704 | 0.953 | 0.584 | 0.191 | 0.000 | 0.238 | 0.584 | 0.377       | 0.339 | 0.584 | 0.151 |       |       |       |       |
| TA    | 1.000 | 0.419 | 0.907(*)| 0.591 | 0.832 | 0.675 | 0.659 | 0.764 | 0.832 | 0.821 | 0.283       | −0.019| 0.732 |
| TDS   | 1.000 | 0.125 | −0.456| 0.532 | 0.866(*)| 0.607 | 0.808 | 0.532 | 0.226 | −0.498| −0.252       | 0.846 |
| TH    | 1.000 | 0.739 | 0.553 | 0.345 | 0.389 | 0.431 | 0.553 | 0.910(*)| 0.380 | 0.050 | 0.584       |
| SO$_4^{2-}$ | 1.000 | 0.608 | −0.033| 0.000 | 0.063 | 0.068 | 0.485 | 0.564 | 0.408 | 0.000 |           |
| Cd    | 1.000 | 0.873(*)| 0.645 | 0.919(*)| 0.873 | 0.269 | −0.341 | 0.055 | 0.080 | 0.845 |           |
| Zn    | 1.000 | 0.791 | 0.643 | 0.568 | 0.375 | −0.645| 0.500 |
| Fe    | 1.000 | 0.999(*)| 0.395 | −0.104| −0.102| 0.791 |
| Cu    | 1.000 | 0.381 | 0.048 | 0.167 | 0.645 |
| Pb    | 1.000 | 0.473 | −0.352| 0.568 |
| Ni    | 1.000 | −0.436| −0.375 |
| Ca    | 1.000 | 0.000 |
| Mg    | 1.000 | 0.000 |
| Cr    | 1.000 | 0.000 |

*Correlation is significant at 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).
A total of 115 culturable bacteria were isolated on the basis of their morphological and biochemical properties. Of these, 20 were selected for further studies due to their ability to tolerate toxicity of six different metals (Zn, Cd, Cu, Ni, Pb, and Cr) and their resistance against 10 different antibiotics. Bacterial isolates viz. *Enterobacter* sp., *Serratia* sp., *Pseudomonas fluorescens* and *Pseudomonas pseudoalcaligenes* were able to grow at very high concentrations of various heavy metals such as Cu, Ni, Pb, and Cr. The minimum growth inhibitory concentrations of these metals were in between 300 and 400 mg/L (Table 3).

*Comamonas* has recovered mainly from Samne ghat and Rajendra Prasad ghat where the concentration of Pb was considerably high. Industrial effluents are thought to be the major source of high Pb in these sites. The abundance of *Comamonas* in these extremely Pb polluted sites might be due to their genomic adaptation and subsequent increased tolerance to Pb. Occurrence and activity of *Comamonas* strains have already been reported in Pb contaminated soils (Konopka et al., 1999). Stoppel and Schlegel, (1995) also reported metal tolerant strain of *Comamonas* sp. Although many *Comamonas* strains has been isolated from clinical samples and regarded as opportunistic pathogens (Willems et al., 1991). The persistent presence of *Serratia, Pseudomonas pseudoalcaligenes, Enterobacter* sp., *Proteus vulgaris* and *Shewanella* sp. in the water sample collected from the Assi ghat and Dashashwamedh ghat was observed throughout year. Along with high MIC towards heavy metals, these isolates also showed MDR against Amp, Chl, Nal, Tet, Van, Imi, Cip, Ery, Pol, and Str (Table 4). Zone of inhibition of different antibiotics (Amp, Chl, Nal, Tet, Van, Imi, Cip, Ery, Pol, and Str) against sensitive bacterial strains were found in the range of 5.02 to 11.23 mm. Except *Serratia, Pseudomonas pseudoalcaligenes, Comamonas*, and *Proteus vulgaris*, all the other strains were sensitive towards imipenem, a β-lactam carbapenem (Yong et al., 2002). Maximum zone of inhibition produced by imipenem in *Aeromonas hydrophila* and *Ochrobactrum intermedium* viz. 11.03 and 11.23 mm, respectively. Therefore, the ability of these bacterial strains to overcome the toxic effects of different drugs pose a serious problem because these drugs are frequently used against bacterial infection. Imipenem is a high end broad spectrum drug mainly used to combat nosocomial
infections. In the Assi ghat, city municipal waste and untreated sewage are the pervasive source of water pollution and also a suitable environment for the growth of human pathogenic bacteria. These bacteria are known for colonizing the respiratory and urinary tract and have also the ability to cause meningitis, arthritis and wound infection. In Dashashwamedh ghat, uncontrolled human interferences and other various activities like boat landing have created water logging condition where harmful bacteria grow in copious amount. Aeromonas, Kurthia, Ochrobactrum, and Brucella were the usual bacterial strains in all the sites throughout year. Morphological and biochemical characteristics of bacterial strains are mentioned in Table 5.

The advent of tools in molecular biology has revolutionized the process of strain identification and saved money, time and energy too. In this study ARDRA was used as a model to examine the capacity of restriction-based techniques for bacterial strain identification, and the possibility of generating phylogenetic information from ARDRA-based dendrograms. Approx. 1.3 kb 16S ribosomal RNA amplified product was observed. ARDRA concerning the 16S ribosomal RNA amplified product of bacterial isolates digested by AluI, HaeIII and AfaI/MspI were reproducible yielded 2–5, 3–7, and 2–4 bands respectively. Band pattern of each isolates represented particular ARDRA genotypes. AluI showed similar banding pattern for Aeromonas hydrophila and Aeromonas sp., Shewanella decolorationis, and Shewanella, Comamonas terrigena and Comamonas sp., Pseudomonas fluorescens, and Pseudomonas pseudoalcaligenes as shown in Figure 1a. Also double restriction endonuclease digestion AfaI/MspI was failed to differentiate most of the bacterial isolates and yielded lowest number of genotypes showing in Figure 2a. However, HaeIII discriminated all the strains on the basis of band pattern and generated species specific patterns as shown in Figure 3a which was further confirmed by sequencing data that all isolates belong to different species. Therefore, it was found that out of all the restriction endonuclease, HaeIII could able to detect interspecies and interstrain variability of cultivable fresh water bacterial strains. Restriction endonuclease viz. HaeIII has also been used for bacterial identification and diversity assessment by many workers (Heyndrickx, Vauterin, Vandamme, Kersters, & De Vos, 1996; Sun et al., 2008).

| Bacterial isolates         | Zn (mg/L) | Cd (mg/L) | Cu (mg/L) | Ni (mg/L) | Pb (mg/L) | Cr (mg/L) |
|----------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Providencia sp.            | 150       | 150       | 200       | 200       | 150       | 150       |
| Shewanella sp.             | 150       | 150       | 150       | 150       | 150       | 150       |
| Shewanella sp.             | 150       | 150       | 150       | 150       | 200       | 150       |
| Shewanella decolorationis  | 200       | 200       | 300       | 200       | 200       | 200       |
| Brucella sp.               | 200       | 150       | 150       | 200       | 200       | 200       |
| Enterobacter sp.           | 200       | 200       | 300       | 350       | 350       | 350       |
| Serratia sp.               | 200       | 200       | 350       | 400       | 350       | 300       |
| Kurthia sp.                | 200       | 200       | 200       | 200       | 150       | 150       |
| Kurthia gibsonii           | 200       | 200       | 200       | 300       | 200       | 150       |
| Rummelbacillus             | 150       | 100       | 150       | 200       | 150       | 150       |
| Comamonas sp.              | 150       | 150       | 150       | 200       | 400       | 150       |
| Comamonas terrigena        | 150       | 150       | 200       | 200       | 400       | 150       |
| Proteus vulgaris           | 150       | 150       | 200       | 200       | 200       | 150       |
| Exiguobacterium sp.        | 200       | 150       | 150       | 200       | 200       | 150       |
| Aeromonas sp.              | 150       | 100       | 150       | 150       | 150       | 150       |
| Stenotrophomonas koreensis | 150       | 100       | 150       | 150       | 150       | 150       |
| Aeromonas hydrophila       | 150       | 100       | 150       | 150       | 150       | 150       |
| Pseudomonas fluorescens    | 200       | 200       | 300       | 350       | 300       | 250       |
| Pseudomonas pseudoalcaligenes | 200   | 200       | 200       | 350       | 300       | 300       |
| Ochrobactrum intermedium   | 100       | 100       | 150       | 100       | 100       | 150       |
### Table 5. Morphological and biochemical characteristics of bacterial isolates

| Bacterial isolates        | Colony Color | Colony texture | Shape       | Gram nature | Catalase | Oxidase | Citrate | Indole | Urease |
|---------------------------|--------------|----------------|-------------|-------------|----------|---------|---------|--------|--------|
| Providencia sp.           | Dull gray    | Opaque         | Rod shape   | G−          | +        | −       | +       | +      | +      |
| Shewanella sp.            | Brownish     | Round          | Rod shape   | G−          | +        | +       | −       | −      | −      |
| Shewanella sp.            | Pale white   | Round          | Rod shape   | G−          | +        | −       | +       | −      | −      |
| Shewanella decolorationis| Pale white   | Round          | Rod shape   | G−          | +        | +       | +       | +      | −      |
| Brucella sp.              | Light gray   | Punctate       | Coccobacilli| G          | +        | +       | −       | −      | +      |
| Enterobacter sp.          | Shiny tan    | Round          | Rod shape   | G−          | −        | −       | +       | −      | −      |
| Serratia marcescens       | Shiny red    | Round          | Rod shape   | G          | +        | −       | −       | −      | +      |
| Kurthia sp.               | Cream        | Round          | Rod shape   | G+          | +        | +       | −       | −      | −      |
| Kurthia gibsonii          | Cream        | Round          | Rod shape   | G+          | +        | +       | −       | −      | −      |
| Rummellibacillus sp.      | Cream        | Round          | Rod shape   | G+          | +        | +       | −       | −      | −      |
| Comamonas sp.             | Cream        | Round          | Rod shape   | G          | +        | +       | −       | −      | −      |
| Comamonas terrigena       | Cream        | Round          | Rod shape   | G          | +        | +       | −       | −      | −      |
| Proteus vulgaris          | Gray         | Round          | Rod shape   | G          | +        | −       | −       | +      | −      |
| Exiguobacterium sp.       | Pale yellow  | Round          | Rod shape   | G+          | +        | −       | −       | −      | −      |
| Aeromonas sp.             | White        | Round          | Rod shape   | G          | +        | +       | −       | −      | −      |
| Stenotrophomonas koreensis| Cream        | Round          | Rod shape   | G          | +        | +       | −       | −      | −      |
| Aeromonas hydrophila      | Cream        | Round          | Rod shape   | G          | +        | +       | −       | −      | −      |
| Pseudomonas fluorescens   | Gray         | Round          | Rod shape   | G          | +        | +       | −       | −      | +      |
| Pseudomonas pseudoalcaligenes | Gray         | Round          | Rod shape   | G          | +        | +       | −       | −      | −      |
| Ochrobactrum intermedium  | Pale         | Round          | Rod shape   | G          | +        | +       | −       | −      | +      |
In order to study evolutionary relatedness, phylogenetic assessment of isolated bacterial strain was performed using ARDRA fingerprints. The AluI fingerprints based phylogenetic tree revealed two major clusters i.e. clusters I and cluster II shown in Figure 1b. The cluster I was divided into subcluster IA and IB. Subcluster IA was again divided into subcluster IA.a and IA.b where cluster IA.a contained Stenotrophomonas as a single genotype. In subcluster IA.b Exiguobacterium sp. and Rummellibacillus sp. belongs to bacilli group formed a close clustering. Cluster IB contained Brucella sp. and Ochrobacterium intermedium formed a tight cluster belongs to alphaproteobacteria group.

Cluster II was bigger and divided into subclusters IIA, IIB and IIC. Cluster IIA showed tight cluster between Stenotrophomonas sp. and Exiguobacterium sp. Although cluster IIB gave clear picture of close cluster between bacilli group members viz. Kurthia sp. and Kurthia gibsonii. Further IIC divided into many subclusters like IIC.a, IIC.b, IIC.c, and IIC.d where intermixing was served very prominently between alpha, beta, gammaproteobacteria and bacilli members.

AfaI/MspI fingerprints based phylogenetic tree showed two major clusters i.e. cluster I and cluster II as in Figure 2b. Cluster I comprised Rummellibacillus sp. as a single genotype. Cluster II was a major cluster divided into subclusters IIA, IIB, and IIC. Cluster IIA showed tight cluster between Stenotrophomonas sp. and Exiguobacterium sp. Although cluster IIB gave clear picture of close cluster between bacilli group members viz. Kurthia sp. and Kurthia gibsonii. Further IIC divided into many subclusters like IIC.a, IIC.b, IIC.c, and IIC.d where intermixing was served very prominently between alpha, beta, gammaproteobacteria and bacilli members.
Dendrogram based on HaeIII fingerprints revealed the presence of 2 clusters out of which the cluster II was the largest one and divided into many subclusters represented members of all the groups like alpha, beta, gammaproteobacteria and bacilli. Whereas cluster I comprised Enterobacter sp. as an outgroup shown in Figure 3b.
The dendrogram generated after combining all DNA fingerprints obtained from ARDRA using AluI, HaeIII and AfaI/MspI showed two clusters i.e. cluster I and cluster II as depicted in Figure 4a. Cluster I revealed members of alphaproteobacteria. Cluster II was a major cluster divided into cluster IIA, IIB, IIC and IID. Cluster IIA further subdivided into subcluster IIA.a, IIA.b and IIA.c. Members of betaproteobacteria showed a tight clustering in cluster IIA.a. Cluster IIA.b comprised members of bacilli group viz. Kurthia sp. and Kurthia gibsonii whereas in cluster IIA.c included members of gammaproteobacteria. Cluster IIB comprised Pseudomonas fluorescens and Pseudomonas pseudoalcaligenes. Cluster IIC represented Stenotrophomonas sp. as a single genotype. Cluster IID subdivided into subcluster IID.a, IID.b and IID.c. Subcluster IID.a included bacilli group members whereas subcluster IID.b and IID.c comprised members of gammaproteobacteria.

Based on the result of ARDRA fingerprinting and phylogenetic characterization of bacterial isolates, we could say that ARDRA is a robust means which can generate species specific pattern for rapid assessment of microbial diversity. Representative strain of each ARDRA fingerprints could be chosen for 16S ribosomal RNA sequencing for obtaining information about what type of microorganism present in the sample. The reliability of ARDRA technique in congruence with the phylogenetic analysis for microbial diversity study was also proved by many workers (Arzu, Nilgun, Birgul, & Cumhur, 2012; Baik et al., 2008). Vaneechoutte and Heyndrickx (2001) mentioned ARDRA technique as a new approach for bacterial identification, phylogeny and taxonomy study. Moyer, Tiedje, Dobbs, and Karl (1996) compared phylogenetic trees based on ARDRA profiles and 16S rDNA sequences and therefore concluded that using different types of restriction endonucleases could be able to yield 76–100% success in obtaining phylogenetic affiliations accurately. Although, more studies on efficiency of ARDRA with other restriction endonucleases required with respect to fresh water bacterial community.
Furthermore, the fingerprints obtained using all the 4 restriction endonucleases viz. *Alu* I, *Hae* III and *Afa*I/*Msp*I were utilized for the PCAPCA in order to check whether phylogenetic tree were in coherence with PCA or not. PCA revealed the presence of seven major clusters and two minor clusters showing in Figure 4b. In the major clusters, Aeromonas sp., Aeromonas hydrophila, *Serratia marcescens*, Providencia sp. and Proteus vulgaris represented members of group gammaproteobacteria settled into one cluster. Members of betaproteobacteria viz. *Comamonas* sp. and *Comamonas terrigena* occupied single cluster, whereas members of bacilli groups were represented into two separate clusters. Members of gammaproteobacteria viz. *Pseudomonas fluorescens*, *Pseudomonas pseudoalcaligenes*, *Shewanella* sp. and *Shewanella decolorationis* were occupied two major clusters. In the major clusters, members of alphaproteobacteria viz. *Brucella* sp. and *Ochrobactrum intermedium* comprised in separate cluster. In minor clusters *Enterobacter* sp. as well as *Stenotrophomonas* sp. represented outgroup. Thus the phylogenetic tree and the PCA gave a clear picture of evolutionary lineage of bacterial isolates.

After comparing ARDRA fingerprints of all the isolated strains, 16S ribosomal RNA amplified products were cloned using competent cell of *E.coli* DH5α. Blue and white colonies were appeared on the petriplate. White colonies represented the recombinants. The cloned 16S ribosomal RNA were sequenced and then sequences were submitted to the NCBI databank where accession numbers were assigned (KP994633, KP994626, KR063129, KR063128, KR063114, KP994630, KP994627, KR063119, KR063118, KR063127, KR063115, KR063116, KP994628, KR063129, KR063111, KR063130, KR063124, KR063123, KR063125, KR063121).

The phylogenetic tree was constructed by using neighbor-joining method (Figure 5). Bootstrap values (%) were based on 1,000 replicates and more than 50% bootstrap values were shown at the branch point. The sequenced 20 bacterial strains grouped into four major classes; alpha, beta, gammaproteobacteria and bacilli. The dominant group was gammaproteobacteria having 60% bacterial isolates, only 10% isolates belongs to alphaproteobacteria, 10% were betaproteobacteria, and 20% were bacilli.

Therefore, it is worth mentioning that combination of physiological, biochemical and molecular methods gives clear cut picture about the existence of multiple metal tolerant and antibiotics resistant bacterial community in fresh water system.
4. Conclusion
Mixing of sewage along with industrial effluents into the Ganga river perturb water quality. Polluted water became a habitat of various pathogenic bacteria having dual properties in rendering multiple heavy metals tolerant capacity as well as antibiotics resistance. Collaboration of physiological, biochemical and molecular techniques could provide complete information about bacterial identification and characterization. Presence of harmful bacteria in the river Ganga invites serious attention from human health perspective since Ganga water is also used for drinking, holy bathing and irrigation purposes.

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