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ORIGINAL PAPER

Antibiotic resistance and genetic diversity in water-borne *Enterobacteriaceae* isolates from recreational and drinking water sources

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Abstract A total of 240 water-borne bacteria including 72 *Escherichia coli*, 83 *Enterobacter*, 30 *Klebsiella*, 36 *Salmonella* and 19 *Shigella* spp. isolates from drinking and recreational water sources were assessed for antibiotic resistance and genetic diversity. *Escherichia coli* (88.89 %) and species of *Enterobacter* (86.75 %), *Klebsiella* (83.33 %) and *Salmonella* (100 %) were resistant to cefadroxil, while >94 % *Shigella* spp. were resistant to cefaclor and cefuroxime. Ofloxacin was the most effective antibiotic against isolates of all the genera. Multiple antibiotic resistance index identified dug well, pond and piped water supplies as high risk sources of enteric pathogens. Random amplified polymorphic DNA analysis and restriction fragment length polymorphism of amplified 16S rRNA gene were studied for genetic relatedness of *Enterobacteriaceae* isolates. Primer P1254 identified 10, 16, 4, 4 and 1 distinct random amplified polymorphic DNA group(s) of *E. coli*, *Enterobacter*, *Klebsiella*, *Salmonella* and *Shigella* species, respectively. Unlike random amplified polymorphic DNA, restriction fragment length polymorphism using *Alu*I and *Hae*III could not segregate isolates in different genetic profiles. 16S rRNA gene of three *Enterobacter* spp. strains from different sources with similar restriction fragment length polymorphism but different random amplified polymorphic DNA patterns was sequenced, and identified as *Enterobacter hormaechei* strains skg0061, 0062 and 0063. The sequence information has been submitted to GenBank (HQ322393-95). Biochemically similar but genetically diverse *Enterobacteriaceae* members from drinking and recreational water sources exhibited varying antibiotic sensitivity. Contamination of water sources with such multiple antibiotic-resistant enteric pathogens poses threat to human health.

Keywords Water sources · Enteric bacteria · 16S rRNA gene · Multiple antibiotic resistance index · Random amplified polymorphic DNA

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Introduction

Water is a vital source of life on the earth. The urban development and improved agriculture practices have been leading to shortage and deterioration of water quality. The underground water is becoming highly polluted due to increased anthropogenic activities. Every effort should be made to achieve safest quality water (WHO 2008). Diarrheal diseases are the major health risk associated with water-borne microbial pathogens. The pathogenic members of *Enterobacteriaceae* causing gastroenteritis are frequently reported from recreational as well as drinking waters (WHO 2008; Bhagat 2011).

Ayodhya-Faizabad, twin holy Hindu pilgrimage Indian cities with 2,643 km^2 area and 2.76 million population are located on the bank of river Saryu (Ghaghra) originating from Himalaya in Nepal. Water in the region is available from river, kunds (holy ponds), ponds, tube well, hand pumps, piped supply and dug wells. The drinking water source is primarily hand pump and piped water while others are used for religious and agricultural activities. The presence of *Enterobacteriaceae* members in water has been considered as an indicator of faecal contamination (Tewari

et al. 2003; Kumar et al. 2012). It is correlated with several undocumented outbreaks of gastrointestinal infection causing diarrheal diseases among native as well as visiting population of twin densely populated cities Ayodhya and Faizabad. The diarrheal diseases associated with *Enterobacteriaceae* are major health problems of the population living with inadequate sanitation and hygiene in developing countries (PulseNetUSA 2004).

Resistance to multiple antibiotics is recognized as an emerging problem worldwide. An indiscriminate use of antibiotics is considered the most important reason for emergence, selection and dissemination of antibiotic-resistant pathogenic bacteria (Sayah et al. 2005). Over the time, selection pressure allows resistant bacteria to evolve specific pattern for resistance to the antimicrobial agents (Troy et al. 2002). The extensive use and abuse of antibiotics in human infectious disease treatment has resulted in co-existence of multiple antibiotic-resistant and -sensitive bacteria in the natural reservoirs. Multiple antibiotic resistance (MAR) indexing is a useful tool for assessment of high-risk environments contaminated with multiple antibiotic-resistant pathogens (Krumperman 1983).

The water-borne enteric pathogens are identified microbiologically, biochemically, serologically and genetically. The conventional microbiological and biochemical methods inadequately differentiate closely related organisms, and are time consuming. Nowadays, various typing techniques of moderate discriminatory ability such as biotyping, serotyping, phage typing and antibiogram typing are in vogue (Kilic et al. 2009).

Molecular approaches are most rapid and definitive for identification of pathogenic enterobacteria (Li et al. 2004). Random amplified polymorphic DNA (RAPD) analysis is widely used to differentiate *Enterobacteriaceae* members, viz., *Salmonella* serotypes (Betancor et al. 2004) and *E. coli* O157:H7 strains (Kim et al. 2005). Heterogeneity of 16S rRNA genes can successfully be used to establish the taxonomic relatedness of microorganisms (Blumberg et al. 1991). There are several techniques based on analysis of 16S rRNA gene including restriction fragment length polymorphism (RFLP) and sequencing. The RFLP analysis of amplified DNA has been proposed as valuable tool for epidemiological typing of microorganisms (Shangkuan et al. 2000; Gray et al. 2001).

In the present study, antibiotic resistance pattern of 240 *Enterobacteriaceae* isolates from different water sources in and around Ayodhya-Faizabad has been studied. RAPD and PCR-RFLP were employed to study the genetic relatedness of the isolates. The relationship between antibiotic sensitivity and genetic diversity of the isolates has been explored. 16S rRNA gene sequencing of three representative *Enterobacter* spp. isolates from different sources and their subsequent phylogenetic analysis was also performed to elucidate contamination of different sources with common *Enterobacteriaceae* members.

Materials and methods

Bacterial isolates

Water-borne *Enterobacteriaceae* cultures (240) were isolated from recreational and drinking water sources, e.g., river, ponds, kunds, hand pumps, piped supply and dug wells. The isolates were identified using standard biochemical tests for the members of *Enterobacteriaceae* as per Bergey's Manual of Systematic Bacteriology (Holt et al. 1993). The identified isolates were *Escherichia coli* (72), *Klebsiella* spp. (30), *Enterobacter* spp. (83), *Shigella* spp. (19) and *Salmonella* spp. (36). These were maintained on nutrient agar slants and stored at 4 °C.

Antibiotic sensitivity assay

Three commonly used classes of antibiotics (μg $disc^{-1}$), fluoroquinolones [ciprofloxacin (5), ofloxacin (5), pefloxine (5), norfloxacin (10)]; cephalosporin [cefadroxil (30), cefazoline (30), cefaclor (30), cefuroxime (30), ceftriaxone (30), cefotaxime (30), ceftazidime (30)] and aminoglycosides [amikacin (30)], were used for testing antibiotic sensitivity. The standard disk diffusion method was used in order to make an assessment of antibiotic resistance pattern among the *Enterobacteriaceae* isolates (Bauer et al. 1966).

One hundred microlitre of bacterial inoculum in log phase (turbidity adjusted to 0.5 McFarland standard) was spread evenly on solidified Müller Hinton agar plate followed by application of commercially available antibiotic discs [6 mm dia., supplied by Oxoid (UK)] using disc dispenser (HiMedia, India). The inoculated Petri dishes were then incubated at 37 °C for 24 h. The inhibition zone was measured using ruled template (HiMedia, India), and the isolates were classified as sensitive/resistant in accordance with performance standards for antimicrobial disk susceptibility tests recommended by Clinical and Laboratory Standards Institute (CLSI). The experiments were performed in triplicate, and average values were considered for patterns of antibiotic resistance or sensitivity.

Multiple antibiotic resistance (MAR) index

The MAR index for the isolates was calculated as per Krumperman (1983) and Hinton et al. (1984). The MAR index was applied to a sample from which several isolates were taken.

The MAR index = $y/(n \times x)$; where y is the aggregate antibiotic resistance score of all isolates from the sample, n is the number of antibiotics tested, and x is the number of isolates from the sample.

Random amplified polymorphic DNA analysis

The genomic DNA was isolated from *Enterobacteriaceae* members as described by Wilson (1997). The purified genomic DNA was used as template for RAPD. Nine primers, viz., P1254: 5′-CCGCAGCCAA-3′, OPB-17: 5′-AGGGAACGAG-3′, OPA-4: 5′-AATCGGGCTG-3′, OPB-15: 5′-CCAGGGTGTT-3′, 1252 (784): 5′-GCGGAA ATAG-3′, OPE-16: 5′-GGTGACTGTG-3′, OPK-01: 5′-CA TTCGAGCC-3′, OPP-03: 5′-CTGATACGCC-3′, CRA23: 5′-GCGATCCCCA-3′ (Neilan 1995; Girão et al. 1999; Betancor et al. 2004) were screened for their suitability in the study. Primer P1254 (5′-CCGCAGCCAA-3′) was selected for RAPD of isolates. Commercially supplied GeNei^{TM} Red Dye PCR Master Mix (2×) was used along with template DNA and primer in reaction mixture. The reaction mixture comprised of template (2 µl, 10 ng), primer (2 µl, 40 pmol), deionized water (8.5 µl) and 12.5 µl Red Dye PCR Master Mix (2×). Amplification was performed in thermal cycler (PTC 125, MJ Research, USA). Primary denaturation was done at 94 °C for 5 min followed by 30 cycles of denaturation (95 °C for 1 min), primer annealing (35 °C for 1 min) and extension (72 °C for 2 min). Final extension was carried at 72 °C for 5 min. The amplified product was electrophoresed on 1.5 % agarose gel for 2.5 h at 75 V with ethidium bromide (EtBr) in the gel. Gel analysis was done on SequentiXGelQuest-DNA fingerprinting software (SequentiX-Digital DNA Processing, Klein Raden, Germany) to compare the RAPD pattern of each isolate and generate phylogenetic trees (Müller et al. 2005). Bacterial isolates were analysed in three independent experiments. The fingerprint of the isolates was established by considering clearly and consistently detected amplified bands of ≥100 bp.

Amplification of 16S rRNA gene

The genomic DNA was used as template for amplification of 16S rRNA gene of bacterial isolates using 27F (5′-AGGGTTCGATCCTGGCTCAG-3′) and 1492R (5′-TA CGGAGACCTTGTTACGACTT-3′) universal primers. The PCR mixture was prepared using 12.5 µl GeNei^{TM} Red Dye PCR Master Mix (2×), 2 µl of template DNA (100 ng), 1 µl of each primer (20 pmol) and 8.5 µl of deionized water to make up to 25 µl of reaction volume. The optimization of annealing temperature was carried out in 96-well gradient PCR machine (Techne, TC-512, UK), and 58.5 °C was selected as annealing temperature. PCR was performed as previously described. Amplified product was electrophoresed on 0.7 % agarose gel (w/v) in Tris–EDTA (TE) amended with EtBr at 50 V for 2 h. The electropherogram was analysed on Doc It, gel documentation system (UVP, UK) for appearance of single band of amplified product measuring approximately 1,450 bp.

PCR-RFLP of 16S rRNA gene

Two restriction endonucleases, *Alu*I and *Hae*III were used for PCR-RFLP of amplified product of 16S rRNA gene (Brunel et al. 1997). The reaction mixture for restriction digestion by *Alu*I (8 U µl^{-1}) and *Hae*III (20 U µl^{-1}) was prepared separately by taking 10 µl of amplified DNA, 1.5 µl of restriction buffer (10×), 2 µl of restriction enzyme and 1.5 µl of water to make final volume of 15 µl reaction mixture. The reaction mixture was incubated for 5 h at 37 °C for complete digestion. The digested DNA was electrophoresed on 1.2 % agarose gel (w/v) in TE amended with EtBr at 70 V for 1 h. The gel was analysed on Doc It, gel documentation system (UVP, UK) for RFLP patterns of digested amplified product of 16S rRNA gene.

16S rRNA gene sequencing

The amplified product of 16S rRNA gene was sequenced on ABI Prism-310 (Applied Biosystems) automated sequencer. The constituents of sequencing reaction were as per manufacturer's recommendation: 1.0 µl terminator ready reaction mix, 1.5 µl sequencing buffer (5×), 40 ng template (PCR product of 16S rRNA gene), 3.2 pmol primer and deionized water to make up final volume of 10 µl. The constituents were mixed well and spinned briefly before setting up in thermal cycler. PCR was carried out in 96-well gradient PCR machine (Techne TC-512, UK) as per the instructions of Applied Biosystems. Five primers were used for sequencing: 1100R (5′-AGGGTTGCGCT CGTTG-3′), 518R (5′-ATTACCGCGGCTGCTGG-3′), 1114F (5′-GCAACGAGCGCAACCC-3′), 27F (5′-AGGG TTCGATCCTGGCTCAG-3′) and 1492R (5′-TACGGAG ACCTTGTTACGACTT-3′).

Phylogenetic analysis of 16S rRNA gene sequence

The sequence obtained was then analysed by BLAST and megaBLAST programs against the database of type strains with validly published prokaryotic names. Fifty sequences with highest scores were then selected for calculation of pairwise sequence similarity using global alignment algorithm, which was implemented at the EzTaxon server (Chun et al. 2007). Phylogenetic neighbours were determined using multiple sequence alignment programme in Clustal W (Thompson et al. 1994). Phylogenetic

relationship among the similarity showing organisms was determined using MEGA 4 software programme (Tamura et al. 2007). The phylogenetic tree was constructed using neighbour-joining (NJ) method and significance of junctions was established using bootstrap (1,000 replicates) method (Felsenstein 1985).

Submission of gene sequence to GenBank

The 16S rRNA gene sequences of isolates were submitted to GenBank database available with National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genbank) using bankit on http://www.ncbi.nlm.nih.gov/BankIt/oldbankit.html (Benson et al. 2007). The accession numbers HQ322393, HQ322394 and HQ322395 were assigned following the submission, and the sequence data were published online by NCBI.

Results and discussion

Antibiotic sensitivity assay

All antibiotics exhibited variable effect on water-borne enteric bacterial isolates (Fig. 1). Ofloxacin exerted most profound effect on all the isolates (92.63 %) with 100 % killing of *Shigella* strains. All *Shigella* isolates were also sensitive to ciprofloxacin while, only 78.95 and 68.42 % isolates were sensitive to amikacin and pefloxine, respectively. The sensitivity of other isolates against ciprofloxacin, amikacin and pefloxine was in the order: 85.72 > 79.64 > 76.46 %, respectively. Majority of *Enterobacter* isolates (>90 %) were sensitive to ofloxacin, ciprofloxacin, amikacin and pefloxine with ofloxacin being most effective against 97.59 % isolates. However, ciprofloxacin, pefloxine and amikacin were less efficacious (66.67–83.33 %) against *E. coli*, *Klebsiella* spp. and *Salmonella* spp. The sensitivity of isolates in our study illustrates broad spectrum activity of fluoroquinolone and aminoglycoside group of antibiotics against the water-borne bacterial isolates. It is customary to include fluoroquinolone as one of the most recommended antibiotics for diarrheal treatment (Jiang et al. 2002). The most effective among the fluoroquinolones (Ofloxacin) affects DNA gyrase which is critical for nucleic acid replication (Drlica and Zhao 1997).

Ceftriaxone, norfloxacin, cefotaxime and ceftazidime had moderate effect on the isolates. While *Klebsiella* (86.67 %) and *E. coli* (83.33 %) were sensitive, 73.68 % *Shigella* isolates were resistant to ceftriaxone. *E. coli* isolates at 83, 79 and 65 % were sensitive, respectively, to cefotaxime, ceftazidime and norfloxacin; while *Enterobacter*, *Klebsiella* and *Salmonella* isolates were moderately affected (50–85.54 %) by these antibiotics. *Shigella* spp. displayed relatively greater resistance against ceftriaxone, cefotaxime and ceftazidime. Cefazoline, cefuroxime, cefaclor and cefadroxil were less effective against all the isolates. Though,

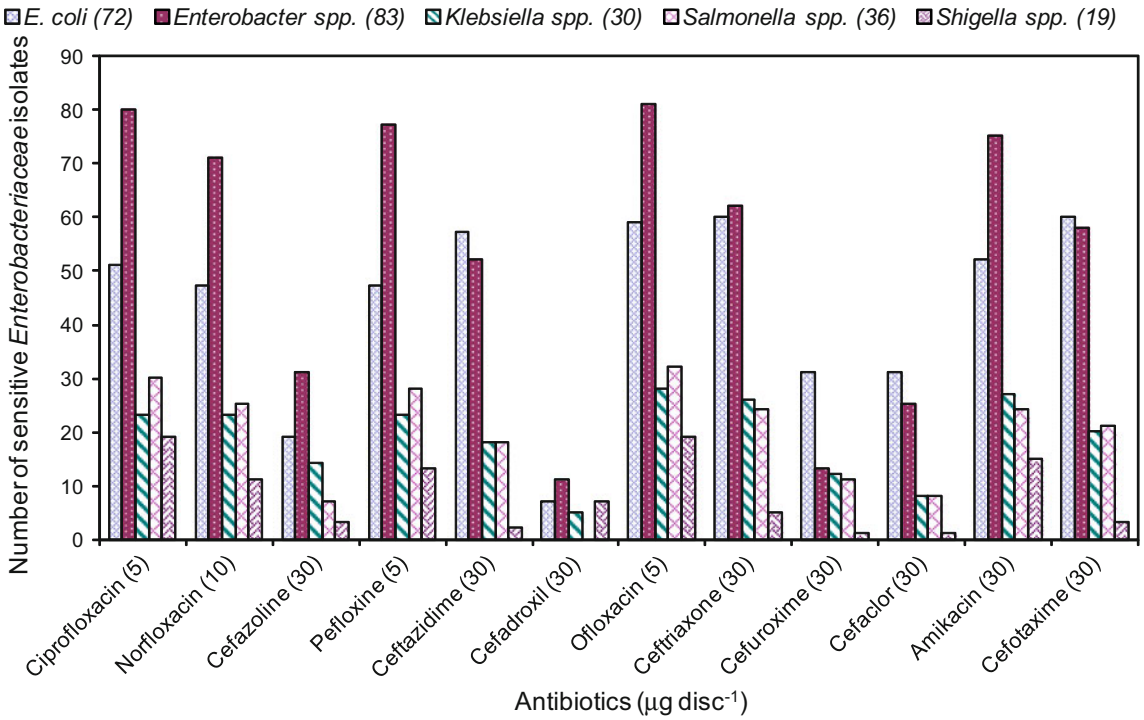

Fig. 1 Antibiotic sensitivity assay of water-borne *Enterobacteriaceae* isolates

cefazoline affected 46.67 % *Klebsiella*, the isolates of other genera were not affected to that extent. Cefadroxil was not effective against *Salmonella* spp. thereby displaying 100 % resistance (Fig. 1). The pattern of resistance against these antibiotics illustrates the differential potential and relevance of the commonly recommended drugs against water-borne gastrointestinal pathogens. Therefore, it is recommended to regularly evaluate the antibiotic sensitivity of commonly reported water-borne enteric pathogens.

The presence of antibiotic-resistant isolates in water bodies is a serious concern for the densely populated developing countries like India. The environmental reservoirs of antibiotic resistance determinants are poorly understood (Allen et al. 2010). The resistance among the isolates might be related to contamination of sources with antibiotic-resistant *Enterobacteriaceae* followed by horizontal or vertical transfer of antibiotic resistance trait. It has also been presumed that much of the hospital/industrial/domestic wastewater does not undergo effective treatment that permits low concentration of antibiotics finding way into water bodies like rivers, lakes, reservoirs, etc. This exposure over period of time provides ideal conditions for transfer of antibiotic resistance (Diwan et al. 2010).

The findings of antibiotic resistance in *Enterobacteriaceae* are extremely significant as they point towards the prevalence of antibiotic-resistant microorganisms in drinking water sources. It has recently been observed that such multiple drug-resistant bacteria 'superbugs' are the cause of worry for nations worldwide (Walsh et al. 2011). These emerging resistant organisms are tough to treat by conventional antibiotics and add to growing cost of treatment for the people in developing countries. Therefore, such studies assume greater significance and call for regular monitoring of water sources to ensure better and healthy world in future.

RAPD analysis of isolates

Escherichia coli and *Enterobacter* spp. exhibited 10 and 16 different RAPD band patterns, respectively. The genetic variation was dominated in hand pump water, as 24 *E. coli* and 11 *Enterobacter* isolates resulted in 5 RAPD patterns each (Table 1). The potable piped water did not reveal much genetic differences in all the isolates of different genera. Surprisingly, species of *Salmonella* and *Shigella* were consistently showing less genetic divergence across all the water sources. The random amplification of Polymorphic DNA of *Klebsiella* spp., *Salmonella* spp. and *Shigella* spp. resulted in four, four and one RAPD patterns, respectively. The dendrograms of *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp. and *Salmonella* spp. isolates showing genetic relationship are presented, respectively, in Fig. 2a–d.

The dendrogram (Fig. 2b) revealed that *Enterobacter* spp. had maximum genetic diversity among all the isolates. RAPD patterns suggest the transmission of bacterial isolates from one to other water sources. The recreational water sources (kund and pond) accounted for nearly 70 % of all genetic patterns observed, while drinking water sources had 38 % genetic divergence. *Enterobacteriaceae* isolates revealed 35 different band patterns of which 42 % were found in drinking water sources (Table 1).

Betancor et al. (2004) segregated *Salmonella enterica* serotypes using the same primer, and concluded that RAPD PCR discriminated true polymorphism unobtainable by serotyping and phenotypic trait characterization of isolates. The RAPD pattern reflected that different isolates were present in more than one drinking and recreational water sources undermining public health risk in the high population density region. Tewari et al. (2003) also reported similar distributional pattern of pathogenic *E. coli* and *Salmonella* serotypes.

Table 1 Distributional pattern of different *Enterobacteriaceae* isolates from various water sources using RAPD

Organism (number of isolates)	Number of RAPD patterns	Patterns present in water sources					
		Hand pump	Piped supply water	Dug well	River	Kund	Pond
E. coli (72)	10 (EC1–10)	EC1, EC2, EC3, EC4, EC5	–	EC2, EC4, EC7	EC9, EC10	EC6, EC7	EC5, EC7, EC8
Enterobacter spp. (83)	16 (EN1–16)	EN2, EN10, EN12, EN13, EN14	EN3	EN15, EN16	EN4	EN1, EN8, EN9, EN10, EN11	EN1, EN2, EN3, EN4, EN5, EN6, EN7
Klebsiella spp. (30)	4 (K1–4)	K2	–	K1, K3, K4	–	K1, K2	K1, K2, K3, K4
Salmonella spp. (36)	4 (S1–4)	S1, S2	S1	–	S3	S3, S4	S1
Shigella spp. (19)	1 (SH1)	SH1	SH1	SH1	–	SH1	SH1

The data presented above are based on consistent observation of identical bands (>100 bp size) present in the gel after amplification. The experiments were repeated thrice to conclude the RAPD patterns observed

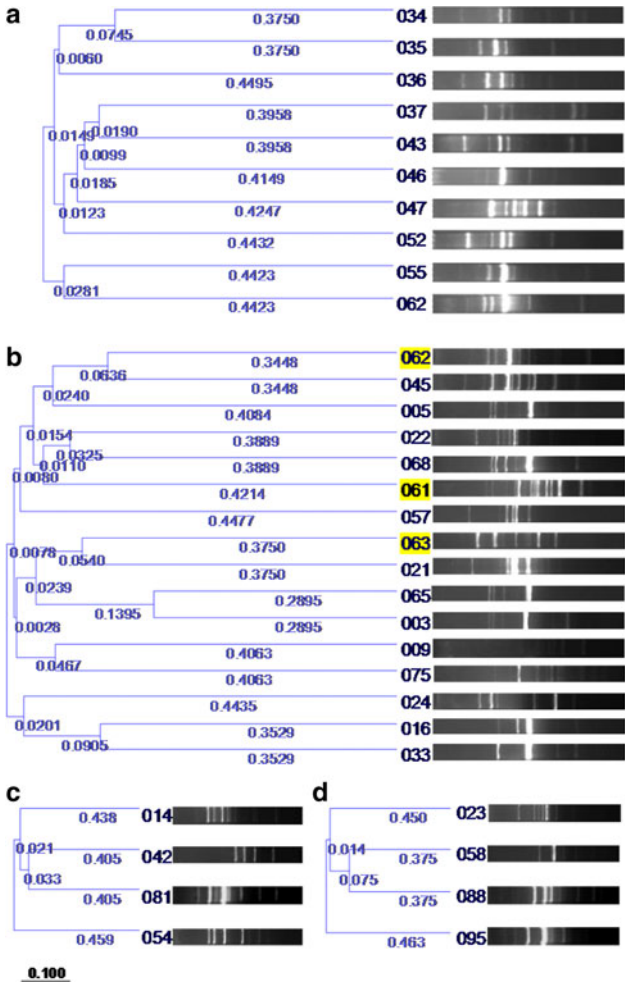

Fig. 2 Dendrograms generated by GelQuest (Ver 3.02) software showing the relationship of **a** 10 *E. coli*, **b** 16 *Enterobacter*, **c** four *Klebsiella* and **d** four *Salmonella* spp. isolates. The analyses of RAPD band patterns were performed using the Jaccard coefficient and UPGMA (unweighted pair group method with arithmetic averages)

The isolates displaying varying resistance/sensitivity patterns to tested antibiotics had similar RAPD profiles. It suggests that genetic alterations which produced resistance do not affect the RAPD profile. This may be due to the acquaintance of antibiotic resistance traits residing on accessory genetic material of the isolates. Similar to our findings, Betancor et al. (2004) demonstrated that antibiotic pressure leading to nalidixic acid resistance, did not alter the RAPD pattern of *Salmonella enterica* phenotypes.

PCR-RFLP of 16S rRNA gene

Restriction digestion (with *Hae*III) of amplified product of 16S rRNA gene resulted in different groups of genetically identical isolates. *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp. and *Salmonella* spp. were discriminated into two groups of each, while *Shigella* spp. members were represented by only one RFLP pattern. *Alu*I digestion could develop two RFLP patterns each of *E. coli*, *Enterobacter*, *Klebsiella* and *Salmonella* spp., while *Shigella* spp., isolates could not be distinguished genetically, and was represented by a single RFLP pattern.

The PCR-RFLP with *Hae*III generates similar genetic profile of various isolates from different genera. While, restriction digestion of PCR amplified product of 16S rRNA gene with *Alu*I showed slightly better, but little polymorphism among the isolates of different genera of *Enterobacteriaceae*. The poor differentiation of isolates must be associated with the conserved sites for restriction digestion of 16S rRNA gene in most of the isolates, as the 16S rRNA gene shares over 99 % homology across different genera. RAPD patterns were suggestive of higher subtypes of various generic isolates, while RFLP of 16S rRNA gene did not reveal as much subtypes of the genera. It may be due to the availability of higher number of annealing sites for RAPD primers on genomic DNA, but the endonucleases restriction sites in 16S rRNA gene of genera are highly conserved (Woese and Fox 1977; Lane et al. 1985).

Similar to our findings, Iriarte and Owen (1996) reported that PCR-RFLP analysis of 23S rRNA gene of *Campylobacter jejuni* strains was not very useful in differentiating isolates at subspecies level. Broda et al. (2000) also reported that restriction digestion of 16S rRNA gene with *Alu*I, *Hae*III and *Taq*I could not distinguish between *Clostridium algidicarnis* and *Cl. putrefaciens*. While, Miteva et al. (2001) reported that amplified ribosomal DNA restriction analysis (ARDRA) is useful for differentiation of bacterial strains at subspecies level due to different *Eco*RI restriction map of 5S rRNA gene. However, contrary to our findings, Brunel et al. (1997) stressed that PCR-RFLP of 16S rRNA gene using restriction enzymes *Alu*I and *Hae*III were effective in differentiating bacterial isolates of different genera including *Enterobacteriaceae*. The characteristic RFLP profiles may be used as marker for identification of genus members (Kullen et al. 1997), but may not be used to distinguish the isolates genetically.

Multiple antibiotic resistance index and genetic diversity

The different water sources contained *Enterobacteriaceae* members resistant not only to a single but multiple antibiotics. These organisms in common parlance are referred as MAR bacteria. The MAR index of 0.83 was highest for *Salmonella* spp. from piped water sources, while the lowest (0.13) MAR index was recorded for *Klebsiella* spp. isolates from hand pumps (Table 2).

The hand pump water with high MAR index reflected greater genetic variation in *E. coli* and *Enterobacter* spp. It

Table 2 Multiple antibiotic resistance (MAR) index of *Enterobacteriaceae* isolates from various sources

Water sources	MAR Index of *Enterobacteriaceae* isolates				
	E. coli	*Enterobacter* spp.	*Klebsiella* spp.	*Salmonella* spp.	*Shigella* spp.
River	0.28 (12)	0.58 (2)	A	0.56 (16)	0.50 (2)
Pond	0.31 (12)	0.36 (36)	0.36 (13)	0.31 (3)	0.75 (2)
Kund	0.33 (12)	0.37 (20)	0.28 (6)	0.40 (10)	0.53 (6)
Hand pump	0.42 (24)	0.23 (11)	0.13 (4)	0.28 (5)	0.58 (2)
Piped supply	0.38 (2)	0.71 (4)	A	0.83 (2)	0.64 (6)
Dug well	0.68 (10)	0.33 (10)	0.62 (7)	A	0.08 (1)

Values in parentheses are the number of isolates

A: no isolates of the species were obtained from the source

is observed that hand pump water is used for drinking purpose, which enhances high risk of water-borne infections. Though the MAR index was high (0.71) for *Enterobacter* spp. in piped supply water, it did not reveal any genetic variation. Similarly, *Shigella* spp. did not reveal diversity apparent in molecular studies despite having high MAR index in all the water sources except dug well. Hand pump and kund water sources showed high genetic divergence of *Salmonella* spp. isolates despite moderate MAR index (0.3–0.4). Maximum diversity of *Klebsiella* spp. was observed in dug well and pond water while MAR index was high (0.62) in dug well only. MAR index suggested dug well, pond and piped supply waters as high risk sources for multi-resistant strains of enterobacteria. The local ponds may apparently be contaminated with antibiotic-resistant bacteria through domestic and industrial waste water discharge. The dug wells are seldom used and therefore, have become dumping sites of domestic waste. The high MAR index of isolates from piped supply might be due to contamination with sewage MAR bacteria through loose connections, rusted and broken pipes passing through underground sewage (WHO 2008).

Hence, it may be concluded that antibiotic selection pressure led to greater genetic diversity, and thus differences in molecular profile of the isolates. Conversely, the reverse was also observed where low genetic diversity was evident despite high MAR index.

Molecular identification and phylogenetic analysis of *Enterobacter* spp.

Three isolates of *Enterobacter* spp. characterized as different RAPD, but same RFLP group members were identified by sequencing 16S rRNA gene. The BLAST and megaBLAST analysis of gene sequences of isolates showed maximum homology (>99.4 %) with *Enterobacter hormaechei* CIP 103441(T). The *Enterobacter hormaechei* is of human origin, which was isolated originally from blood, wounds, sputum, and was named in the honour of Estenio Hormaeche, an Uruguayan microbiologist who (with P. R. Edwards) proposed and defined the genus Enterobacter. The species is negative for indole production but produces H_2S. Originally, most of the isolates were reported moderately sensitive to several antibiotics but resistant to ampicillin, cefoxitin and cephalothin (O'Hara et al. 1989). The studied isolates were designated as *Enterobacter hormaechei* strains skg0061, 0062 and 0063 from pond, hand pump and kund, respectively (Fig. 3). Sequence information was submitted in GenBank database NCBI with accession numbers HQ322393, HQ322394 and HQ322395, respectively, for *Enterobacter hormaechei* strains skg0061, 0062 and 0063. The phylogenetic neighbours were analysed by Clustal W, and NJ tree was constructed using MEGA 4 software.

The 16S rRNA gene sequencing followed by database search is frequently used for identification and taxonomic study of bacterial isolates (Amann et al. 1994). Identification of genus and species by 16S rRNA gene sequencing has been useful (Janda and Abbott 2007). The genotypic characterization using conserved sequences such as the small-subunit (16S) rRNA gene provides the basis for ongoing evaluation and restructuring of bacterial systematics (Kirschner et al. 1993; Ludwig and Schleifer 1994). The present study revealed the contamination of different water sources with identical *Enterobacteriaceae* members.

Conclusion

The water-borne *Enterobacteriaceae* isolates from various sources in study region were resistant to antibiotics of one or more classes. Fluoroquinolones were most effective antibiotics against the isolates, while cefadroxil was least effective. High risk sources (dug well, pond, piped supply water) contaminated with multiple antibiotic-resistant *Enterobacteriaceae* members were identified by MAR

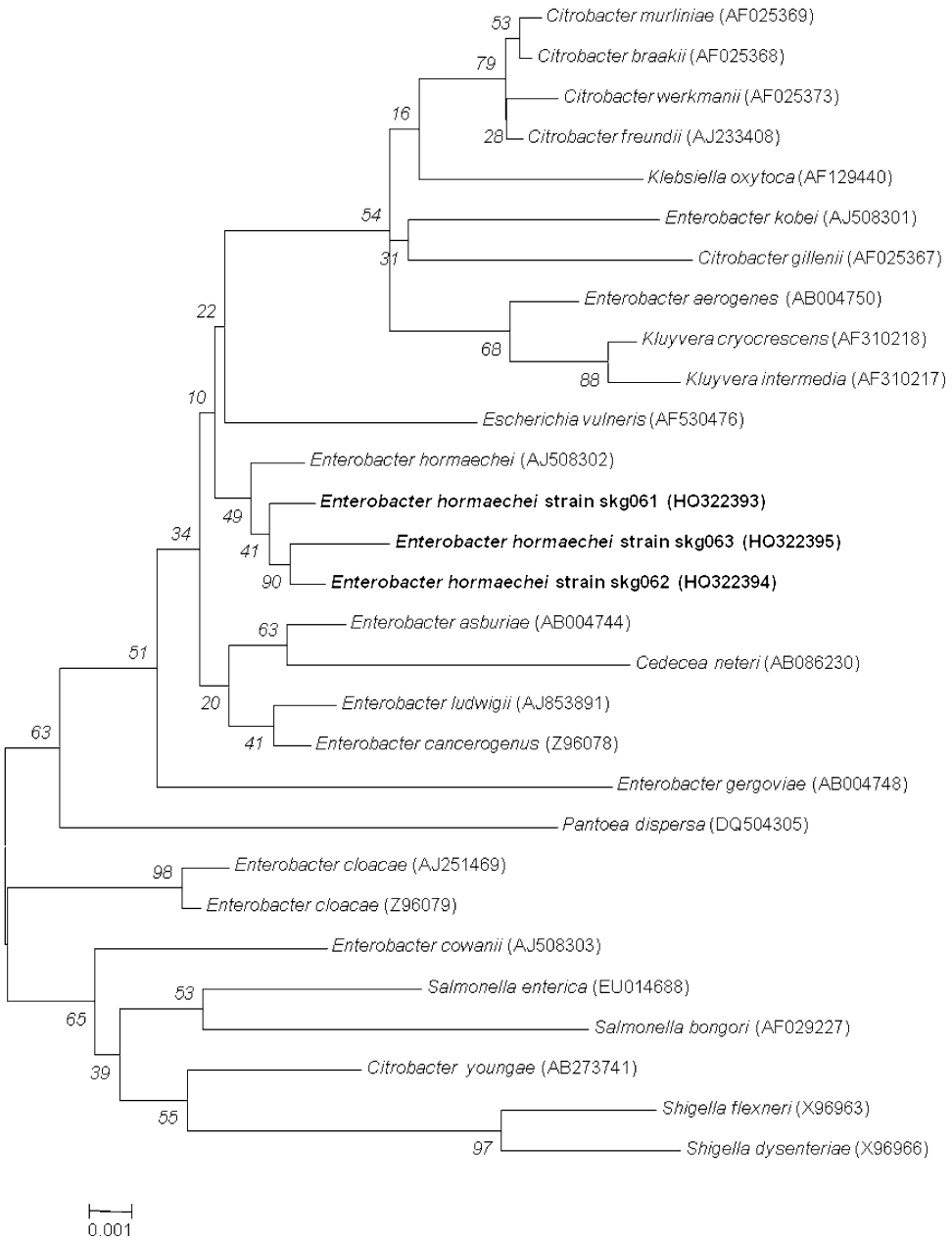

Fig. 3 The phylogenetic tree is constructed to show evolutionary relationships of *Enterobacter hormaechei* strains skg0061 (HQ322393), 0062 (HQ322394) and 0063 (HQ322395) using neighbour-joining method. Bootstrap values for 1,000 replicates are shown at branch points. Branches corresponding to partitions reproduced in <50 % bootstrap replicates are collapsed. The evolutionary distances were computed using the maximum composite likelihood method, and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option)

indexing. The isolates having identical RAPD profile showed varied antibiotic sensitivity patterns. *Enterobacter* spp. isolates from different sources having identical PCR-RFLP but different RAPD patterns were identified as strains of *Enterobacter hormaechei* by 16S rRNA gene sequencing and phylogenetic study.

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Conflict to interest The authors declare that they have no conflict to interest.

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