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Class 1 and Class 2 Integrons and Plasmid-Mediated Antibiotic Resistance in Coliforms Isolated from Ten Rivers in Northern Turkey†

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We aimed to determine the molecular mechanisms of antibiotic resistance in coliforms isolated from ten rivers in northern region of Turkey. A total of 183 isolates were tested for antimicrobial susceptibility by disk diffusion and agar dilution methods. Resistance to ampicillin, streptomycin, trimethoprim, tetracycline, and chloramphenicol was detected in 58%, 51.9%, 24%, 28.4%, and 12.5%, respectively. Twelve (6.5%) phylogenetically distant organisms were detected to harbor self-transmissible plasmids ranging 52 to >147 kb in sizes. Resistances to ampicillin, tetracycline, trimethoprim, streptomycin, and nalidixic acid were commonly transferable traits. Transferable nalidixic acid-resistant strains harbored *qnrS* gene, which was the first report of plasmid-mediated quinolone resistance in bacteria of environmental origin in Turkey. Fourteen and five coliforms harbored class 1 and class 2 integrons, respectively, and some of them were located on transferable plasmids. Sequence analyses of variable regions of the class 1 and 2 integrons harbored various gene cassettes, *dfrA1*, *dfr2d*, *dfrA7*, *dfrA16*, *dfrA17*, *aadA1*, *aadA5*, bla_{OXA-30}, and *sat1*. A gene cassette array, *dfrA16* has been demonstrated for the first time in a *Citrobacter koseri* isolate. Class 1 and class 2-bearing strains were clustered in different groups by BOX-PCR fingerprinting. Rivers in the northern Turkey may act as receptacle for the multi-drug resistant enterobacteria and can serve as reservoirs of the antimicrobial resistance determinants in the environment. The actual risk to public health is the transfer of resistance genes from the environmental bacteria to human pathogens.

***Keywords*:** river, coliform, integron, resistance plasmid

Antibiotic resistance has been detected in different aquatic environments including rivers (Ash *et al.*, 2002). Introduction of antimicrobial compounds into the aquatic environment via medical therapy, agriculture, and animal husbandry has resulted in selective pressures on bacterial residents (Col and O'Connor, 1987).

Multidrug-resistant bacterial strains in various environments have become a significant public health concern. This situation has been reported mostly due to the transfer of the antimicrobial-drug resistance (R) determinants, mediated by genetic elements such as integrons, transposons, and plasmids (Recchia and Hall, 1995; Carattoli, 2001; Rowe-Magnus and Mazel, 2002). Integrons are genetic elements carrying determinants of site-specific recombination and an expression system, which integrates single or groups of mobile antibiotic resistance gene cassettes (Hall and Collis, 1995). They have been found in the chromosome as well as in different plasmids and transposons (Hall and Stokes, 1993).

The most frequently reported are class 1 and class 2 integrons, which have been demonstrated to contribute to the spread of antimicrobial resistance genes among enterobacteria (Fluit and Schmitz, 2004). Class 1 integrons have been identified on transposable elements such as mercury resistance transposon Tn*21* (Grinsted *et al.*, 1990). Class 2 integrons are present on transposon Tn*7*, and it is known to carry three classic gene cassettes, *dfrA1*, *sat1*, and *aadA1*, which confer resistance to trimethoprim, streptothricin, and streptomycin/spectinomycin, respectively (Hansson *et al.*, 2002). Class 1 and/or class 2 integrons have been reported in clinical isolates of the *Enterobacteriacea* family (Leverstein-van Hall *et al.*, 2001), in bacteria from food (Sunde, 2005) and also in aquatic environments (Roe *et al.*, 2003); however, there have been very limited studies (Petersen *et al.*, 2000; Mukherjee and Chakraborty, 2006) in aquatic environments such as creeks or rivers.

Investigations on antibiotic resistance in the aquatic habitat have concerned bacteria of fecal origin because they are used as pollution indicators and may be associated with infectious diseases (Jones *et al.*, 1986).

Only a limited number of studies (Ozgumus *et al.*, 2007) have examined integrons and R-plasmids in aquatic enterobacteria in Turkey. The major concern of this study was about the aquatic *Enterobacteriaceae* family containing class 1 and class 2 integrons and R plasmids which may be potential reservoirs of antibiotic-resistance genes in the environment. This is the first report regarding a detailed investiga-

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tion on molecular characterization of antibiotic resistance in enterobacterial species isolated from rivers in northern Turkey which is rich in natural fresh water sources.

Materials and Methods

Water sampling and bacterial isolation

Water samples in sterile glass bottles were collected aseptically from ten rivers in different districts in northern-east coastal region of Turkey during one year from December 2003 to December 2004 and transported on ice to the Microbiology & Molecular Biology Research Laboratory of Rize University, Rize, Turkey. The samples were processed within 8 h of collection. Coliforms were enumerated by multiple-tube fermentation method (AWWA, 1998). Fecal growth was achieved by monitoring the acidification and gas production during growth in Brilliant green broth (Oxoid, UK) at 44±0.5°C for 24±3 h, and sub-cultured on Eosine Methylene Blue agar (Oxoid, UK) plates from the fermentation tubes in order to isolate the coliforms.

Bacterial strains

A total of 183 strains of *Escherichia coli* (n=35), *Klebsiella pneumoniae* (n=35), *Klebsiella oxytoca* (n=12), *Enterobacter* spp. (n=23), *Citrobacter koseri* (n=20), *Citrobacter freundii* (n=3), and *Proteus vulgaris* (n=2) were included in this study. Bacteria were identified to the species level using biochemical reactions as previously described (Brenner, 1986). All coliforms identified to species level were stored in 20% of glycerol at -35°C until next use.

Antimicrobial susceptibility testing

The susceptibility tests were carried out by the standard disk diffusion method, and the results were interpreted as described in Clinical Laboratory Standards Institute (CLSI, 2003a). The following antibiotic disks (Oxoid, UK) were used: tetracycline (30 μg), nalidixic acid (30 μg), gentamicin (10 μg), ofloxacin (5 μg), neomycin (30 μg), netilmicin (30 μg), kanamicin (30 μg), streptomycin (10 μg), ampicillin (10 μg), amikacin (30 μg), trimethoprim (5 μg), and chloramphenicol (30 μg). Minimum inhibitory concentrations (MICs) of ampicillin, tetracycline, trimethoprim, streptomycin, nalidixic acid, gentamicin, and amikacin, which powder of the antibiotics were from Sigma (USA) to the transconjugants and transformants were performed by agar dilution method as described in CLSI (2003b) guidelines.

Plasmid isolations and resistance transfer

Conjugation assays were performed by broth mating method (Rice *et al.*, 1990). Equal volumes (1 ml) of cultures of the antibiotic resistant coliforms as donor and *E. coli* K12 strain C600 (F^- *thr leu thi* Rif^r) as the recipient, grown with agitation in Luria-Bertani (LB) broth were mixed and incubated for 18 h at 35°C without shaking. The transconjugants were selected on Eosine Methylene Blue agar (Oxoid, UK) supplemented with 200 μg/ml of rifampin (Hoecst, Germany) to inhibit donors and 30 μg/ml of ampicillin, netilmicin, kanamicin, gentamicin, tetracycline, chloramphenicol or 25 μg/ml of trimethoprim (Sigma) to inhibit recipient. The frequency of transfer was expressed relative to the number of donor cells.

Total plasmid DNAs were isolated from the enterobacterial isolates by alkaline extraction method (Kado and Liu, 1981) and transformed into the competent *E. coli* K12 strain JM101 ($RecA^-$) cells by heat shock method as previously described (Ausubel *et al.*, 1995). Transformants were selected on LB agar containing 30 μg/ml of ampicillin, netilmicin, kanamicin, gentamicin, tetracycline, chloramphenicol or 25 μg/ml of trimethoprim (Sigma). Plasmid DNAs were electrophoresed on 0.8% agarose gel containing 0.5 μg/ml ethidium bromide (Sigma) and visualized with UV light. The approximate sizes of the plasmids were estimated by comparison to plasmid marker (*E. coli* V517) (Macrina *et al.*, 1978) and the reference strain *E. coli* R39, kindly supplied by Dr. Luca Guardabassi (Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Denmark).

PCR for integrons

To prepare DNA templates for PCRs, coliforms, transconjugants, and transformants were inoculated into 3 ml LB broth and incubated for 20 h at 37°C with shaking. Cells from 1.5 ml of the overnight culture were harvested by micro-centrifugation (13,000×g, 10 min). After decanting the supernatant, the pellet was re-suspended in 500 μl of deionized water. The cells were lysed by boiling for 10 min. The debris was removed by centrifugation (13,000×g, 10 min). A 1 μl of supernatant was used as template for PCR amplifications. All PCR reactions were carried out in a Mastercycler Personal thermal cycler (Eppendorf, USA) using *Taq* polymerase, nucleotides and buffers purchased from MBI Fermentas (Vilnius, Lithuania).

The presence of integrons were examined by PCR with specific oligonucleotide primers 5'-CS; 5'-GGCATCCAAGC AGCAAG-3' and 3'-CS; 5'-AAGCAGACTTGACCTGA-3', amplifying the variable regions of class 1 (Lévesque *et al.*, 1995) and class 2 integrons with specific oligonucleotide primers hep51; 5'-GATGCCATCGCAAGTACGAG-3' and hep74; 5'-CGGGATCCCGGACGGATGCACGATTTGTA-3' (White *et al.*, 2001). Reaction compositions and cycling parameters of the PCRs were performed as previously described (Lévesque *et al.*, 1995; White *et al.*, 2001). The PCR products were then electrophoresed on 1% agarose gel containing 0.5 μg/ml ethidium bromide (Sigma) and visualized with UV light.

BOX-PCR assay

BOX-PCR fingerprints were obtained with the primer BOXA1R; 5'-CTACGGCAAGGCGACGCTGACG-3' (Versalovic *et al.*, 1994). Conditions for PCR were optimized and performed as described (Seurinck *et al.*, 2003). DNA fragments were separated at 60 V for 3 h on 1% agarose gels containing ethidium bromide. Gel was visualized under UV light. Patterns were compared by eye and considered identical when the positions of all bands matched. Differences in band intensity were ignored. All amplified BOX-PCR bands were scored for their presence (1) or absence (0) (data not shown). A similarity matrix was generated by using the Sneath and Sokal methodology (Sneath and Sokal, 1973). The Jaccard index citation was used (similarity: a / $a+b$, where a is the homolog bands in two genotypes and

where *b* is the number of non-homolog fragments in two genotypes). The similarity dendogram was generated using the NTSYS-pc with the unweighted pair group method with arithmetic means (UPGMA) tree building method (Rohlf, 1990).

DNA sequencing and bioinformatic analysis

After PCR products of the integrons were purified from the agarose gel by using QIAQuick® Purification Kits (QIAGEN, UK) prior to sequencing, they were cloned into the pGEM-T Easy vector according to the manufacturer's instructions (Promega, USA). Recombinant plasmids carrying amplicons of class 1 and class 2 integrons were sent to Macrogen Inc. (Korea) for sequencing by using two primers (SP6 promoter primer and T7 promoter primer) complementary to the sequence of the plasmid vector pGEM-T Easy. Data from sequencing was compared with those available in the GenBank database by using the alignment search tool, BLAST (Altschul *et al.*, 1997), accessible from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST), and by the multiple sequence alignment program, CLUSTAL W, accessible from the European Bioinformatics Institute website (http://www. ebi.ac.uk/clustalw).

Results

Total resistance to antibiotics

From the bacterial cultures, 183 enterobacteria (88 *E. coli*, 47 *Kelbsiella* spp., 23 *Enterobacter* sp., 23 *Citrobacter* sp., and two *Proteus vulgaris*) were tested for the antimicrobial susceptibility. Resistance to ampicillin in *Klebsiella* spp. and *Enterobacter* sp. did not take into account while calculating resistance rate due to possessing intrinsic resistance to ampicillin. Based on the results from *E. coli*, *Citrobacter* sp., and *Proteus vulgaris*, resistance to ampicillin which was the most common resistance phenotype among the isolates was observed in 66 (58%) of 113 strains (Fig. 1). Resistance to aminoglycosides such as streptomycin (51.9%), neomycin (46.4%), gentamicin (42%) was quite high. The lowest resistance was for amikacin (18.5%) among aminoglycosides tested. Resistance to chloramphenicol (12.5%), ofloxacin (4.9%), nalidixic acid (16.3%), and trimethoprim (24%) was also observed (Fig. 1).

Plasmid transfer studies

Resistances to ampicillin, tetracycline, trimethoprim, and some of the aminoglycosides such as streptomycin and amikacin were also plasmid-mediated traits. Transconjugants and transformants showed high-level resistance to the drugs ranging from the MICs of 16 to 2,048 μg/ml (Table 1), indicating that the resistance determinants was well expressed in host cells. Plasmid transformation studies revealed that fifteen transformants harboring non-conjugative plasmids about 2.6 to 147 kb encoded antibiotic resistance such as resistances to ampicillin, trimethoprim, streptomycin, tetracycline or nalidixic acid, as seen in Table 1. Nalidixic acid-resistant strains *E. coli* KD55 and *K. pneumoniae* KD100 were detected to carry *qnrS* gene (Table 1). Conjugation experiments were performed on 130 of 183 isolates. Twelve (6.5%) different species of enterobacterial isolates like *E. coli*, *Citrobacter* sp., *Klebsiella* spp., or *Proteus* sp. were detected to harbor self-transmissible plasmids ranging 52 to >147 kb in sizes (Table 1). Conjugal transfer frequency was in the range of 3×10^{-6} to 10^{-2} (data not shown). Some strains, for example *E. coli* KD17, *Citrobacter freundii* KD18, *Citrobacter koseri* KD66, *Klebsiella oxytoca* KD84 or *Proteus vulgaris* KD171, contained two or three self-transmissible large plasmids with different molecular sizes (Table 1).

Integron detection and characterization

All 183 enterobacterial isolates were screened for the presence of class 1 and class 2 integrons by specific PCRs. The amplicon lengths, corresponding to the approximate sizes of

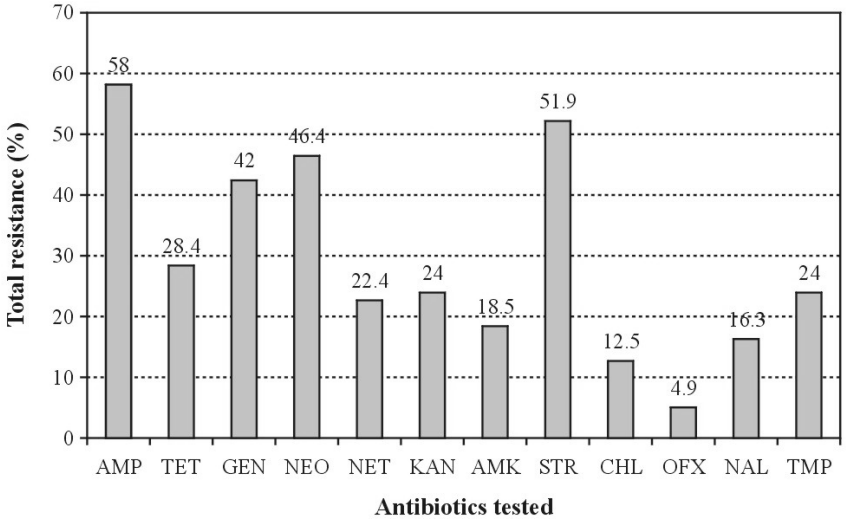

Fig. 1. Frequency of total resistance to antibiotics in 183 coliform bacteria isolated from ten rivers. For the abbreviations, see footnote Table 1.

Table 1. Antibiotic resistance patterns, integron and R plasmid contents of the representative coliforms and MICs of the antibiotics to R^+ *Escherichia coli* K-12 strains JM101 and C600

Donor	R^+ *Escherichia coli* K-12 strain	Antibiotic resistance phenotype	Integron content of donor	Plasmid(s) content (kb) of R^+ cell[c]	MIC (μg/ml) of selected antibiotics* to R^+ *E. coli* K-12 strains JM101 or C600						
					AMP	TET	TMP	STR	NAL	GEN	AMK
Enterobacter spp. KD5	JM101	**AMP**[a]		5.5	>2048	0.5	0.5	0.5	0.5	0.5	0.5
Escherichia coli KD8	C600	**TET** STR, CHL, **AMP**, TMP	Class 1	>**147**	>2048	128	0.5	0.5	0.5	0.5	0.5
Escherichia coli KD9	JM101	TET, **AMP**, **TMP**	-	6.5	>2048	0.5	512	0.5	0.5	0.5	0.5
Escherichia coli KD16	JM101	**AMP**	-	3.8	>2048	0.5	0.5	0.5	0.5	0.5	0.5
Escherichia coli KD17	C600	**TET**, STR, **AMP**	-	>**147, 80**	>2048	128	0.5	0.5	0.5	0.5	0.5
Citrobacter freundii KD18	C600	**AMP**	-	>**147**, **130**, 66	>2048	0.5	0.5	0.5	0.5	0.5	0.5
Escherichia coli KD21	JM101	**AMP**	-	3.2	>2048	0.5	0.5	0.5	0.5	0.5	0.5
Escherichia coli KD34	JM101	GEN, NEO, NET, KAN, STR, **AMP**, AMK	-	5.5	>2048	0.5	0.5	0.5	0.5	0.5	0.5
Escherichia coli KD36	JM101	TET, NAL, GEN, OFX, NEO, NET, STR, CHL, **AMP**, AMK, **TMP**	Class 1 Class 2	6.7	>2048	0.5	16	0.5	0.5	0.5	0.5
Escherichia coli KD39	C600	**TET**, GEN, NEO, NET, STR, **AMP**, **TMP**, AMK	Class 1	>**147**	>2048	128	512	0.5	0.5	0.5	0.5
Citrobacter koseri KD42	C600	**TET**, GEN, NEO, NET, KAN, STR, **AMP**	-	**80**, 6, 5, 2.5, 1	>2048	128	0.5	0.5	0.5	0.5	0.5
Escherichia coli KD48	C600	**TET**, GEN, **STR**, **AMP**	-	61, **52**	>2048	64	0.5	128	0.5	0.5	0.5
Escherichia coli KD55	C600	TET, **NAL**[b], **GEN**, OFX, NEO, NET, KAN, **STR**, **AMP**, **TMP**	Class 1	**88**, **80**	>2048	0.5	512	512	128	1024	0.5
Escherichia coli KD60	C600	**AMP**, TMP	-	>**147**, **75**, 4	>2048	0.5	0.5	0.5	0.5	0.5	0.5
Citrobacter koseri KD66	C600	GEN, NEO, STR, **AMP**	-	>**147**, **73**, 7.4	>2048	0.5	0.5	0.5	0.5	0.5	0.5
Klebsiella oxytoca KD70	JM101	NET, STR, **AMP**, **TMP**	-	2.6	>2048	0.5	512	0.5	0.5	0.5	0.5
Escherichia coli KD73	C600	GEN, NEO, NET, **AMP**, **STR**, AMK	-	>**147**, >147, **130**, **95**	>2048	0.5	0.5	512	0.5	0.5	0.5
Escherichia coli KD74	JM101	GEN, NEO, STR, **AMP**	-	6.7, 3.8	>2048	0.5	0.5	0.5	0.5	0.5	0.5
Klebsiella oxytoca KD84	C600	**TET**, GEN, OFX, NEO, NET, KAN, STR, **AMP**, **TMP**	Class 1	>**147**, >147, **81**, **61**	>2048	64	64	0.5	0.5	0.5	0.5
Enterobacter spp. KD94	JM101	GEN, CHL, **AMP**, **TMP**	-	6.7, 3.8	>2048	0.5	512	0.5	0.5	0.5	0.5
Citrobacter koseri KD95	JM101	TET, GEN, **AMP**, **TMP**	-	6.7, 3.8	>2048	0.5	512	0.5	0.5	0.5	0.5
Klebsiella pneumoniae KD100	JM101	**TET**, **NAL**[b], GEN, OFX, NEO, KAN, **STR**, **AMP**, AMK	-	147	>2048	64	0.5	64	128	0.5	0.5
Escherichia coli KD104	JM101	TET, NEO, STR, CHL, **AMP**	-	2.8	>2048	0.5	0.5	0.5	0.5	0.5	0.5
Klebsiella pneumoniae KD106	JM101	NEO, STR, **AMP**	-	150	>2048	0.5	0.5	0.5	0.5	0.5	0.5
Escherichia coli KD145	JM101	NAL, GEN, OFX, STR, **AMP**, **TMP**	Class 1 Class 2	7.3	>2048	0.5	512	0.5	0.5	0.5	0.5
Escherichia coli KD167	JM101	TET, CHL, **AMP**	-	10	>2048	0.5	0.5	0.5	0.5	0.5	0.5
Proteus vulgaris KD171	C600	TET, **GEN**, NAL, OFX, NEO, KAN, **STR**, CHL, **AMP**, **AMK**, **TMP**	Class 1	>**147**, >147, **81**	>2048	0.5	512	256	0.5	2048	32
Escherichia coli K-12 strain C600	-	RIF	-	-	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Escherichia coli K-12 strain JM101	-	-	-	-	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5

* AMP, ampicillin; TET, tetracycline; GEN, gentamicin; NEO, neomycin; NET, netilmicin; KAN, kanamycin; AMK, amikacin; STR, streptomycin; CHL, chloramphenicol; OFX, ofloxacin; NAL, nalidixic acid; TMP, trimethoprim; RIF, rifampicin

[a] Abbreviated antibiotic names in bold indicate plasmid-mediated resistance

[b] Plasmid-mediated nalidixic acid resistance was encoded by *qnrS* gene

[c] Numbers in bold indicate the approximate molecular size of self-transmissible plasmids

Table 2. Characteristics of class 1 and class 2 integron-bearing enterobacterial isolates

Strain	Organism	Antibiotic[a] resistance phenotype	Integron class /size (bp)	Gene cassette array and order	Inserted gene cassette into plasmid	EMBL accession no.
KD8	*Escherichia coli*	TET, STR, CHL, AMP, TMP	Class 1 / 769	*dfrA7*	+	EU339236
KD27	*Escherichia coli*	TET, NAL, GEN, OFX, NEO, NET, STR, CHL, AMP, AMK, TMP	Class 2 / 2224	*dfrA1-sat1-aadA1*	-	EU339237
KD36	*Escherichia coli*	TET, NAL, GEN, OFX, NEO, NET, STR, CHL, AMP, AMK, TMP	Class 1 / 2013 Class 2 / 2224	*bla*$_{OXA-30}$-*aadA1* *dfrA1-sat1-aadA1*	- -	EU339234 EU339237
KD39	*Escherichia coli*	TET, GEN, NEO, NET, STR, AMP, TMP, AMK	Class 1 / 1586	*dfrA1-aadA1*	+	-[b]
KD46	*Escherichia coli*	TET, NAL, GEN, OFX, NET, KAN, STR, AMP, TMP	Class 1 / 769	*dfrA7*	-	EU250576
KD48	*Escherichia coli*	TET, NAL, GEN, OFX, NEO, NET, KAN, STR, AMP, TMP	Class 1 / 1663	*dfrA17-aadA5*	+	-[c]
KD54	*Escherichia coli*	TET, NAL, GEN, OFX, NEO, NET, KAN, STR, CHL, AMP, TMP	Class 1 / 561	*dfr2d*	-	EU339233
KD61	*Citrobacter koseri*	GEN, STR, AMP, TMP	Class 1 / 741	*dfr16*	-	EU158182
KD62	*Escherichia coli*	TET, GEN, NEO, STR, AMP, TMP	Class 1 / 769	*dfrA7*	-	EU250577
KD65	*Klebsiella pneumoniae*	TET, GEN, NEO, STR, CHL, AMP, TMP	Class 1 / 1586	*dfrA1-aadA1*	-	-[b]
KD84	*Klebsiella oxytoca*	TET, GEN, OFX, NEO, NET, KAN, STR, AMP, TMP	Class 1 / 1586	*dfrA1-aadA1*	+	-[b]
KD121	*Escherichia coli*	TET, STR, CHL, AMP, TMP	Class 2 / 2224	*dfrA1-sat1-aadA1*	-	EU339237
KD145	*Escherichia coli*	NAL, GEN, OFX, STR, AMP, TMP	Class 1 / 1663 Class 2 / 2224	*dhfrA17-aadA5* *dfrA1-sat1-aadA1*	- +	-[c] EU339237
KD171	*Proteus vulgaris*	TET, GEN, NAL, OFX, NEO, KAN, STR, CHL, AMP, AMK, TMP	Class 1 / 1586	*dfrA1-aadA1*	+	-[b]
KD187	*Klebsiella pneumoniae*	TET, STR, AMP, TMP	Class 1 / 769	*dfrA7*	-	EU339235
KD189	*Escherichia coli*	TET, NAL, STR, CHL, AMP, TMP	Class 2 / 2224	*dfrA1-sat1-aadA1*	-	EU339237
KD191	*Proteus vulgaris*	TET, NEO, CHL, AMP, TMP	Class 1 / 1586	*dfrA1-aadA1*	-	-[b]

[a] AMP, ampicillin; TET, tetracycline; GEN, gentamicin; NEO, neomycin; NET, netilmicin; KAN, kanamycin; AMK, amikacin; STR, streptomycin; CHL, chloramphenicol; OFX, ofloxacin; NAL, nalidixic acid; TMP, trimethoprim
[b] Similar to *dfr1-aadA1* gene cassette array (GenBank accession no. DQ875876) at nucleotide level
[c] Similar to *dfrA17-aadA5* gene cassette array (GenBank accession no. DQ875874) at nucleotide level

the inserted DNA cassette, varied from 0.5 to 2.2 kb for class 1 integrons and 2.2 kb for class 2 integrons. However, two *E. coli* strains (KD36 and KD145) carried both class 1 and class 2 integrons (Table 2). A 0.7-kb amplicon of class 1 integron recorded in five coliform strains (KD8, KD46, KD, KD61, KD62, and KD187), which three of them were *E. coli* and other two were *Citrobacter koseri* and *Klebsiella pneumoniae*. A 1.5-kb amplicon of class 1 integron were detected in five coliform strains (KD39, KD65, KD84, KD171, and KD191), which two of them were *Proteus vulgaris* and the other isolates as *E. coli*, *K. pneumoniae*, and *K. oxytoca* were also detected. A 0.5-kb and a 1.6-kb amplicon of class 1 integron recorded in two *E. coli* strains KD48 and KD54, respectively. In total, fourteen (7.6%) of the coliforms carried detectable class 1 integron structure, and five had class 2 integrons with the same size that all were harbored in *E. coli* strains. Class 1 integrons in four *E. coli* strains (KD8, KD39, KD48, and KD145), in one *Klebsiella oxytoca* strain (KD84) and a class 2 integron in one *Proteus vulgaris* strain (KD171) located on the plasmids (Table 1). Characterization of gene cassettes revealed a significant association between the nature of the gene cassettes and the corresponding antibiotic resistance phenotype of the isolates (Table 1 and 2). Class 1 and 2 integron-specific PCR products cloned in a pGEM-T Easy Vector were subjected to DNA sequencing and gene cassettes were identified by sequence analysis. All class 1 integron-carrying isolates expressed resistance to trimethoprim and to sulfamethoxazole, a sulfanomide (data not shown). The most common carriage by integron-positive isolates involved dihydrofolate reductase gene cassettes such as *dfrA1*, *dfr2d*, *dfrA7*, *dfrA16*, and *dfrA17* which encodes dihydrofolate reductase enzymes conferring resistance to trimethoprim, with aminoglycoside adenyltransferase A genes such as *aadA1* and *aadA5* which encodes aminoglycoside adenyltransferase A enzyme conferring resistance to streptomycin/spectinomycin antibiotics. Six strains carried only one gene cassette, and one strain (*E. coli* KD36) had a gene cassette array, *aadA1* and *bla*$_{OXA30}$ gene encoding a β-lactamase enzyme conferring resistance to β-lactam antibiotics such as ampicillin (Table 2). Resistance to other antibiotics like chloramphenicol, nalidixic acid, and tetracycline did not correspond to identified gene cassettes. The sequence derived from the 769 bp amplicon of three *E. coli* strains KD8, KD46, and KD62 (GenBank accession no. EU339236, EU250576, and EU250577, respectively) and one *K. pneumoniae* strain KD187 (GenBank accession no. EU339235) showed *dfrA7* gene. The sequence derived from the 1,586 bp amplicon of one *E. coli* strain KD39, two *Proteus vulgaris* strains KD171 and KD191, one *K. pneumoniae* strain KD65 and one *K. oxytoca* strain KD84 showed *dfrA1-aadA1* gene cassette array. The sequence derived from the 1,663 bp amplicon of two *E. coli* strains KD48 and KD145 showed

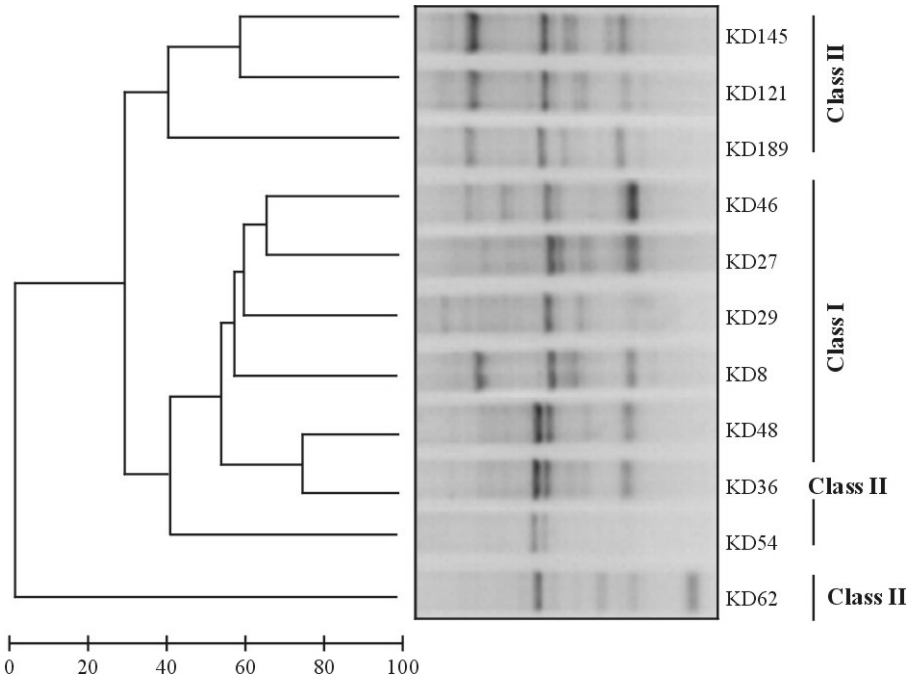

Fig. 2. The similarity dendogram of DNA fingerprinting using BOXA1R primer of *Escherichia coli* isolates containing class 1 and class 2 integrons isolated from river water using the UPGMA method.

dfrA17-aadA5 gene cassette array. The resulting sequence from *E. coli* KD54 (GenBank accession no. EU339233) showed *dfr2d* gene cassette which encodes dihydrofolate reductase type 2 enzyme conferring resistance to trimethoprim. Analysis of the nucleotide sequences of the common class 2 integrons derived from the 2,224 bp amplicon of five *E. coli* strains KD27, KD36, KD121, KD145, and KD189 (GenBank accession no. EU339237) showed *dfrA1* gene which encodes dihydrofolate reductase conferring resistance to trimethoprim, *sat1* gene which encodes streptothricin acetyltransferase 1 conferring resistance to streptothricin antibiotic and *aadA1* gene which encodes aminoglycoside adenyltransferase A conferring resistance to streptomycin/spectinomycin antibiotics.

BOX-PCR fingerprinting

We genotyped eleven strains carrying class 1 and class 2 integrons and/or transferable antibiotic resistance by BOX-PCR fingerprinting. Analysis yielded patterns of amplification product for all isolates, and these products varied in molecular weights, as shown in Fig. 2. To evaluate the strain diversity of *E. coli* from river water samples, dendrogram of BOX fingerprintings was constructed by using the UPGMA method. Significant clusters in each dendrogram were identified (Fig. 2), and *E. coli* strains containing class 1 and class 2 integrons were discriminated from each other. Two major clusters were obtained. One corresponded to class 1 and class 2 integrons and second corresponding to the *E. coli* strain KD62 containing class 2 integron. The class 1 and class 2 integron-containing strains' cluster was also divided into two major sub-clusters; one contained the strains harboring class 2 integron, and the other had the whole strains carried class 1 integrons along with the strain KD36 carrying class 2 integron.

Discussion

The increase in the number of resistant and multidrug-resistant strains of bacteria is a major concern worldwide, particularly with the decline in the number of new antibiotics available for treatment in clinical settings. The prevalence of bacterial resistance to antibiotics in the aquatic environment has received comparatively little attention. In the current study, we found that antibiotic-resistant coliforms were widespread in water samples from ten rivers in northern Turkey. Our recent studies showed that drinking waters in public use in the same region were contaminated with multiple antibiotic-resistant *E. coli*, and these water sources are supplied mainly from the local rivers after processing such as chlorination (Alpay-Karaoglu *et al.*, 2007; Ozgumus *et al.*, 2007). It has been reported that in natural environments, resistant organisms can be indigenous or introduced through natural or anthropogenic causes (Hu *et al.*, 2008).

In all rivers sampled during one year, high fecal coliform counts (most probable number, >1,100 CFU/100 ml) were observed according to the results of multiple-tube fermentation assays (data not shown). The results presented here reflect that there is a serious anthropogenic pollution in the rivers, probably resulting from the intensive urbanization present around the rivers. Moreover, some of the domestic sewage inputs of these settlements discharge into these local streams. Thus, much of the isolates exhibited high-level antibiotic resistance. However, Toroglu *et al.* (2005) has been reported that 40% of the coliforms isolated from a river in southern Turkey showed multiple antibiotic resist-

ance (MAR). On the other hand, Pathak and Gopak (2008) have reported that coliforms identified as *E. coli*, *Klebsiella* sp., *Enterobacter* sp., and *Citrobacter* sp. were present in treated drinking water samples. However, we detected the same coliform pattern in river water samples.

Recently, MAR for kanamycin, nalidixic acid, tetracycline, and trimethoprim have been shown in fecal coliforms isolated from both stool samples from humans and treated drinking water in India (Pathak and Gopak, 2008). Similarly, *E. coli* isolates from drinking water sources in Jordan have been detected high resistance levels for ampicillin, trimethoprim/sulfamethoxazole, gentamicin, and tetracycline (Shehabi *et al.*, 2006). However, all class 1 integron-carrying strains were resistant to sulfamethoxazole (data not shown), a sulfonamide antibiotic which is an indicative of the *sul1* gene conferring resistance to sulfonamides (Hall and Collis, 1995). Our results are in general agreement with that reported by latter two investigators.

The occurrence of coliforms with high-level resistance to ampicillin and to other antibiotics has been proposed to reflect human influence in the environment (Andersen and Sanda, 1994). High-level of resistance to ampicillin (58%) was in agreement with that reported by other investigators from China (Hu *et al.*, 2008), from Australia (Watkinson *et al.*, 2007), and from India (Pathak and Gopak, 2008). A previous study by Koksal *et al.* (2007) from Turkey exhibited much lower level at 48%. The mechanism of resistance to ampicillin could probably be mediated by TEM-type β-lactamases encoded by $bla_{\text{TEM-1}}$ genes, which is commonly carried by Tn*3* transposon, highly widespread and emerging in clinical isolates of *Enterobacteriaceae* family as suggested by Roy (1999).

Transconjugant and transformant of two strains (KD55 and KD100) expressed high-level resistance to nalidixic acid in MIC of 128 μg/ml. We tested these strains for the presence of plasmid-mediated quinolone resistance genes, *qnrA*, *qnrB*, and *qnrS* by PCR (data not shown). We found that these strains carried *qnrS* gene. Although plasmid-mediated quinolone resistance has been known to confer low-level resistance (Nordmann and Poirel, 2005), some strains have also been shown to express high-level resistance to nalidixic acid in MIC of 128 μg/ml in Latin America (Castanheira *et al.*, 2007), like those in the current study. They suggested that the multi-copy plasmids could result in the over-expression of the *qnr* genes, and this could cause high-level resistance to nalidixic acid.

The high MIC values of the antibiotics against to the transconjugants and the transformants suggests that coliforms from the rivers could probably be a pool of the antibiotic resistance determinants, which contains movable genetic structures such as transposons and/or integrons, and also reflects that there is always a risk of interchange of antibiotic resistance elements between resident bacterial populations and human pathogens. The results are epidemiologically similar to those of the surveys of the rivers in India (Mukherjee and Chakraborty, 2007) and from South Africa (Biyela *et al.*, 2004).

Class 1 and/or 2 integrons have been reported in clinical isolates of *E. coli* (Solberg *et al.*, 2006), resistant coliforms from human and animal sources (Van Essen-Zanderbergen *et al.*, 2007), non-coliform pathogenic bacteria (Chang *et al.*, 2007), *E. coli* from human stools (Phongpaichit *et al.*, 2008), bacteria from food (Sunde, 2005), avian pathogenic *E. coli* strains (Kim *et al.*, 2007) and also in aquatic environments (Roe *et al.*, 2003). Together, class 1 and 2 integrons accounted for 17 (13%) of 130 multidrug-resistant coliform isolates in this study. This prevalence was almost the same with that reported by Roe *et al.* (2003), who showed that 16% of *E. coli* isolates from irrigation water and sediments contained class 1 and 2 integrons, but was far lower than that reported from Jordan (Shehabi *et al.*, 2006), who has been shown that 36% of *E. coli* isolates from drinking waters carried class 1 integron integrase genes. The residence of class 1 and 2 integrons associated with R plasmids was agreement with that reported by Mukherjee and Chakraborty (2007). The class 1 and class 2 integrons associated with R plasmids may contribute to the horizontal dissemination of antibiotic resistance gene cassettes, resulting in important information on the mechanism of acquisition of multidrug-resistance genes in contaminating bacteria in different aquatic and also in clinical environments. Interestingly, in the present study, we observed that the source of class 1 and 2 integron-bearing strains were mostly different from each other according to the BOX-PCR fingerprinting. Besides, our previous study (Ozgumus *et al.*, 2007) showed that tap and spring water sources in the same region were possibly contaminated by the same sources of *E. coli* strains.

We found a 767 bp class 1 integron carrying a gene cassette, *dfrA7*, harbored by three coliforms. This gene cassette has been demonstrated for the first time in a *K. pneumoniae* strain isolated from river waters. On the other hand, we showed for the first time a gene cassette, *dfrA16* (GenBank accession no. EU158182) inserted in a 741 bp class 1 integron in a *Citrobacter koseri* isolate. Similar studies regarding the identification of integrons harbored by coliform isolates have been reported from Indian (Mukherjee and Chakraborty, 2006) and South African rivers (Biyela *et al.*, 2004). The presence of class 1 and class 2 integrons in different species of *Enterobacteriaceae* family is significant in the context of gene transfer and dissemination of resistance gene cassettes in the environment and maybe to humans' microbiota or pathogenic organisms. These results indicate that class 1 and class 2 integrons are widespread in aquatic enterobacteria, and they may act as a receptacle for the movable genetic elements coding antibiotic resistance traits and spread of bacterial antibiotic resistance genes in the environment.

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References

Alpay-Karaoglu, S., O.B. Ozgumus, E. Sevim, F. Kolayli, A. Sevim, and P. Yesilgil. 2007. Investigation of antibiotic resistance profile and TEM-type β-lactamase gene carriage of ampicillin-resistant *Escherichia coli* strains isolated from drinking water. *Ann. Microbiol.* 57, 281-288.

Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389-3402.

Andersen, S.R. and R.A. Sanda. 1994. Distribution of tetracycline resistance among gram-negative bacteria isolated from polluted and unpolluted marine sediments. *Appl. Environ. Microbiol.* 60, 908-912.

Ash, R.J., B. Mauck, and M. Morgan. 2002. Antibiotic resistance of gram-negative bacteria in rivers, United States. *Emerg. Infect. Dis.* 8, 713-716.

Ausubel, F.M., R. Brient, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1995. Short Protocols in Molecular Biology, 2nd ed., John Willey & Sons, New York, N.Y., USA.

AWWA-American Water Works Association (AWWA). 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, American Water Works Association and Water Environment Federation, Washington, D.C., USA.

Biyela, P.T., J. Lin, and C.C. Bezuidenhout. 2004. The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. *Water Sci. Technol.* 50, 45-50.

Brenner, D.J. 1986. Facultatively anaerobic gram-negative rods, p. 408-516. *In* N.R. Krieg and J.G. Holt (eds.), Bergey's Manual of Systematic Bacteriology, Vol. 1. Williams & Wilkins, Baltimore, USA.

Carattoli, A. 2001. Importance of integrons in the diffusion of resistance. *Vet. Res.* 32, 243-259.

Castanheira, M., A.S. Pereira, A.G. Nicoletti, A.C.C. Pignatari, A.L. Barth, and A.C. Gales. 2007. First report of plasmid-mediated *qnrA1* in a ciprofloxacin-resistant *Escherichia coli* strain in Latin America. *Antimicrob. Agents Chemother.* 51, 1527-1529.

Chang, L.L., H.H. Lin, C.Y. Chang, and P.L. Lu. 2007. Increased incidence of class 1 integrons in trimethoprim/sulfamethoxazole-resistant clinical isolates of *Stenotrophomonas maltophila*. *J. Antimicrob. Chemother.* 59, 1038-1045.

CLSI-Clinical and Laboratory Standards Institute (CLSI). 2003a. Performance standards for antimicrobial disk susceptibility tests; 8th ed., Approved standard M2-A8., CLSI, Wayne, PA, USA.

CLSI-Clinical and Laboratory Standards Institute (CLSI). 2003b. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Vol. 23, No. 2. 6th ed. Approved standard M7-A6, CLSI, Wayne, PA, USA.

Col, N.F. and R.W. O'Connor. 1987. Estimating worldwide current antibiotic usage: Report of task force I. *Rev. Infect. Dis.* 9, S232-243.

Fluit, A.C. and F.J. Schmitz. 2004. Resistance integrons and super-integrons. *Clin. Microbiol. Infect.* 10, 272-288.

Grinsted, J., F. De La Cruz, and R. Schmitt. 1990. The Tn*21* subgroup of bacterial transposable elements. *Plasmid* 24, 163-189.

Hall, R.M. and C.M. Collis. 1995. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol. Microbiol.* 15, 593-600.

Hall, R.M. and H.W. Stokes. 1993. Integrons: novel DNA elements which capture genes by site-specific recombination. *Genetica* 90, 115-132.

Hansson, K., L. Sundström, A. Pelletier, and P.H. Roy. 2002. IntI2 integron integrase in Tn*7*. *J. Bacteriol.* 184, 1712-1721.

Hu, J., J. Shi, H. Chang, D. Li, M. Yang, and Y. Kamagata. 2008. Phenotyping and genotyping of antibiotic-resistant *Escherichia coli* isolated from a natural river basin. *Environ. Sci. Technol.* 42, 3415-3420.

Jones, J.G., S. Gardener, B.M. Simon, and R.W. Pickup. 1986. Antibiotic resistant bacteria in Windermere and two remote upland tarns in the English Lake district. *J. Appl. Bacteriol.* 60, 443-453.

Kado, C.I. and S.T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* 145, 1365-1373.

Kim, T.E., Y.W. Jeong, S.H. Cho, S.J. Kim, and H.J. Kwon. 2007. Chronological study of antibiotic resistances and their relevant genes in Korean avian pathogenic *Escherichia coli* isolates. *J. Clin. Microbiol.* 45, 3309-3315.

Koksal, F., N. Oguzkurt, M. Samasti, and K. Altas. 2007. Prevalence and antimicrobial resistance patterns of *Aeromonas* strains isolated from drinking water samples in Istanbul, Turkey. *Chemotherapy* 53, 30-35.

Leverstein-van Hall, M.A., A.T.A. Box, H.E.M. Blok, A. Paauw, A.C. Fluit, and J. Verhoef. 2001. Evidence of extensive interspecies transfer of integron-mediated antimicrobial resistance genes among multidrug-resistant *Enterobacteriaceae* in a clinical setting. *J. Infect. Dis.* 186, 49-56.

Lévesque, C., L. Piche, C. Larose, and P.H. Roy. 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob. Agents Chemother.* 39, 185-291.

Macrina, F.L., D.J. Kopecko, K.R. Jones, D.J. Ayers, and S.M. McCowen. 1978. A multiple plasmid containing *Escherichia coli* strain: convention source of size reference plasmid molecules. *Plasmid* 1, 417-420.

Mukherjee, S. and R. Chakraborty. 2006. Incidence of class 1 integrons in multiple antibiotic-resistant Gram-negative copiotrophic bacteria from the River Torsa in India. *Res. Microbiol.* 157, 220-226.

Mukherjee, S. and R. Chakraborty. 2007. Conjugation potential and class 1 integron carriage of resident plasmids in river water capiotrophs. *Acta Microbiol. Immunol. Hung.* 54, 379-397.

Nordmann, P. and L. Poirel. 2005. Emergence of plasmid-mediated resistance to quinolones in *Enterobacteriaceae*. *J. Antimicrob. Chemother.* 56, 463-469.

Ozgumus, O.B., E. Celik-Sevim, S. Alpay-Karaoglu, C. Sandalli, and A. Sevim. 2007. Molecular characterization of antibiotic resistant *Escherichia coli* strains isolated from tap and spring waters in a coastal region in Turkey. *J. Microbiol.* 45, 379-387.

Pathak, S.P. and K. Gopak. 2008. Prevalence of bacterial contamination with antibiotic-resistant and enterotoxigenic fecal coliforms in treated drinking water. *J. Toxicol. Environ. Health A* 71, 427-433.

Petersen, A., L. Guardabassi, A. Dalsgaard, and J.E. Olsen. 2000. Class 1 integrons containing a *dhfrI* trimethoprim resistance gene cassette in aquatic *Acinetobacter* spp. *FEMS Microbiol. Lett.* 182, 73-76.

Phongpaichit, S., K. Wuttananupan, and N. Samasanti. 2008. Class 1 integrons and multidrug resistance among *Escherichia coli* isolates from human stools. *Southeast Asian J. Trop. Med. Public Health* 39, 279-287.

Recchia, G.D. and R.M. Hall. 1995. Gene cassettes: a new class of mobile element. *Microbiology* 141, 3015-3027.

Rice, L.B., S.H. Willey, G.A. Papanicolaou, A.A. Medeiros, G.M. Eliopoulos, R.C. Moellering, and G.A. Jacoby. 1990. Outbreak of ceftazidime resistance caused by extended-spectrum β-lactamases at a Massachusetts chronic care facility. *Antimicrob. Agents Chemother.* 34, 2193-2199.

Roe, M.T., E. Vega, and S.D. Pillai. 2003. Antimicrobial resistance markers of class 1 and class 2 integron bearing *Escherichia coli* from irrigation water and sediments. *Emerg. Infect. Dis.* 9, 822-826.

Rohlf, F.J. 1990. NTSYS-pc numerical taxonomy and multivariate analysis system, p. 1-145. Version 1.8, Applied Biostatistics, New York, N.Y., USA.

Rowe-Magnus, D.A. and D. Mazel. 2002. The role of integrons in

antibiotic resistance gene capture. *Int. J. Med. Microbiol.* 292, 115-125.

Roy, P.H. 1999. Horizontal transfer of genes in bacteria. *Microbiol. Today* 26, 168-170.

Seurinck, S., W. Verstraete, and S.D. Siciliano. 2003. Use of 16S-23S rRNA intergenic spacer region PCR and repetitive extragenic palindromic PCR analyses of *Escherichia coli* isolates to identify nonpoint fecal sources. *Appl. Environ. Microbiol.* 69, 4942-4950.

Shehabi, A.A., J.B. Odeh, and M. Fayyad. 2006. Characterization of antimicrobial resistance and class 1 integron found in *Escherichia coli* isolates from human stools and drinking water sources in Jordan. *J. Chemother.* 18, 468-472.

Sneath, P.N.A. and R.R. Sokal. 1973. Numerical taxonomy. W.H. Freeman and Company, San Francisco, USA.

Solberg, O.D., R.M. Ajiboye, and L.W. Riley. 2006. Origin of Class 1 and 2 integrons and gene cassettes in a population-based sample of uropathogenic *Escherichia coli. J. Clin. Microbiol.* 44, 1347-1351.

Sunde, M. 2005. Prevalence and characterization of class 1 and class 2 integrons in *Escherichia coli* isolated from meat and meat products of Norwegian origin. *J. Antimicrob. Chemother.* 56, 1019-1024.

Toroglu, S., S. Dincer, and H. Korkmaz. 2005. Antibiotic resistance in Gram-negative bacteria isolated from Aksu River in (Kahramanmaras) Turkey. *Ann. Microbiol.* 55, 229-233.

Van Essen-Zandbergen, A., H. Smith, K. Veldman, and D. Mevius. 2007. Occurrence and characteristics of class 1, 2 and 3 integrons in *Escherichia coli*, *Salmonella* and *Campylobacter* spp. in the Netherlands. *J. Antimicrob. Chemother.* 59, 746-750.

Versalovic, J., M. Schneider, F.J. de Bruijn, and J.R. Lupski. 1994. Genomic fingerprinting of bacteria with repetitive sequence-based polymerase chain reaction. *Methods Cell. Biol.* 5, 25-40.

Watkinson, A.J., G.R. Micalizzi, J.R. Bates, and S.D. Costanzo. 2007. Novel method for rapid assessment of antibiotic resistance in *Escherichia coli* isolates from environmental waters by use modified chromogenic agar. *Appl. Environ. Microbiol.* 73, 2224-2229.

White, P.A., C.J. McIver, and W.D. Rawlinson. 2001. Integrons and gene cassettes in the *Enterobacteriaceae*. *Antimicrob. Agents Chemother.* 45, 2658-6261.