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Antibiotic Resistance Profiles of Enteric Bacteria Isolated from Kucukcekmece Lagoon (Istanbul–Turkey)

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Abstract

The aim of this study is to find out the density of the fecal bacteria and to analyze resistance to antimicrobials of Gram negative bacilli isolated from the Kucukcekmece Lagoon, Istanbul. Samples were taken monthly from June 2006 to June 2008 and a total of 232 Gram negative bacilli were isolated. Chloramphenicol, tetracycline, nalidixic acid, ampicillin, imipenem, ceftazidime, amikacin, streptomycin and amoxicillin + clavulanic acid were used in antimicrobial susceptibility tests. Susceptibility to trimethoprim + sulfamethoxazole was also examined in only integron-bearing organisms. The antibiotic resistance tests resulted in bacteria being the most resistant against ampicillin (76.29%) and the most sensitive against amikacin (93.56%). Of 232 isolates, 20 (8.6%) coliforms harbored class 1 and/or class 2 integrons. DNA sequencing showed that variable regions of the integrons harbored various gene cassettes; *dfrA12*, *dfrA15*, *dfrA17*, *aadA1*, *aadA2*, *aadA5*, bla_{OXA-30} and *sat2*. Integrons were found in bacteria from all sampling areas except 12 and D3.

In this study, the determination of bacterial identification of the species of Gram negative bacilli and their Antibiotic Resistance Profiles in the Kucukcekmece Lagoon for the first time was investigated. A finding indicates that there is a heavy fecal pollution in this lagoon environment, which might probably be resulted from the intensive anthropogenic facilities. The risk to public health could be the transfer of antibiotic resistance determinants from the bacterial isolates to normal microbiota bacteria of humans unless the effective precautions such as water treatment plants are taken.

Keywords: Gram negative bacilli, antibiotic resistance, integron, Kucukcekmece Lagoon, Istanbul, Turkey.

Küçükçekmece Lagünü'nden (İstanbul- Türkiye) İzole Edilen Enterik Bakterilerin Antibiyotik Direnç Profilleri

Özet

Bu çalışmanın amacı, Küçükçekmece Lagünü'ndeki (İstanbul) fekal bakteri yoğunluğunu bulmak ve izole edilen Gram negatif basillerin antibiyotiklere direncini analiz etmektir. Örnekler, Haziran 2006'dan Haziran 2008 tarihleri arasında aylık olarak alınmış ve toplam 232 Gram negatif basil izole edilmiştir. Antimikrobial hassasiyet testleri için kloramfenikol, tetrasiklin, nalidiksik asit, ampisilin, imipenem, seftazidim, amikasin, streptomisin ve amoksisilin+klavulanik asit kullanılmıştır. Trimetoprim+sulfametoksazola yatkınlık da sadece integron taşıyan organizmalar için incelenmiştir. Antibiyotik direnç sonuçlarına göre bakterilerin en dirençli olduğu antibiyotik ampisilin (%76,29) ve en hassas olduğu antibiyotik ise amikasin (%6,44) olarak bulunmuştur. 232 izolatın 20 si (%8,6) sınıf 1 ve / veya sınıf 2 integron barındırmıştır. DNA sekanslama sonucunda, integronlara ait değişken bölgelerin barındırdığı gen kasetlerinin, DfrA12, dfrA15, dfrA17, aadA1, aadA2, aadA5, blaOXA-30 ve sat2 olduğu tespit edilmiştir. Bakterilere ait bu integronlar 12 ve D3 nolu istasyonlar dışındaki tüm örnekleme bölgelerinde bulunmuştur.

Bu çalışmada, Küçükçekmece Lagünü'nde Gram negatif bakteri türlerinin identifikasyonu ve antibiyotik direnç profili ile ilgili çalışma ilk kez yapılmıştır. Muhtemelen yoğun antropojenik girdilerin sonucu olarak Lagün'de ağır bir fekal kirlilik olduğu görülmüştür. Atık su arıtma tesisleri gibi etkili önlemler alınmazsa, insanların normal mikrobiotasında yer alan bakteri izolatlarına, antibiyotik direnç genlerinin aktarımı ile halk sağlığı riski oluşması olasıdır.

Anahtar Kelimeler: Gram negatif basiller, antibiyotik direnci, integron, Küçükçekmece Lagünü, İstanbul, Türkiye.

Introduction

The study of water system ecology and life focuses on scientific studies of physical, chemical and biological conditions in fresh waters such as lakes, ponds and streams. Except some microorganisms,

which take part in the floras of the marine ecosystems and contribute to some kind of biogeochemical circles, some other microorganisms arising from humans and animals that are present in water and have pathogen effects, are also available. Besides, well-known organisms that come from the living bodies carrying the disease and then moving into the water may be present in the soil and also participate directly to sea ecosystems through organisms' faeces (Logan 1994).

Antibiotic resistance has been detected in various aquatic environments (Ash *et al.*, 2002). Introduction of antibiotics into the aquatic environments via medical therapy, agriculture and animal husbandry has resulted in selective pressure on bacterial populations (Col and O'Connor, 1987). A large part of the antibiotics consumed ends up in wastewater, and in the wastewater the antibiotics may exert selective pressure for or maintain resistance among microorganisms. Antibiotic resistant bacteria and genes encoding antibiotic resistance are commonly detected in wastewater, often at higher rates and concentrations compared to surface water. Wastewater can also provide favorable conditions for the growth of a diverse bacterial community, which constitutes a basis for the selection and spread of antibiotic resistance (Börjesson, 2009, Kümmerer, 2009b, 2009c).

Multidrug-resistant bacteria in different environments have become a major public health concern due to the transfer of the antimicrobial-drug resistance (R) determinants in bacterial population. This is generally mediated by genetic elements such as integrons, transposons and plasmids (Recchia and Hall, 1995; Carattoli, 2001; Rowe-Magnus and Mazel, 2002). Integrons are genetic structures carrying antibiotic resistance 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 resistance plasmids and transposal elements (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 the family *Enterobacteriaceae* and non-fermenters (Fluit and Schmitz, 2004). Class 1 integrons have been identified transposons such as Tn*21* (Grinsted *et al.*, 1990). Class 2 integrons are present on transposon Tn*7*. Class 1 and/or class 2 integrons have been shown in clinical isolates of the *Enterobacteriacea* family (Leverstein-van Hall *et al.*, 2001) and also in aquatic environments (Roe *et al*., 2003).

The incidence of antibiotic resistance bacteria in aquatic environments has increased dramatically as a consequence of the widespread use of antibiotics by humans (Baya *et al*., 1986; Kümmerer, 2009b, 2009c). During recent decades, antibiotics have been widely used as therapy for bacterial infections in humans and animals, and as growth promoters in agriculture and aquaculture, increasing the percentages of antibiotic resistance bacteria in various environments and may cause problems in therapy by selecting resistance bacteria (Silva *et al*., 2006).

In this study, the determination of bacterial identification of the species of Gram negative bacilli in the Kucukcekmece Lagoon for the first time was investigated. This study focuses on enteric bacteria, their density and the bacteriological pollution, together with their contents of antibiotic resistance gene cassettes inserted into integrons.

Materials and Methods

Study Site and Sample Collection

The Kucukcekmece Lagoon is in the western part of the city of Istanbul. The Lagoon has a surface area of 15.22 km^2 and the coordinates are 41º00′ N - 28º43′ E (Figure 1). The lagoon is connected to the Marmara Sea (Deniz) via a narrow channel. Despite this connection, the lagoon's main water sources are underground springs, three nutrient-rich streams (Sazlıdere (D1, D2, D3), Ispartakule (E2, E3) and Nakkas), and several ephemeral brooks. Therefore, the lagoon (10, 11, 12 and 13) has been subjected to heavy nutrient inputs because of poor sanitary treatment of wastewater associated with human population growth around the lake.

The water samples were collected monthly from ten different stations along the Kucukcekmece Lagoon for a period of two year from June 2006 to June 2008 in a cloudless and bright day. The water samples were collected between 8-10 am in surface with the bacteriologic sampler bottles (500 ml) in sterilized dark glass bottles. Five water samples were taken from surface at the junction point between the stream and the lagoon and the other five samples were taken from various points within the Lagoon and from the surface and different depths (50 cm, 1 m, 5 m, 10 m and occasionally 15 m). Samples were transported to the laboratory in icebox and subjected to bacteriological examination with in 4 hours of collection. The physical measurements have been designated in the categories of temperature, pH, salinity and dissolved oxygen (in situ) with a multiprobe model.

Isolation and Identification of Bacterial Isolates

The bacteriological analyses started within 4 hours after being received and after a five tube most probable number (MPN) method (Lauryl Sulfate Broth (Merck 1.10266)) was used. For the detailed analyses, the standard water and wastewater methods were applied (APHA, 2005). Water samples were plated on eosin methylene blue agar (EMB, Merck 1.01347) and chromogenic UTI agar (HiMedia, MV1353). To identify of the species in

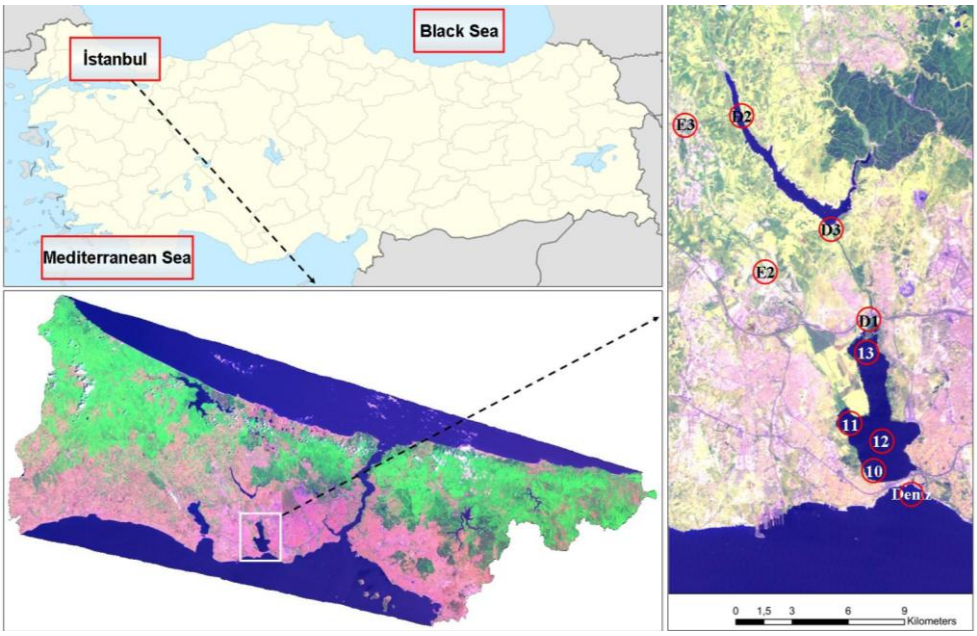

Figure 1. Location of Kucukcekmece Lagoon showing sampling sites (41º 00´ N - 28º 43´ E).

Enterobacteriaceae, typical colonies were selected and confirmed by IMVIC tests.

Susceptibility Testing

The minimum inhibition concentration was determined by the disk diffusion method in Mueller-Hinton medium in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2003). This method allows for the determination of total levels of antibiotic resistance from typical colonies, thereby detecting the most resist phenotypes present. Nine antimicrobial agents were selected as representatives of important classes of antimicrobials: ampicillin (AM, 10 µg), amoxicillin-clavulanic acid (AMC, 10 µg), tetracycline (TE, 30 µg), chloramphenicol (CM, 30 µg), nalidixic acid (NA, 30 µg), amikacin (AC, 30 µg), streptomycin (S, 10 µg), imipenem (I, 10 µg), ceftazidime (CAZ, 10 µg). The results were separately interpreted by using the breakpoints from the CLSI guidelines for the family *Enterobacteriaceae* and non-fermenters (CLSI, 2003).

PCR for Integrons

To prepare DNA templates for PCR, bacterial isolates were inoculated into LB broth and incubated for 20 h at 37ºC with shaking. Cells from 1.5 ml of the overnight culture were collected 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 and 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 amplified from the bacterial isolates by using the specific oligonucleotide primers for class 1 integrons; 5'-CS, 5'-GGCATCCAAGCAGCAAG-3' and 3'-CS, 5'-AA GCAGACTTGACCTGA-3' and for class 2 integrons; hep51: 5'-GATGCCATC GCAAGTACGAG-3'and hep74: 5'-CGGGATCCCGGACGGATGCACGA TTT GTA-3', amplifying the variable regions of the integrons. Reaction composition and cycling parameters were used the methods previously described for class 1 integrons (Lévesque *et al.*, 1995) and for class 2 integrons (White *et al.*, 2001). The PCR products were then electrophoresed on 1% agarose gel containing 0.5 µg/ml ethidium bromide (Sigma, St. Louis, MO) and visualized with UV light.

DNA Sequencing and Bioinformatic Analysis

After PCR products of the integrons were purified from the agarose gel by using QIAQuick® Purification Kits (QIAgen, Crawly, UK) prior to sequencing, they were cloned into the pGEM-T Easy vector according to the manufacturer's instructions (Promega, Madison, USA). Recombinant plasmids carrying amplicons of class 1 and class 2 integrons were sent to Macrogen Inc., Seoul, 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, CLUSTALW, accessible from the European Bioinformatics Institute website (http://www.ebi.ac.uk/clustalw).

Statistical Analyses

The antibiotics resistances are expressed as percentage. The statistical significance of differences in antibiotic resistance and required statistical analyses were applied by using SPSS® version 13 program and MVSP 3.1.

Results

As a result, the expected seasonal variations of the values from the measured physical parameters were displayed in Figures. As can be seen in Figure 2, air and lagoon water temperature values varied between 4°C and 34.8°C and between 8.3°C and 26.7°C, respectively. The temperature variation between sampled periods reached up to 10ºC. Higher water temperatures were recorded in the summer period of 2007 (Figure 2). However, mean surface temperature did not differ considerably among the sites (P<0.001). All stations waters, except for Ispartakule stream, were well oxygenated. The pH data obtained suggested a slightly alkali and mean values 7.6-8.4 (Figure 3). The values did not show any considerable sudden changes which could have affected the bacterial growths. Similar results also were obtained for salinity. Salinity dates' were differences between stream and lagoon stations. These changes have been effective on the distributions of bacterial species (P<0.001).

The statistical analysis (ANOVA) that was conducted in order to display the differences among the stations split the stations into four major groups. The differences in water temperature, salinity and DO that draws attention during sampling in the stations, were also determined to be statistically significant as a result of the evaluation of the data. The stations with a statistically significant difference among themselves (P<0.01) are provided in Figure 4. The results provided in this figure indicated Stations E2, D2, D3 and E3 (stream group); as well as Stations D1, 10 and 11 (lagoon group) as two distinct groups. The Kucukcekmece Lagoon is connected to the Marmara

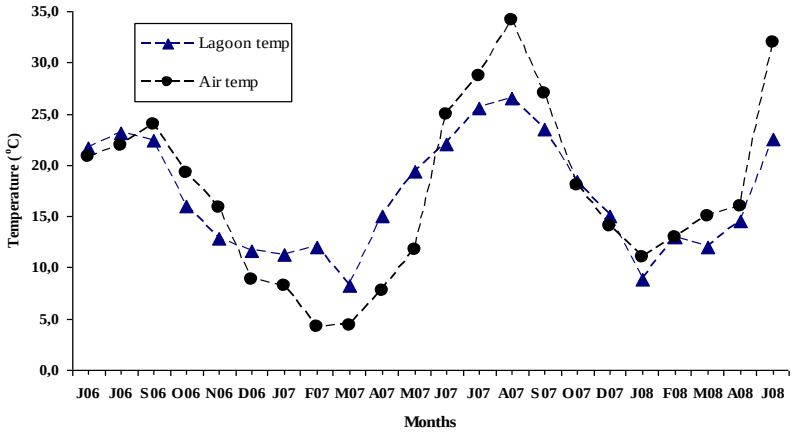

Figure 2. Monthly variations of temperature at sampling stations.

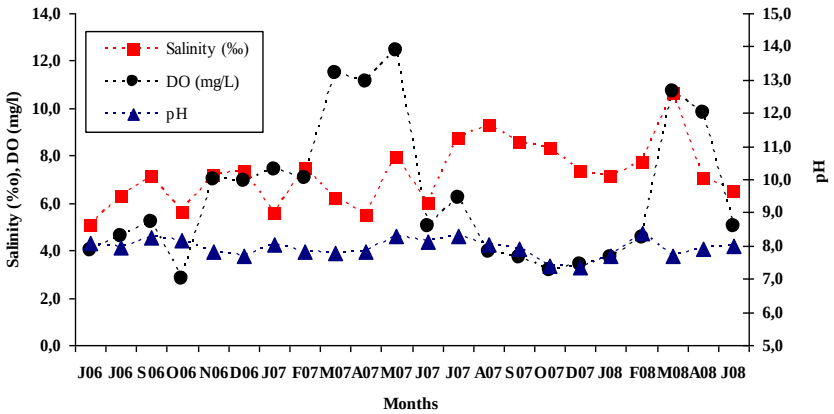

Figure 3. Monthly variations of pH, salinity (‰) and DO (mg/L).

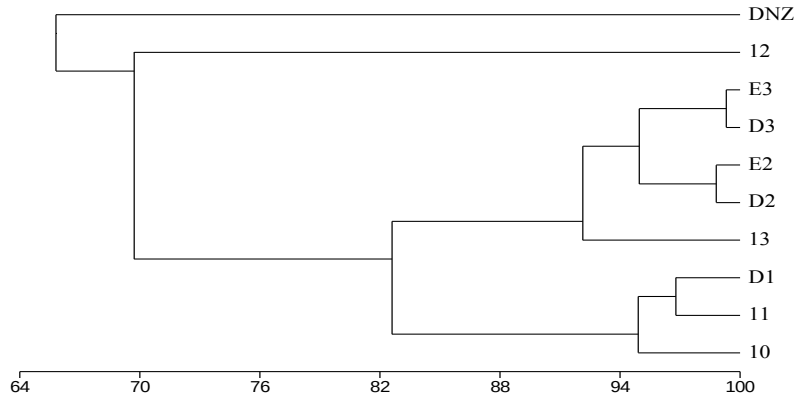

Figure 4. Bray-Curtis similarity dendrogram for the sampling stations.

Sea via a narrow channel, and station Deniz (DNZ) was selected such that the lagoon ecosystem and its connection with the sea could be represented. Station 12 was selected such that it was located at the middle of the lagoon, thus unaffected from the presence of point sources.

In the present study, the bacterial identification of enteric bacteria and antibiotic resistance tests were determined in the Kucukcekmece Lagoon. Total of 232 bacterial isolates were identified. Antibiotic resistance was observed to varying degrees in all stations (Table 1). Using a direct antibiotic susceptibility assay, specific resistance levels (%) in all water samples examined in ascending frequency of resistance to the following 9 agents ampicillin (AM), amoxicillin-clavulanic acid (AMC), tetracycline (TE), chloramphenicol (CM), nalidixic acid (NA), amikacin (AC) (17%), streptomycin (S), imipenem (I), ceftazidime (CAZ) were showed. The highest resistance rate was observed for ampicillin in lagoon (74.4%), sea (84.6%) and stream (77.6%). The lowest resistance rates were for amikasin 5.8%, 7.7 %, 7.1% and imipenem 8.3%, 7.7% and 5.1%, respectively. And percentage of the bacterial isolates showing resistance to aminoglycosides such as streptomycin (23.1%, 23.1%, 17.3%) was quite high frequency. Total average resistance of AM (76.3%) > AMC (36.9%) > S (20.7%) > NA and TE (16.8%) > CAZ (16.4%) > CM (9.4%) > I (6.8%) > AC (6.4%) were also determined (Figure 5).

Wide differences in resistance percentages were observed for particular antimicrobials among bacterial genera, as was the case for tetracycline and for genera such as *Serratia* and *Klebsiella*. Moreover, resistance percentages for different antimicrobials greatly differed within the same bacteria, among *Klebsiella* sp. Resistance percentages were significantly different between stations within the same bacterial genus, with the exception of *Serratia* and *Klebsiella* sp. isolates which were resistant to amoxicillin-clavulanic acid and chloramphenicol, respectively (Table 2).

All 232 Gram negative isolates were screened for the presence of class 1 and class 2 integrons by specific PCR. The amplicon lengths, corresponding to the approximate sizes of the inserted DNA cassette, varied from 0.7 to 1.9 kb for class 1 integrons and 2.2 kb for class 2 integrons. In total, 20 (8.6%) of the coliforms carried detectable class 1 integron structure, and one (0.4%) had class 2 integrons that was harbored in unidentified Gram negative bacterium (Table 2).

Class 1 and class 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*, *dfrA12*, *dfrA15* and *dfrA17* which encodes dihydrofolate reductase enzymes conferring resistance to trimethoprim with aminoglycoside adenyltransferase genes such as *aadA1*, *aadA2* and *aadA5* which encodes aminoglycoside adenyltransferase enzyme conferring resistance to streptomycin/spectinomycin antibiotics. One strains carried only one gene cassette, and one isolate (non-fermenting Gram negative bacterium) 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. Analysis of the nucleotide sequences of the common class 2 integrons derived from the 2224 bp amplicon of unidentified Gram negative bacterium showed *dfrA1* gene which encodes dihydrofolate reductase conferring resistance to trimethoprim, *sat2* 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 (Table 2).

Table 1. Antibiotic resistance rates in bacteria isolated from lagoon, sea and stream

	No. of resistance to antibiotics* (%)								
	AM	AC	S	I	TE	CM	NA	CAZ	AMC
					Lagoon				
10	19(15.7)	2(1.7)	6(5.0)	3(2.5)	5(4.1)	3(2.5)	4(3.3)	5(5.0)	8(9.7)
11	25(20.7)	3(2.5)	8(6.6)	3(2.5)	4(3.3)	2(1.7)	9(7.4)	3(3.0)	7(8.5)
12	29(24.0)	2(1.7)	10(8.3)	2(1.7)	9(7.4)	7(5.8)	5(4.1)	6(5.9)	11(13.4)
13	17(14.0)	0(0.0)	4(3.3)	2(1.7)	1(0.8)	0(0.0)	2(1.7)	4(4.0)	3(3.7)
Tot R	90(74.4)	7(5.8)	28(23.1)	10(8.3)	19(15.7)	12(9.9)	20(16.5)	18(17.8)	29(35.4)
Tot S	31(25.6)	114(94.2)	93(76.9)	111(91.7)	102(84.3)	109(90.1)	101(83.5)	83(82.2)	53(64.6)
Total	121	121	121	121	121	121	121	101	82
					Sea				
Sea	11(84.6)	1(7.7)	3(23.1)	1(7.7)	3(23.1)	2(15.4)	4(30.8)	1(9.1)	5(50.0)
Tot R	11(84.6)	1(7.7)	3(23.1)	1(7.7)	3(23.1)	2(15.4)	4(30.8)	1(9.1)	5(50.0)
Tot S	2(15.4)	12(92.3)	10(76.9)	12(92.3)	10(76.9)	11(84.6)	9(69.2)	10(90.9)	5(50.0)
Total	13	13	13	13	13	13	13	11	10
					Stream				
D1	19(19.4)	2(2.0)	5(5.1)	1(1.0)	4(4.1)	4(4.1)	3(3.1)	6(7.2)	5(6.9)
D2	13(13.3)	0(0.0)	4(4.1)	0(0.0)	5(5.1)	1(1.0)	6(6.1)	3(3.6)	5(6.9)
D3	12(12.2)	2(2.0)	2(2.0)	3(3.1)	4(4.1)	1(1.0)	1(1.0)	2(2.4)	5(6.9)
E2	16(16.3)	1(1.0)	4(4.1)	0(0.0)	3(3.1)	1(1.0)	4(4.1)	2(2.4)	5(6.9)
E3	16(16.3)	2(2.0)	2(2.0)	1(1.0)	1(1.0)	1(1.0)	1(1.0)	0(0.0)	7(9.6)
Tot R	76(77.6)	7(7.1)	17(17.3)	5(5.1)	17(17.3)	8(8.2)	15(15.3)	13(15.7)	27(37.0)
Tot S	22(22.4)	91(92.9)	81(82.7)	93(94.9)	81(82.7)	90(91.8)	83(84.7)	70(84.3)	46(63.0)
Total	98	98	98	98	98	98	98	83	73

* AM: ampicillin, AC: amikacin, S: streptomycin, I: imipenem; TE: tetracycline, CM: chloramphenicol, NA: nalidixic acid, CAZ: ceftazidime; AMC: amoxicillin+clavulanic acid

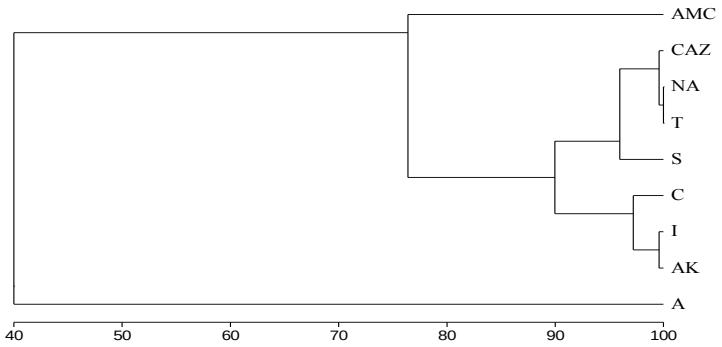

Figure 5. Bray-Curtis similarity dendrogram for total average of each antibiotics

Discussion

In this study, for the first time in literature, the density and bacterial identification of the species of *Enterobacteriaceae* and non-fermentative Gram negative bacteria in Kucukcekmece Lagoon were determined. When the results at the narrow channel of the Kucukcekmece Lagoon and the Marmara Sea were examined, it was found that the values were over the standards. It is interesting to note that there were no refining activities, even though the anthropogenic pollutants were at the level of threatening the human health. High resistance levels to certain groups of antibiotics detected in the bacteria isolated from these aquatic environments supports this hypothesis. In the period of the investigation, the bacterial species were altered; on the other hand their density values did not show a change. When the lagoon stations which displayed different bacterial compositions were gathered in groups, the results were changing significantly every month.

It's known that characteristics of fecal coliforms and *E. coli*, which are indicators for fecal contamination, depend on environmental conditions and the presence of industrial effects. In fact, during the observations of the sampling, it was seen that the streams with the low flow-speeds such as Ispartakule Stream, were not only exposed to industrial pollution, but also to anthropogenic pollution. Stream Ispartakule, which showed a monotonous structure by *E. coli* in the period of November-December-February, turned into a structure where all kind of species were changing by environmental factors. It is said that, especially, until the seawaters get cold in

Table 2. Epidemiological properties of the bacterial isolates containing integrons

No	Species	Date isolated	Site isolated	Gene cassettes / size (bp)	Resistance phenotype*	GenBank Accession No.
13b	Non-fermentative Gram negative bacterium	29.10.06	Lagoon	*dfrA12*, *aadA2* / 1913	SXT, STR	GQ280255
10a	*Escherichia coli*	24.02.07	Lagoon	*dfrA15* / 739	STR	GQ280260
D1b	Unidentified bacterium	21.09.06	Stream	*dfrA15* / 739	SXT	GQ280262
Deniz2	Unidentified bacterium	23.03.07	Sea	*aadA1* / 1009	STR	-[a]
11/50b	*Enterobacter* spp.	23.10.06	Lagoon	*aadA2* / 792	STR	-[b]
10/1b	Non-fermentative Gram negative bacterium	29.11.06	Lagoon	*dfrA17*, *aadA5* / 1664	SXT, STR	-[c]
10/1a	Non-fermentative Gram negative bacterium	23.10.06	Lagoon	bla_{OXA-1}, *aadA1* / 2013	AMP, STR	-[d]
D2b	*Escherichia coli*	29.11.06	Stream	*dfrA17*, *aadA5* / 1664	SXT, TE, NA, STR	-[c]
Deniz1a	*Enterobacter* spp.	23.03.07	Sea	*aadA1* / 1009	STR	-[a]
E2a	Unidentified bacterium	24.02.07	Stream	*dfrA15* / 793	SXT	-[e]
E2a	Unidentified bacterium	18.01.07	Stream	*dfrA15* / 793	SXT	GQ280261
10a	Unidentified bacterium	23.10.06	Lagoon	*dfrA1*, *sat2*, *aadA1* / 2224	SXT, STR	-[f]
D1b	Unidentified bacterium	21.09.06	Lagoon	*dfrA15*, *aadA1* / 1594	SXT, STR	GQ280259
E3	*Escherichia coli*	23.03.07	Stream	*dfrA12*, *orfF*, *aadA2* / 1913	SXT, STR	GQ280256
10/1b	Unidentified bacterium	29.06.07	Lagoon	*dfrA17*, *aadA5* / 1664	SXT, STR	-[c]
E2b	*Enterobacter* spp.	24.02.07	Stream	*dfrA17* / 767	SXT	-[g]
13/50a	*Escherichia coli*	29.11.06	Lagoon	*dfrA1*, *aadA1* / 1586	SXT, STR	-[h]
D1b	Unidentified bacterium	21.09.06	Stream	*dfrA15* / 793	SXT	GQ280262
Deniz a1	*Enterobacter* spp.	23.03.07	Sea	*aadA1* / 1009	STR	-[a]
10/1c	Unidentified bacterium	29.10.06	Lagoon	*dfrA12*, *orfF*, *aadA2* / 1913	AMP, SXT, STR	GQ280257
D1a	*Escherichia coli*	21.09.06	Lagoon	*dfrA12*, *orfF*, *aadA2* / 1913	AMP, TE, C, NA, SXT, STR	GQ280258

*AMP, ampicillin; TE, tetracycline; C, chloramphenicol; NA, Nalidixic acid; SXT, trimethoprime-sulfamethoxazole; STR, streptomycin.
[a] Similar to *aadA1* gene cassette array (GenBank accession no. FJ001871) at nucleotide level.
[b] Similar to *aadA2* gene cassette array (GenBank accession no. FJ001869) at nucleotide level.
[c] Similar to *dfrA17-aadA5* gene cassette array (GenBank accession no. FJ001849) at nucleotide level.
[d] Similar to bla_{OXA-30}-*aadA1* gene cassette array (GenBank accession no. EU339234) at nucleotide level.
[e] Similar to *dfrA15* gene cassette array (GenBank accession nos. GQ280260, GQ280261 and GQ280262) at nucleotide level.
[f] Similar to *dfrA1-sat2-aadA1* gene cassette array (GenBank accession no. EF543147) at nucleotide level.
[g] Similar to *dfrA17* gene cassette array (GenBank accession no. FJ001851) at nucleotide level.
[h] Similar to *dfrA1-aadA1* gene cassette array (GenBank accession no. FJ001874) at nucleotide level

November, almost all the streams would be represented just by a unique species. However, when the environmental factors started to change, particularly, in the periods of temperature increase, different species were observed. Similarly, in the rainfall periods, being added to the washed solid inputs, the seasonal species appeared.

Due to negative effects of anthropogenic pollutant carried by the Sazlıdere (D1, D2, D3) and Ispartakule (E2, E3) treatment plant, the density of the enteric bacteria have been determined as the higher values almost every months. At the same time, effects of physiochemical parameters and rain should be considered. On the other hand, the reason of the increase in the enteric bacteria level at the stations of E2 and E3 in different period is the untreated wastewater which is the source of Ispartakule Stream and human activities. The negative effects of these bacterium groups on coastal ecosystem that reach the sea directly has been searched by means of investigating antibiotic resistance plasmids (Ozgumus *et al*., 2009; Alhaj *et al*., 2007; Danishta *et al*., 2010). No refining activities were observed, although anthropogenic pollutants were at a level to threaten the human health.

As to make a general evaluation, the most intensively apparent species was *E. coli*. Second one was *Citrobacter*. The distribution of this genus was found to be 9% in this study. The species of *Enterobacter* and *Citrobacter* were apparent as well, when *E. coli* was not present. This was expected since they are more resistant against environmental conditions than *E. coli*. Many researchers also determined the same findings and reached the same results (Garrity, 2001; Holt *et al*., 1994). Therefore *E. coli* became more apparent species in such type of streams because they are affected by the industrial pollutants in the stream. Meanwhile, some species have never been considered in evaluations, in spite of their apparent presence in some periods. Some of these species were non-fermentating bacteria; such as Gram negative (which live unrestrictedly in nature) and sporeless bacilli. The most well-known and apparent species are *Acinetobacter* spp., *Pseudomonas* spp. *and Stenotrophomonas* spp. (Logan, 1994; Garrity, 2001). The detailed analysis of these species were not conducted in this study, however they were evaluated as a group. For non-identified Gram negative in biochemical test, enteric was excluded from the bacteria. It is important to note that Gram negative had different environmental bacteria however fecal coliform bacteria was not detected in this test.

In addition to this study, there is an ongoing

investigation about the negative effects of these bacterium groups on coastal ecosystem that reach the sea directly. We know that resistance genes are transferred within a population in the environment (Schmidt *et al*., 2000). Therefore, environmental bacteria should not be regarded as being devoid of antibiotic resistance determinants simply because they are physically removed from clinical settings. Such bacteria have natural intrinsic resistance, as well as having the ability to acquire determinants from agricultural run-off and human wastewater discharge, which may contain antibiotic resistance organisms, as well as sublethal concentrations of metabolically active antibiotic. The tracking of such organisms to their source may help determine the source of fecal pollution in aquatic ecosystems (Moore *et al*., 2010). In our study, 74.4% lagoon, 84.6% sea and 77.6% stream isolates were resistant to ampicillin. One possible explanation for wide variation in ampicillin resistance among reported studies may be due to the composition of bacterial species in different environments and the exchange of R factors. For example, the species composition of a sample has been shown to be influenced by the frequency of fecal input, type and proportion of input, and type of recipient water. Furthermore, a high proportion of ampicillin-resistant *Klebsiella* could transfer resistance factors to other members of the *Enterobacteriaceae* (Parveen *et al*., 1997).

Especially, the ability of dissemination and molecular epidemiology of the resistance and virulence genes between related bacterial species such as *E. coli*, *Citrobacter* sp. and *Enterobacter* sp. has been tried to be searched by means of investigating antibiotic resistance plasmids, transposons and also integron gene cassettes.

In the current study, we found that antibiotic-resistant Gram-negative bacteria were widespread in water samples from lagoon, sea and stream. Many studies (Mukherjee and Chakraborty, 2006; Kim *et al*., 2008; Ozgumus *et al*., 2009) demonstrated that various aquatic environments have the importance as a reservoir for antibiotic resistance genes and resistant bacterial pathogens. A recent study (Ozgumus *et al*., 2009) showed that rivers in northern Turkey were contaminated with multiple antibiotic-resistant *E. coli*, and these strains were detected to harbore *aadA*- and *dfr*-derived gene cassettes inserted into class 1 and class 2 integrons. Laroche *et al*. (2009) also reported the same gene cassette arrays from an estuary in France. Like those in the current study, class 1 integron gene cassettes, such as *aadA1*, *dfrA12-orfF-aadA2*, and *dfrA17-aadA5* were also detected in Korean rivers (Kim *et al*., 2008). The results presented here reflect that there is a serious anthropogenic pollution in different aquatic environments such as rivers or estuary, probably resulting from the intensive urbanization present around those places.

In this study was used to qualitatively estimate the diversity of antibiotic resistance present in waterborne bacteria through a generic approach, by identifying the most resistant organism present in water, by employing a direct antibiotic susceptibility method. Likewise, the basis for the employment of this method was to determine the presence of the most resistant organisms from potentially mixed bacterial populations originating from clinical specimens, so that the most resistant phenotypes could be treated appropriately. Several publications reported that the potential for environmental waterborne organisms to be promiscuous and exchange their resistance genetic determinants, with each other and more importantly, with transient pathogens entering the water system, is a cause for concern (Kümmerer, 2004, 2009a, 2009b, 2009c); Moore *et al*., 2010). In addition, investigations about the negative effects of these bacterial groups on coastal ecosystems that reach the sea directly should be planned. Especially, the ability of dissemination and molecular epidemiology of the resistance and virulence genes between related bacterial species such as *E. coli*, *Citrobacter* sp. and *Enterobacter* sp. and Gram negative non-fermenting bacteria should be monitorized. Furthermore, health risk assessment is required to help define the ecological significance of antibiotic resistance waterborne organisms, but equally to assess the fate of natural environmental organisms.

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