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Antibiotic and Heavy Metal Resistance in Bacteria Isolated from the Eastern Mediterranean Sea Coast

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Abstract In this study it aimed to determine the microbial diversity, level of antibiotic resistance patterns and distribution of heavy metal resistance of bacterial isolates from the Eastern Mediterranean Sea coast. The resistance of 255 Gram-negative bacterial isolates to 16 different antibiotics and to 5 heavy metals was investigated. The most common strains isolated from all samples were *Citrobacter koseri* (9.0 %), *Escherichia coli* (8.2 %) and *Pantoea agglomerans* (8.2 %). Our results revealed a high incidence of resistance to ampicillin (74.0 %), streptomycin (70.0 %) and cefazolin (48.3 %). The multiple antibiotic resistance (MAR) index ranged from 0.2 to 0.75. Isolates showed tolerances to different concentrations of heavy metals. Our results show that the Eastern Mediterranean Sea coast has a significant proportion of antibiotic and heavy metal resistant pathogens, or opportunist Gram-negative bacteria, and these bacteria may result in a potential public health hazard.

Keywords Mediterranean Sea · Antibiotic resistance · Gram-negative bacteria · Heavy metal resistance

Bacteria present in the aquatic environment can be divided into two groups: one of indigenous bacteria and the other a result of contamination by hospital discharge, such as human or animal feces commonly introduced into the aquatic environment. Sewage-polluted water contains large amounts pathogen bacteria. These bacteria have several ways of infecting humans including by being swallowed, inhaled or coming in contact with a wound. The potential for development of antibiotic-resistant bacteria has raised social concerns leading to intensive investigation of the influence of antibiotics on human and ecosystem health (Kim et al. 2011). Antibiotics are poorly absorbed in human and animal digestive systems, resulting in a majority of them being excreted unchanged in feces and urine, which eventually find their way into the environment through the disposal of sewage, hospital wastewater and animal waste (Schlusener and Bester 2006). In the last decade, several studies have reported that antibiotic resistance patterns are becoming a global problem (Matyar et al. 2004; Stachowiak et al. 2010). Resistance can be intrinsic, i.e., associated with reduced penetration of the antibiotic into the cell, or resistance can result from active processes, such as changes in the transport of antibiotics into or from the cell, from modifications of cellular target molecules or from the production of enzymes that modify and deactivate the antibiotic (Hermansson et al. 1987). Antibiotic resistance genes commonly transfer via conjugation or transformation. Conjugative gene transfer mediated by plasmids with a broad host range is generally believed to be a common and widespread mechanism for the transfer of genes across a broad phylogenetic range of bacteria. Metals are toxic to microorganisms, inhibiting enzymatic activities, disrupting membrane functions, and damaging nucleic acids (Gadd 1992). As a result of increasing industrialization, water pollution due to heavy metals has posed serious problems in many aquatic systems because bacteria can acquire resistance after exposure to these agents (Jana and Bhattacharya 1988). The Mediterranean Sea is connected to the Atlantic Ocean, surrounded by the Mediterranean region and almost completely enclosed by land: on the north by Anatolia and Europe, on the south by Africa, and on the east by the Levant. The sea is technically a part of

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the Atlantic Ocean although it is usually identified as a completely separate body of water. It covers an approximate area of 2.5 million km^2.

In this study, it is focused on the Eastern Mediterranean Sea coasts (Mersin, Karatas and Iskenderun). This is the first study tracking antimicrobial and heavy metal resistance in bacteria in the sea water of Mersin and Karatas. The Mersin and Karatas sea-sides are important centers for tourism. At the same time, industrial and domestic waste is discharged near these regions. Iskenderun Bay is important for industrial productions. Domestic waste, including hospital waste, is discharged into the bay. In addition, iron and steel factories, a fertilizer factory, a refinery and a coal-fired power plant discharge a high amount of processed or unprocessed waste into the bay. Despite these environmental pressures, the bay remains an important region for fishing and aquaculture. Thus, the aims of the present study were to identify Gram negative bacteria isolated from coastal areas of the Eastern Mediterranean Sea (Mersin, Karatas and Iskenderun), to determine the antibiotic and heavy metal resistance of these bacteria and to investigate the relationship between antibiotic and heavy metal resistance.

Materials and Methods

In this study samples were collected from May to October 2009 from regions of the Mediterranean Sea on the southern coast of Turkey (Fig. 1); the geographic coordinates of the sampling sites were Mersin: 34°33′06.5″E, 36°45′34.42″N; Karatas: 35°23′06.31″E, 36°33′42.35″N; and Iskenderun: 36°09′09.05″E, 36°35′29.13″*N*. Water samples were collected 0–20 cm below from the surface using sterile 250 mL bottles (APHA 2005). All samples were brought to the laboratory in an ice chest, and processed within 4 h of collection. A total of 51 samples were examined for the presence of Gram negative bacteria (18, 16 and 17 samples from Mersin, Karatas and Iskenderun, respectively).

Bacteria were isolated using the spread plate technique. To determine total counts, plate counts of Gram-negative bacteria were made using MacConkey Agar (McC) (Merck), inoculated with appropriate dilutions from the sample homogenates, and incubated for 24–72 h at 35°C. To evaluate the incidence of resistant bacteria, media supplemented with ampicillin (50 μg mL^{-1}), streptomycin (25 μg mL^{-1}), tetracycline (25 μg mL^{-1}) and chloramphenicol (25 μg mL^{-1}) were used. A total of 255 Gram-negative bacterial isolates were randomly selected from the plates containing antibiotics: 87 isolates from Mersin, 80 from Karatas and 88 from Iskenderun. The isolates were purified onto McC agar and then maintained in nutrient

Fig. 1 Sampling regions

agar (Oxoid). All of the isolates were characterized by phenotypical characteristics, namely Gram staining, oxidase and catalase reactions, motility, OF glucose and gelatin liquefaction tests (Lemos et al. 1985). Isolates were then identified using the Becton–Dickinson Crystal E/NF identification software (BBL, Md, USA).

Antibacterial susceptibility testing was performed by an agar diffusion test (Bauer et al. 1966), using Mueller–Hinton agar (Difco). A total of 16 different antibiotic discs belonging to 9 classes were used in this study, including ampicillin (AM, 10 μg), amikacin (AN, 30 μg), streptomycin (S, 10 μg), gentamicin (GM, 10 μg), kanamycin (K, 30 μg), imipenem (IPM, 10 μg), meropenem (MEM, 10 μg), cefazolin (CZ, 30 μg), ceftizoxime (ZOX, 30 μg), cefuroxime (CXM, 30 μg), cefepime (FEP, 30 μg), chloramphenicol (C, 30 μg), nitrofurantoin (F/M, 300 μg), nalidixic acid (NA, 30 μg), tetracycline (TE, 30 μg) and trimethoprim-sulphamethoxazole (SXT, 1.25 and 23.75 μg). The isolates were determined to be sensitive according to the information supplied by the manufacturer (BBL, Md, USA).

Reference strains of *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, as recommended by NCCLS (2002), were used as control organisms for verification of the antibacterial effect of the discs. All discs were purchased from Becton–Dickinson (BBL, Md, USA).

For all isolates, we calculated the MAR index values (a/b, where 'a' represents the number of antibiotics the isolate was resistant to, and 'b' represents the total number of antibiotics the isolate was tested against). A MAR index value >0.2 is observed when isolates are exposed to high risk sources of human or animal contamination, where antibiotics use is common; in contrast a MAR index value < or = 0.2 observed when antibiotics are seldom or never used (Krumperman 1983).

The MIC of five different heavy metals (Cd^{+2}, Cr^{+3}, Cu^{+2}, Pb^{+2} and Mn^{+2}) was determined for each isolate using Mueller–Hinton agar (Difco) containing each metal in concentrations ranging from 12.5 µg/mL to >3,200. Five different heavy metals were used in the compounds $CdCl_2.2H_2O$, $CuSO_4.5H_2O$, $CrCl_3$, $Pb(NO_3)_2$, and $MnCl_2.2H_2O$ (Merck). The isolates were considered resistant if the MIC values exceeded that of the control organism. An *Escherichia coli* K-12 strain was used as the control organism as described by Akinbowale et al. (2007) and Ansari and Malik (2007).

Results and Discussion

In this study, a total of 255 isolates were obtained representing 19 Gram-negative bacterial genus and their 35 species (Table 1). A high percentage of the bacteria isolated belonged to the genus *Citrobacter* (16.47 %), which was especially prevalent in the Mersin region. In the environment, *Citrobacter* are commonly found in water and soil. They also colonize the gastrointestinal tract and therefore can cause urinary tract, respiratory, intra-abdominal, wound and central nervous system (CNS) infections (Samonis et al. 2009).

In this study, a second prevalent genus, *Vibrio* (8.6 %), was isolated intensively from Iskenderun. Chelossi et al. (2003) showed in their study performed in the Western Mediterranean that oxidase-positive fermenting strains were prevalent and belonged to the group of *Vibrionaceae*. The genus *Vibrio* constituted some of the most virulent pathogens to man, including *V. parahaemolyticus*, which is responsible for many fatal epidemics of food poisoning whose virulence mechanisms were studied in detail by various authors (Nishibuchi and Kaper 1995). The third prevalent species were *E. coli* (8.2 %) and *P. agglomerans* (8.2 %). *E. coli* and *P. agglomerans* were recovered intensively from Karatas (76.2 % and 57.1 % respectively). *E. coli* is an indicator of fecal contamination in aquatic environments. *P. agglomerans* is the most frequent species associated with human infections among *Pantoea* spp. (Gavini et al. 1989).

As shown in Fig. 2, among the all isolates, a high percentage of bacteria were resistant to ampicillin (87.1 %), streptomycin (82.4 %) and cefazolin (56.9 %). The trend of resistance among all isolates for sixteen antibiotics was found to be the following: AM > S > CZ > FM > CXM > K > SXT > GM > AN > NA > C = TE > IPM > CZ > MEM > ZOX. Among isolates from Karatas, resistances to streptomycin and cefazolin were higher than among the isolates from Mersin and Iskenderun, but ampicillin-resistance among bacteria from Iskenderun was higher than those from Mersin and Karatas. The high degree of resistance to ampicillin in the present study was similar to the findings of Pontes et al. (2009). Pontes et al. found a total of 232 ampicillin-resistant Gram-negative isolates among

Table 1 Distribution of bacteria isolated from three different regions

Species	Region Mersin	Karatas	Iskenderun	n
Acinetobacter baumannii	1			1
Acinetobacter lwoffi	2			2
Aeromonas caviae	2			2
Aeromonas hydrophila	3	8	1	12
Burkholderia cepacia		5	11	16
Chryseobacterium indologenes		2		2
Citrobacter freundii	12	5	2	19
Citrobacter koseri	23			23
Enterobacter aerogenes	1			1
Enterobacter cloacae	10			10
Enterobacter gergoviae	1			1
Escherichia coli	4	16	1	21
Flavimonas oryzihabitans			2	2
Klebsiella oxytoca	5	3	1	9
Klebsiella pneumoniae ssp pneumoniae	6			6
Klebsiella pneumoniae ssp ozaenae		2	3	5
Klebsiella pneumoniae ssp rhinoscleromat	3	1	11	15
Pantoea agglomerans	7	12	2	21
Pasteurella multocida	1			1
Proteus penneri		1		1
Pseudomonas aeruginosa			14	14
Salmonella choleraesuis ssp choleraesuis		1		1
Salmonella paratyphi A		4	3	7
Salmonella spp		1		1
Salmonella typhi		1		1
Serratia ficaria	1		1	2
Serratia fonticola		3		3
Serratia marcescens		6		6
Shigella species	1	6	7	14
Sphingomonas paucimobilis			1	1
Vibrio alginolyticus			1	1
Vibrio metschnikovii	1		1	2
Vibrio parahaemolyticus	1	2	16	19
Yersinia enterocolitica group	1	1	10	12
Yersinia pestis	1			1
Total	87	80	88	255

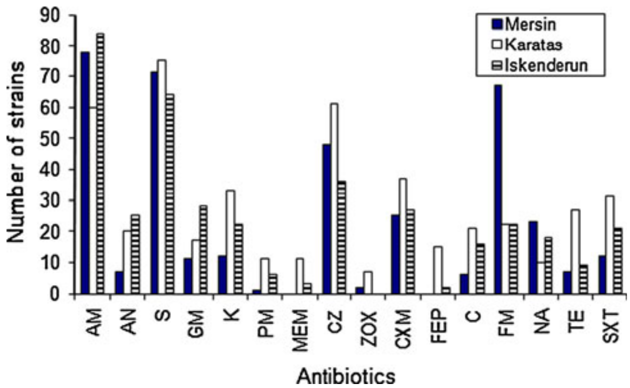

Fig. 2 Antibacterial resistance of bacteria isolated from three regions along the Mediterranean Sea

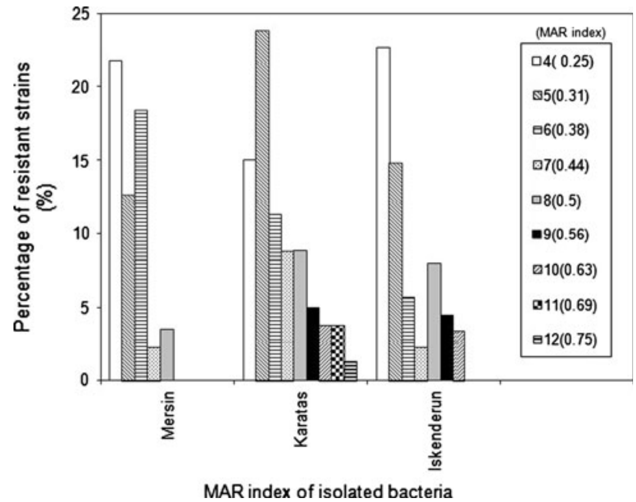

Fig. 3 MAR index of isolated bacteria

272 isolates (85.3 %) in their study. While among the Karatas isolates, 18.8 % and 13.8 % of bacteria were resistant to cefepime and meropenem, respectively, no bacteria from Mersin were resistant to these antibiotics. At the same time, 8.8 % of bacteria from Karatas were resistant to ceftizoxime, but no bacteria from Iskenderun were resistant to ceftizoxime. These results show resistance to carbapenems, third- and fourth-generation cephalosporins, was relatively infrequent among the isolates. Matyar et al. (2008) found in their study, performed at Iskenderun Bay, that a high percentage of bacteria were resistant to streptomycin (100 %), cefazolin (89.8 %), ampicillin (83.7 %) and trimethoprim-sulfamethoxazole (69.4 %), whereas a low percentage of bacteria were resistant to cefepime (12.3 %) and meropenem (14.3 %).

Bacteria isolates resistant to four or more antibiotics were designated as multiple- antimicrobial resistant. A high frequency of multi-resistant bacteria was found for most of the isolates from all the three regions: Karatas (81.3 %), Iskenderun (61.4 %), and Mersin (58.6 %). The MAR index values ranged from 0.25 to 0.75 for Karatas isolates, from 0.25 to 0.63 for Iskenderun isolates and 0.25–0.5 for Mersin isolates (Fig. 3). Pontes et al. (2009) found in their study, performed in a tropical region, a high frequency of multiresistant bacteria. Pathak and Gopal (2007) found *E. coli* isolates from glacial water resistant for seven out of ten antibiotics. Chitanand et al. (2010) reported that the MAR index ranged from 0.15 to 0.48 in their study performed in aquatic environment in India. In general, the marine environment may be a powerful incubator for new combinations of virulence properties due to the extremely large overall population of bacteria and efficient mixing time scales. These natural phenomena may be further enhanced by human activity, such as increased sewage input and ballast water transport (Ruiz et al. 2000).

In the present study, resistance to five heavy metals (Cd^{+2}, Cr^{+3}, Cu^{+2}, Pb^{+2} and Mn^{+2}) was investigated for all isolates. As shown in Table 2, trends in heavy metal resistance vary depending on the sample sites: Mersin, $Cd > Cu > Cr = Pb > Mn$; Karatas, $Cd > Cu > Cr = Mn > Pb$; and Iskenderun, $Cu > Cd > Mn > Cr > Pb$. Altug and Balkis (2009) found that the resistance to 7 heavy metals was in the order of $Cu > Mn > Ni > Zn > Pb > Cd > Fe$ for sea water isolates. Resistance to five heavy metals was as follows for Mersin, Karatas and Iskenderun isolates, respectively: to cadmium, 82.3 %, 58.8 % and 95.5 %; to chromium, 2.3 %, 2.5 %, and 4.5 %; to copper, 67.8 %, 41.3 %, and 97.7 %; lead, 2.3 %, 1.3 % and 2.3 %; and to manganese, 1.1 %, 2.5 % and 6.8 %. The minimal inhibitory concentrations (MIC) of the isolates ranged from 12.5 µg/mL to 3,200. The Iskenderun isolates showed higher resistance cadmium, copper, chromium and manganese than did the Mersin and Karatas isolates. Resistance to lead was similar between Iskenderun and Mersin isolates. Tolerance to the highest MIC (3,200µg/mL) of copper was found only among the Iskenderun isolates. It is known that there are significant levels of accumulated heavy metals in crustacean and fish tissues from Iskenderun Bay (Çoğun et al. 2005; Türkmen et al. 2006) and in mussel tissues from Mersin (Karayakar et al. 2007). Iskenderun Bay has a coal-fired power plant, iron and steel factories and an oil refinery. The highest heavy metal resistance detected in the Iskenderun isolates could be a result of contamination from these sources.

Metal-resistant isolates from Mersin also showed a high resistance to three antibiotics: ampicillin, streptomycin and nitrofurantoin. Similarly the Karatas isolates that were metal resistant also showed a high resistance to three antibiotics: ampicillin, streptomycin and cefazolin. In contrast, heavy-metal resistant Iskenderun isolates showed resistance to only two antibiotics: ampicillin and streptomycin. The association between antibiotic resistance and resistance to heavy metals is very common in the same

Table 2 Heavy metal tolerance in bacteria from three different regions

Metal/environment	Metal concentrations (μg/mL) with number of tolerant isolates											Resistant	
	n	12.5	25	50	100	200	400	800	1,600	3,200	>3,200	n	(%)
Cadmium					[a]								
Mersin	87		2	3	10	33	26	11	2			72	82.3
Karatas	80		3	10	20	20	15	10	2			47	58.8
Iskenderun	88				4	23	29	29	3			83	95.5
Copper						[a]							
Mersin	87		2		5	21	28	19	12			59	67.8
Karatas	80			5	19	23	20	10	3			33	41.3
Iskenderun	88				2		11	49	25	1		86	97.7
Chromium								[a]					
Mersin	87			25	31	25	1	3	2			2	2.3
Karatas	80			11	24	25	13	5	2			2	2.5
Iskenderun	88		3	5	27	30	15	4	4			4	4.5
Lead									[a]				
Mersin	87	1	3	17	28	26	7	2	1	2		2	2.3
Karatas	80			19	19	21	15	4	1	1		1	1.3
Iskenderun	88	3	1	1	4	2	30	40	5	2		2	2.3
Manganese									[a]				
Mersin	87				11	35	17	21	2	1		1	1.1
Karatas	80				20	21	15	17	6	2		2	2.5
Iskenderun	88	1	1	4	16	15	18	16	11	6		6	6.8

[a] Minimal inhibition concentration of standard strain *Escherichia coli* K12

organism (also in the same plasmid, transposon, or integron), demonstrating that industrial pollution most likely selects for antibiotic resistance and vice versa (Baker-Austin et al. 2006).

In this study, antibiotic and heavy metal resistance patterns of Gram-negative bacteria in the Eastern Mediterranean Sea were investigated. Our results suggest that the Karatas region in particular is a reservoir for antibiotic-resistant bacteria and that the Iskenderun region is a reservoir for heavy-metal-resistant bacteria. The increasing numbers of antibiotic- and heavy-metal-resistant bacteria could be a result of gene transfer activities. Bacteria from different sources, such as humans, animals and soil, are able to transfer or exchange their resistance genes. At the same time, water contaminated with antibiotics, disinfectants, pesticides, and heavy metals might encourage selective activities and result in antibiotic and heavy metal resistance.

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