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Antibiotic Levels and heavy metal resistance in gram-negative bacteria isolated from seawater, Iskenderun Organized Industrial Zone

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Abstract

Gram-negative bacteria were isolated from the territorial seawater of Iskenderun Organized Industrial Zone in Iskenderun Bay, to measure their resistance levels against to antibiotic and heavy metal. Sixteen species of bacteria were identified by using VITEK II System. Total of 18 antibiotics disc and four heavy metals were applied for the resistance test.

Antibiotic resistances of all isolates were at high percentages for eritromisine (94.4%), ampicilline (72.7%), streptomycin (68.3%), cefazolin (64.6%) and carboksipeneme (57.1%). The highest resistant strain was resistant against to 17 antibiotics, while the weakest resistant of two isolates were sensitive to all antibiotics. MAR index values were found to be higher than 0.2 for 94.9% of all isolates.

Heavy metal resistances of all isolates were found to be in high percentages as 100% for cadmium, %100 for copper, 90.7% for manganese, and 67.7 % for lead.

High resistances of examined bacteria against to both antibiotics and heavy metals indicated a dense and multisource pollution in the bay. The pollution in territorial waters of the bay may threat the aquatic life consequently, the public health.

Keywords: Antibiotic, Bacteria, Heavy Metal, Iskenderun Bay, Public Health

INTRODUCTION

Turkey is covered by seas on three sides with 8333 km long coastal line with a total area of 46, 583 hectares of sandy beaches and tourism and fishing are considerable income sources for the country. Iskenderun Bay, with 65 km long and 35 km wide and total surface area is 2 275 km^2, covers approximately 4 % of Mediterranean Sea by the 9 5 km^3 water volume.

The bay has a dynamic and efficient structure by the large segment of the bottom currents and wind movements with a reverse flow and pollutants from the ocean. The accumulation of pollutants by both domestic and international sources shows increased and threatened concentrations over time without recycling feature which causing the ecological deterioration. The main domestic pollutants were coming from industrial facilities (primarily to the iron and steel plants), residential units, ship traffic, fertilizer, coal and oil transport wastes. As a result of these events, eutrophication is formed, which can have a negative impact on the marine and coastal environment. The negative effects of eutrophication on marine eco systems includes: algal blooms, increased sedimentation and oxygen consumption, oxygen depletion in the seawater.

There are many studies which show the accumulation of heavy metal pollution in marine organisms [3,4,5,6,7,8]. According to Tepe [7], heavy metals accumulated in substantially high levels can be very toxic for fish and aquatic environment.

Over the last few decades the aquatic environment has been contaminated by persistent pollutants of domestic origin. Antibiotic contamination has been identified as a concern in coastline, due to discharges from urban sewage, agricultural and intensive tourism activities. Several recent studies demonstrate that use of antibiotics in all parts of the sea and seafood production chains contribute to the increasing level of antibiotic resistance among the sea-borne pathogenic bacteria. A lot of microbial ecology studies have reported that heavy metal and antibiotic resistance are becoming a global importance [8,9,10]. In addition, it is well known that plasmids isolated from marine bacteria carry resistance to heavy metals and antibiotics [11]. The presence of plasmids in marine sediment and water-column bacteria is well documented [12,13,14]. Moreover, heavy metal resistance genes are often found on plasmids and transposons [15,16].

The purpose of the present study to examine the levels of antibiotic and heavy metal resistance in gram-negative bacteria, isolated from seawater, along the coastline of Iskenderun Organized Industrial Zone, Iskenderun Bay

(Turkey), and based on the results to determine the pollution level of the bay which have effects on both aquatic organisms and public health.

MATERIALS AND METHODS

Sampling

The study was carried out the northeast coast of the Iskenderun Bay. Seawater samples were collected from both the coastline and the open seawater of industrial zone under sterile conditions. Seawater samples were collected 0–20 cm below from the surface, using 250 ml sterile bacteriological sample bottles and brought to the laboratory in an ice chest. All of these sampling were performed within 4 hours [17].

Bacterial Isolation and Antibiotic Resistance Test

Gram-negative bacteria from the seawater were isolated using the spread plate technique. To isolation gram-negative bacteria were made using MacConkey Agar (Merck), inoculated with appropriate dilutions from the sample homogenates, and incubated for 24–72 h at 35 ° C then maintained in nutrient agar (Oxoid).

Antibiotic resistance of bacterial strains was determined by the agar diffusion test [18] using Mueller–Hinton agar (Difco) and 18 antibiotic discs representing 11 classes of antibiotics: Amikacin (AN, 30 µg), ampicillin (AM, 10 µg), nalidixic acid (NA, 30 µg), chloramphenicol (C, 30 µg), tetracycline (TE, 30 µg), nitrofurantoin (F/M, 300 µg), streptomycin (S, 10 µg), gentamicin (GM, 10 µg), imipenem (IPM, 10 µg), cefazolin (CZ, 30 µg), meropenem (MEM, 10 µg), cefuroxime (CXM, 30 µg), cefepime (FEP, 30 µg), trimethoprim-sulphamethoxazole (SXT,1.25 and 23.75 µg), ciprofloxacin (CIP, 5 µg), carboxypenicillin (CB, 100 µg), cefotaxime (CTX, 30 µg), erythromycin (E, 15 µg).

The entire surface of the Mueller–Hinton agar plate (diameter, 90 mm) (Difco) was covered with the required inoculums, and the plate was air dried for 15 min before the disks were laid on the surface and incubation was performed for 18 h at the required temperature. The verification of the antibacterial effect as the reference strain *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used [18].

We calculated the MAR index values for all isolates (*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 of equal or less than 0.2 was defined as those antibiotics were seldom or never used for the animal in terms of treatment whereas the MAR index value higher than 0.2 is considered that animal have received high-risk exposure to those antibiotics [19].

Determination of the MIC of heavy metals and bacterial identification

The Minimal Inhibitory Concentration (MIC) for each bacterial isolate for 4 heavy metals was determined using Mueller–Hinton agar (Difco) containing Cd^{+2}, Cu^{+2}, Pb^{+2} and Mn^{+2} at concentrations ranging from 25 µg/ml to 3200 µg/ml. Medium was sterile filtered into aqueous solutions of metal (Whatmann, 0.2 µm in diameter) passing through the sterilization process is added. Molecular formula of metals used are as follows; $CdCl_2.2H_2O$, $CuSO_4.5H_2O$, $Pb(NO_3)_2$ and $MnCl_2.2H_2O$ (Merck). The isolates were measured resistant if the MIC values exceeded that of the *E. coli* K-12 strain which was used as the control [20]. Furthermore, all isolates were screened by colonial morphology, Gram stain, Oxidation/Fermentation of glucose and motility. The strains were further identified with VITEK 2 system (BioMeriéux, France). The VITEK 2 system was used according to the manufacturer's instructions; ID-Gram Negative cards (ID-GN cards; BioMeriéux, France) were used for identification.

RESULTS

The resistance frequency to various antimicrobials and results of identification for bacteria are presented in Table 1. The strains (161 gr negative bacteria) and their amounts were *Acinetobacter baumannii complex* (4), *Actinobacillus ureae* (10), *Aeromonas salmonicida* (5), *Bordetella trematum* (2), *Budvicia aquatica* (13), *Burkholderia mallei* (9), *Escherichia coli* (46), *Francisella tularensis* (9), *Moraxella osloensis* (2), *Pasteurella canis* (2), *Pasteurella pneumotropica* (3), *Pseudomonas fluorescens* (15), *Pseudomonas luteola* (26), *Shigella group* (2), *Sphingobacterium thalpophilum* (7) and *Sphingomonas paucimobilis* (6), respectively.

Among the seawater isolates, a high percentage of bacteria were resistant to E 94.4%, AM 72.7%, S 68.3%, CZ 64.6% and CB 57.1%, whereas a low percentage of bacteria were resistant to TE 49.7%, SXT 42.2%, GM 34.8%, CXM 34.8%, NA 32%, FM 28%, CTX 27.3%, FEP 19.3%, CIP 15.5%, IPM 14.9%, MEM 9.9%, C 8.7% and AN 8.1% [Figure 1]. Only one strain isolated from seawater, was resistant to 17 antibiotics (resistant to all antibiotics except chloramphenicol). The strain was identified as *Actinobacillus ureae* with VITEK II System. Two isolates were sensitive to all antibiotic disks. These isolates were identified as *Acinetobacter baumannii complex* and *Pseudomonas luteola*.

Levels of multiple antibiotic resistant isolates were examined in terms of expressions for the MAR, 75.4% of all isolates MAR value of > 0.2. Over 98% of the isolates were resistant: 6.8% to one antibiotic, 0.9% to two antibiotics, 8.7% to three, 11% to four, 7.5% to five antibiotics, 4.3% to six, 3.1% to seven, 8.7% to eight, 12% to nine, 4.3% to ten, 3% to eleven, 6.2% to twelve, 11% to thirteen, 1.2% fourteen and 0.6% to seventeen.

A heavy metal resistance of Cd (100%) = Cu (100%) > Mn (90.7%) > Pb (67.7%) was observed in all isolates [Table 2] and the heavy metal resistance profiles of isolates to species level are demonstrated in Table 3.

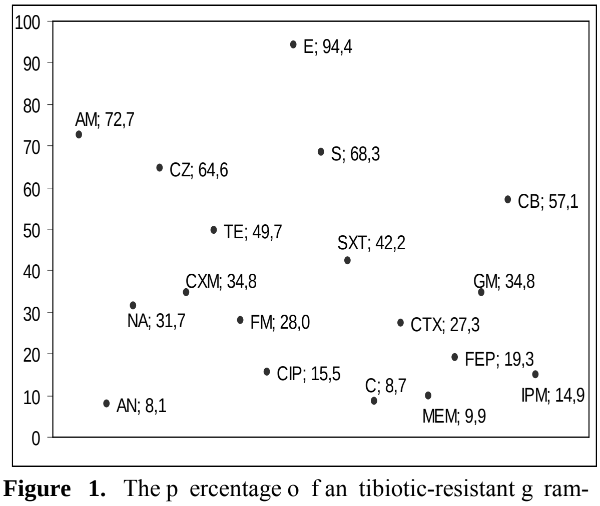

Figure 1. The percentage of antibiotic-resistant gram-negative bacteria

Table 1. Results of identification and antibiotic resistance profiles

Species (n)	Percentage of antibiotic resistant bacteria																	
	AM	**AN**	**NA**	**CZ**	**CXM**	**TE**	**FM**	**CIP**	**E**	**S**	**SXT**	**C**	**CTX**	**MEM**	**FEP**	**GM**	**CB**	**IPM**
Acinetobacter baumannii complex (4)	25		25	50	25	25	75		25			25						
Actinobacillus ureae (10)	100	10	10	80	50	100	70	10	100	100	100	20	50	60	40	100	100	60
Aeromonas salmonicida (5)	100		20	40	40	80	80		100	100	100	20	40	20	20	80	100	60
Bordetella trematum (2)	50					50	50		100	50								
Budvicia aquatica (13)	77		15	69	23	76,9	62		85	77	62		23	23	7,69	46,2	46,2	23,1
Burkholderia mallei (9)	100			78	44	100	78		100	100	100		33	22	22,2	88,9	100	88,9
Escherichia coli (46)	74	13	59	76	52	58,7	4,3	45,7	100	76	57	8,7	54	2,2	43,5	45,7	67,4	4,35
Francisella tularensis (9)	78	11	11	33	22	22,2		11,1	100	33		11	22				55,6	
Moraxella osloensis (2)	50		50	50					100	50								
Pasteurella canis (2)				50					100	100								
Pasteurella pneumotropica (3)	100			33	67	66,7	67		100	100	100		67	67		66,7	66,7	33,3
Pseudomonas fluorescens (15)	53	13	47	80	20	26,7	13	13,3	100	80	20	6,7	6,7	6,7	13,3	6,67	20	6,67
Pseudomonas luteola (26)	85	12	19	46	27	19,2	31		88	42	3,8	7,7				3,85	57,7	
Shigella group (2)	100		50	50		50	50		100	100	50	50	50		50	50	100	
Sphingobacterium thalpophilum (7)	57		43	71	29	42,9			86	57	29	14				28,6	42,9	
Sphingomonas paucimobilis (6)			14	71	14	14,3			86	29							14,3	

Table 2. The percentage of isolates resistant to heavy metals

Heavy Metal	Levels of resistance	Concentration (μg/ml)						Total Resistance
		3200	1600	800	400	200	100	
Cd	n	49	39	65	1	7		161
	m	30,4	24,2	40,4	0,6	4,3	*	100
Mn	n	90	56	6	9			146
	m	55,9	34,8 *	3,7	5,6			90,7
Cu	n	32	30	88	11			161
	m	19,9	18,6	54,7	6,8	*		100
Pb	n	96	13	42	10			109
	m	59,6	8,1 *	26,1	6,2			67,7

n number of resistance isolates m percentage of resistance isolates * MIC of standard strain *E.coli* K12

Table 3. Results of heavy metal resistance profiles with species level

Species (n)	Pb	Cu	Mn	Cd
Acinetobacter baumannii complex (4)	0	100	100	100
Actinobacillus ureae (10)	100	100	100	100
Aeromonas salmonicida (5)	100	100	100	100
Bordetella trematum (2)	100	100	100	100
Budvicia aquatica (13)	53,8	100	69,2	100
Burkholderia mallei (9)	100	100	100	100
Escherichia coli (46)	67,3	100	84,7	100
Francisella tularensis (9)	33,3	100	100	100
Moraxella osloensis (2)	0	100	100	100
Pasteurella canis (2)	50	100	100	100
Pasteurella pneumotropica (3)	100	100	100	100
Pseudomonas fluorescens (15)	60	100	100	100
Pseudomonas luteola (26)	73	100	100	100
Shigella group (2)	100	100	100	100
Sphingobacterium thalpophilum (7)	28,5	100	42,8	100
Sphingomonas paucimobilis (6)	66,6	100	100	100

DISCUSSIONS

A total of 161 Gr (-) bacteria were isolated, identified with VITEK II compact system and examined for antibiotic, heavy metal resistance. The extremely high level of antibiotic resistance observed in these bacteria at Table 1.

A high incidence of antibiotic and heavy metal resistance in *Aeromonas spp.* and *Pseudomonas spp.* were reported by Matyar et al. [21] from Iskenderun Bay. According to Matyar et al., all isolates showed high resistance to S 35.7%, GM 35.7%, GM 35.7%, IPM 14.2%, FEP 14.2%, FM 50%, TE 21.4% and SXT 50%, respectively. These rates in present study 100%, 80%, 60%, 20%, 80%, 80% and 100%, respectively. By comparison, our results indicate higher rates of all antibiotics. In addition, other studies Matyar et al. [22] indicated that bacteria isolated from gill, some fish species of Iskenderun Bay, showed high resistance to AM (66.7%) and CZ (47.3%) whereas, no isolates showed resistance to IPM and CXM.

A previous study [10] reported that levels of antibiotic resistance among the 30 isolated from Baltic Sea, AM 12%, C 8.3%, CIP 4.2%, E 6.3% and CXM 8.3% respectively. Another study examined that level of antibiotic resistance from fresh water resources in Malaysia respectively, TE 78.4%, E 53.8%, NA 57% and PM 65.4% [9]. The data on these two studies compare with our study E, AM, CIP and CXM resistance of all isolates at least three times higher than.

The results of antibiotic resistance in this study indicate that the industrial zone is also polluted directly or indirectly by domestic and hospital waste water, industrial waste. Moreover, unnatural increases in antibiotic resistant bacteria in seawater indicate that unconscious use of different groups of antibiotics. Taken the results of present study demonstrated that, local people encouraged to conscious consumption of antibiotics. Otherwise, this situation may have negative consequences for people health.

One hundred sixty one bacterial strains isolated from organized industrial zone near the Iskenderun Bay were also tested for their resistance to four different heavy metals; the resistance value for all bacteria presented in Table 3. According to Matyar et al. [21], who reported a high incidence of heavy metal resistance in *Aeromonas spp*., heavy metal resistant rate of Cd 14.3%, Cu 100%, Mn 7.1% and Pb 71.4%, respectively. In addition, other studies of Matyar et al. [22] demonstrated that high resistance to cadmium 60.2%, copper 50.5%, manganese 8.6% and lead 6.5% from intestinal bacteria. These rates in present study Cd (100%) = Cu (100%) > Mn (90.7%) > Pb (67.7%), respectively. At the same study reported that heavy metal resistance rate of cadmium 52%, copper 45.3%, lead 3% and 5.3% from all isolates. Sevgi et al. [23] used ICP-AES to detect heavy metal contents of industrial soils in Mersin (Turkey). It was reported that very high levels of Cr and Ni. Furthermore; this study demonstrated that levels of copper resistance among *Pseudomonas spp*. 26 % was found. Yilmaz [24] studies same area of Iskenderun Bay, reported that high heavy metals concentrations in the tissues of fish samples. A lot of studies are largely depending on accumulating high levels of heavy metals in different tissues of the fish and marine invertebrates [6,7]. Moreover, it is well known that heavy metals accumulated in considerably high levels can be very toxic for fish and fish product. Furthermore, most of the marine products may accumulate heavy metals and later times pass them to human beings by consumption.

The bacterial isolates of this study were resistant to most of heavy metals and antibiotics. In addition, the most important findings of present study are increasing in antibiotics and heavy metals resistance from marine gram-negative bacteria in the gulf of Iskenderun. This may be due to the seawater sources of the Iskenderun Bay that were highly contaminated with antibiotic and heavy metal residues. Moreover, this negative situation is a significant problem for local people. Because some microorganisms are found in marine products, which have a very critical role in food chain, and this may reveal general public health problems. Our result showed that humans' activities might contribute to the level of heavy metal and antibiotic resistant bacteria living such zones. In future, this area may receive large amounts of heavy metals and antimicrobial agents due to industrial activities, domestic and hospital wastewater. Both local people and fishermen should be informed about these adverse conditions.

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