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Heavy Metal Resistances of *Enterobacteriaceae* from Aksu River (Turkey) Polluted with Different Sources

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In this study, the heavy metal contamination of Aksu river, which have urban, industrial and agricultural effluents and also resistance frequency against heavy metals, belonging to *Enterobacteriaceae* (representative of the human and animal commensal flora) (66 isolates) and *Pseudomonas* sp., (only one isolate) were investigated. The samples were taken from five different sample stations settled on Aksu river and its brooks. The level of heavy metals, namely nickel, cadmium, copper and chromate, in water samples were measured by using a Perkin-Elmer 3110 atomic absorption spectrophoto-meter. Resistance frequency of the isolates was analyzed by agar dilution method. The highest resistances against all concentrations of all metals were found out *Klebsiella* sp. strains. Highest ratio of heavy metal resistance in the isolates was determined in 1 mM nickel (97 %), copper (88 %), cadmium (61 %) and chromate (25 %) concentration, respectively. No resistant bacteria was observed at 7 mM Cu and Cd and 5 mM Ni and Cr concentrations. These results suggest that releasing of urban and industrial wastewater into running surface waters without treatment processess increases the bacterial resistance against heavy metals. Therefore, the infectious diseases and heavy metal resistance are spreaded on large areas.

Key Words: Aksu river, *Enterobacteriaceae*, Heavy metal resistance, Agar dilution method, Pollution.

INTRODUCTION

The production of heavy metals has increased rapidly due to industrial developments[1]. Therefore, the contamination of soils, sediments and waterways with metals are remarkably issues[2-4]. Nowhere has such contamination been more problematic than at the primary sources for industrially major metals. Metal mining has been conducted for over two millennia with particularly tragic results as human populations and the demands for metals increase[5-8].

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Toxic metal wastes from defense-related activities, industry and municipal sources have routinely came in the environment through disposal in landfill sites or by accidental release such as Chernobyl. These practices have resulted in surface contamination problems, transport to groundwater and/or bioaccumulation of radionuclides and toxic metals[9-12]. Metals such as Cs, Sr, Cd and to a lesser extent, Co are prevalent in soils near industrial centers[11,13] at concentrations up to 50 mg of Cs/g, 350 mg of Cd/g and 500 mg of Sr/g[12]. Cocontaminants, toxic metals are often inhibitory to other bioremediative processes, *e.g.*, hydrocarbon degradation[14].

Increased numbers of metal-tolerant bacteria[15-19], *Actinomycetes*[20] and fungi[20-22] have been seen as a result of heavy metal pollution by the traditional plate count technique. The mechanism of resistance to heavy metals is commonly based on the novel membrane transport systems that expel the toxic ions (including cobalt, nickel, zinc and probably copper and chromium) from the bacterial cytoplasm[23]. Once heavy metals go into the environment, biological systems including microorganisms can accumulate and introduce the metals into food webs[24]. Toxic metals are mobilized from industrial activities and fossil fuel consumption and eventually are accumulated through the food chain leading to serious ecological and health problems[25]. Some heavy metals (*i.e.* Ni) are toxic even at low concentrations[26,27] and they are an important source of contamination in industrial societies[25].

Kahramanmaras, is a developing city, located in the southern part of Turkey, with the 315,000 inhabitants. The economy of the Kahramanmaras mainly depends on the textile, yarn industry and agriculture. The Aksu river (28.3 m^3/s), located in southern Turkey, receives the wastewater discharge from the sewage of the city of Kahramanmaras. The sewage system of the city is not filtered, as well as it is not chlorinated before it reaches into the Aksu river[28].

In the present study, four different heavy metal (nickel, cadmium, copper and chromate) pollution in Aksu river in Kahramanmaras in Turkey using plate count technique were analyzed.

EXPERIMENTAL

Study area and sampling stations: Five different sampling stations were chosen along the pollution gradient in the Aksu river[29].

1st Station: The Aksu river on side of city's garbage dump. **2nd Station:** On the Erkenez brook carries domestic, industrial waste and especially waste from slaughter that dispersed into the Aksu river. **3rd Station:** On the Oklu brook, transport sewage of city into the Aksu river. **4th Station:** On the Karasu brook carries especially industrial waste, but also agricultural and domestic waste that runs into the Aksu river. **5th Station:** On the Aksu river flows into the intensive agricultural activities area.

Water sample collection: Water samples (100 mL) were collected at all stations in January, February, April, May, June, July, August, October, November 2001, using sterile screw-capped glass bottles and stored in cold bags at 4 °C until analysis in the laboratory within 2 h after collection.

Isolation of coliform microorganisms: Triplicate plates with EMB were inoculated with appropriated dilutions from the water samples. Representative colonies were purified an EMB-agar. Organisms which exhibited a yellow-green metallic sheen on EMB-agar were incubated at 37 °C for 24 ± 4 h. Preliminary identification of strains obtained in pure culture was based on Gram staining, respiration fermentation tests and biochemical tests (IMVIC tests)[30]. *Pseudomonas* spp. was isolated by incubation at 42, 37 and 20 °C on *Pseudomonas* selective medium (Oxoid CM 457). Isolate was then tested at these temperatures for casein hydrolysis and pigment production on milk-agar with cetrimide[31] and for a positive oxidase reaction. Complete identification of *Enterobacteriaceae* was achieved by use of the tests in Bergey's Manual of Determinative Bacteriology[32].

Determination of heavy metal resistance of isolates: The heavy metal ion resistance of the isolates of water samples was determined by means of the agar dilution method. Heavy metal salt solutions, such as $NiCl_2 \cdot 6H_2O$, $CdCl_2 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$, $K_2Cr_2O_7$, were used in a final concentration of 1 to 7 mM in the supplemented Plate Count Agar plates. Heavy metal resistance was also tested by direct plating of isolates on heavy metal-containing media and also by spot inoculation of up to 50 isolates on triplicate plates of medium containing heavy metal. This compromise was necessary since it was impracticable to determine MIC values for so many isolates and heavy metal[33].

Determination of heavy metal resistance of water samples: Metal concentrations in these water samples were measured using atomic absorption spectrophotometer (Perkin-Elmer 3110), with reference to appropriate standard solutions.

RESULTS AND DISCUSSION

According to the results of heavy metal ion analysis of water samples, different rates of heavy metal pollution (except manganese) in whole sampling stations were found (Table-2). Water quality criters showing contamination rate in river (ppm) is indicated in Table-1[34].

Contamination rate of water was entranced 1st site is by Cu, Fe, Ni, Pb, 2nd site is by Fe, Ni and Pb, 3rd site is by Cu, Zn, Ni and Pb, 4th site is by Ni and Pb and 5th site is by Fe, Ni and Pb heavy metal ions. Water samples taken whole stations were ranked heavy contamination rate by lead. Waters of stations of 1, 4 and 5 were ranked as contamination, station of 2 and 3 were ranked as heavy contamination by nickel. Waters of station of 1, 3 and 5 were ranked as low contamination by copper ions (Table-2).

TABLE-1
WATER QUALITY CRITERIA OF CONTAMINATION RATE IN RIVER (ppm) [Ref. 34]

Contamination rate	Cu	Fe	Zn	Mn	Ni	Pb
Clear	0.02	0.3	0.2	0.1	0.02	0.01
Low contamination	0.05	1.0	0.5	0.5	0.05	0.02
Contamination	0.20	5.0	2.0	3.0	0.20	0.05
Heavy contamination	> 0.20	> 5.0	> 2.0	> 3.0	> 0.20	> 0.05

TABLE-2
HEAVY METAL IONS RESULTS IN AKSU RIVER (ppm)

Site no.	Cu	Fe	Zn	Mn	Ni	Pb
1	0.063	1.658	0.165	0.012	0.096	1.562
2	0.031	2.321	0.179	0.045	0.485	4.688
3	0.063	0.663	0.484	0.019	0.258	2.344
4	0.031	0.331	0.221	0.019	0.126	1.562
5	0.063	1.160	0.262	0.019	0.900	1.562

67 Bacterial isolates were selected and identified from water samples and their heavy metal Maximum Tolerance Concentration (MTC) were determined. All isolates were found to be least tolerant to chromium and nickel with MTC 4 mM (Table-3).

TABLE-3
MAXIMUM TOLERANCE CONCENTRATION IN ISOLATES

Conc.	$NiC1_2 \cdot 6H_2O$			$CdCl_2 \cdot H_2O$			$CuSO_4 \cdot 5H_2O$			$K_2Cr_2O_7$		
	P	G	R %	P	G	R %	P	G	R %	P	G	R %
1 mM	67	65	97.0	67	41	61.0	67	59	88.0	67	17	25.0
2 mM	67	39	58.0	67	23	34.0	67	29	43.0	67	7	10.0
3 mM	67	23	34.0	67	15	22.0	67	16	24.0	67	2	3.0
4 mM	67	1	1.5	67	12	18.0	67	8	12.0	67	1	1.5
5 mM	67	0	0	67	11	16.0	67	5	7.5	67	0	0
6 mM	–	–	–	67	5	7.5	67	2	3.0	–	–	–
7 mM	–	–	–	67	0	0	67	0	0	–	–	–

Conc. = Concentration, P = Planting, G = Growing, R % = Percentage of resistance, n = 3 (Replication number of experiment).

97 % of the strains were shown to possess tolerance 1 mM, 58 %; 2 mM, 34 %; 3 mM and 1.5 % 4 mM nickel (Table-3). Ni was selected as a model metal for screening. Since Ni is a potent carcinogenic metal as Cr and Cd and even toxic at relatively low concentration[35].

61 % of the strains were observed to possess tolerance 1 mM, 34 % 2 mM, 22 % 3 mM, 18 % 4 mM, 16 % 5 mM and 7.5 % 6 mM cadmium (Table-3). Diaz-Ravina *et al.*[36] determined an increase in tolerance to the metals (Cu, Cd, Zn, Ni) added to soil and observed for the bacterial community obtained from unpolluted soil. Lejeune *et al.*[37] showed a strain of Alcaligenes eutrophus resistant to highly Cd^{2+} ions.

88 % of the strains showed tolerance 1 mM, 43 % 2 mM, 24 % 3 mM, 12 % 4 mM, 7.5 % 5 mM and 3 % 6 mM copper (Table-3). Cervantes *et al.*[38] detected that the presence of high concentrations of Cu^{2+} ions in the environment promotes the selection of microorganisms possessing genetic determinants for copper resistance. There are some bacterial species that can tolerate high levels of copper. It also has been reported by Trevors[39] that copper resistance is plasmid-encoded in *Escherichia coli*, *Proteus vulgaris* and a *Pseudomonas syringea* isolates.

25 % of the isolates possessed tolerance 1 mM, 10 % 2 mM, 3 % 3 mM and 1.5 % 4 mM chromium (Table-3). Ohtake *et al.*[40] showed *Pseudomonas* have genes of plasmids that basic of control resistances to (CrO_4^{2-}) ions that decrease of CrO_4^{2-} take by cells. Efstathiou and McKay[41] determined *Streptococcus lactis* have genes of plasmids that control resistances to (CrO_4^{2-}) ions. Unaldi *et al.*[42] detected maximum tolerance concentration in water isolated *Pseudomonas* spp was 1.7 mM to chromium, 21 mM copper, 20 mM nickel and 10 mM cadmium. Due to water taken from garbage dump, levels of the MTC were very high.

It is frequently thought that these resistances arose as a result of human pollution in recent decades. Furthermore, long term exposure to metals imposes a selection pressure that favours the proliferation of microbes, which are tolerant or resistant to this stress. Development of the metal-resistant pollution in a contaminated soil can result from; (i) vertical gene transfer, (ii) horizontal gene transfer, (iii) and selection pressures on spontaneous mutants (due to the presence of metals). Transposable elements carrying mercury resistance genes have been linked to the distribution of this trait in nature[43]. Unaldi *et al.*[42] transferred copper and nickel resistance determinants from *Pseudomonas* sp to *E. coli* AB3505 in 2×10^{-5} frequency.

The isolated 67 strains were identified in our laboratory following Bergey's Manuel of Determinative Bacteriology[32]. 45 Isolates were identified as *Escherichia coli*, 20 isolates were identified as *Klebsiella* spp., one isolate was identified as *Citrobacter* spp. and one isolate was identified as *Pseudomonas* spp (Table-4).

The highest resistance against 1 mM nickel concentration was determined in *E. coli* (98 %) strains except for *Citrobacter* and *Pseudomonas* spp (These genus were representing only one member). Nevertheless, the highest resistance against 2, 3 and 4 mM nickel concentrations was designated in *Klebsiella*

TABLE-4
HEAVY METAL RESISTANCES IN ISOLATES

	Conc.	*E. coli*			*Klebsiella spp*			*Citrobacter spp*			*Pseudomonas spp*		
		P	G	R %	P	G	R %	P	G	R %	P	G	R %
$CdCl_2 \cdot H_2O$	1 mM	45	24	53	20	16	80	1	0	0	1	1	100
	2 mM	45	14	31	20	9	45	–	–	–	1	0	0
	3 mM	45	7	16	20	8	40	–	–	–	–	–	–
	4 mM	45	6	13	20	6	30	–	–	–	–	–	–
	5 mM	45	6	13	20	5	25	–	–	–	–	–	–
	6 mM	45	3	7	20	2	10	–	–	–	–	–	–
	7 mM	45	0	0	20	0	0	–	–	–	–	–	–
$NiC1_2 \cdot 6H_2O$	1 mM	45	44	98	20	19	95	1	1	100	1	1	100
	2 mM	45	23	51	20	15	75	1	0	0	1	1	100
	3 mM	45	11	24	20	11	55	–	–	–	1	1	100
	4 mM	45	0	0	20	1	5	–	–	–	1	0	0
	5 mM	–	–	–	20	0	0	–	–	–	–	–	–
$CuSO_4 \cdot 5H_2O$	1 mM	45	38	84	20	19	95	1	1	100	1	1	100
	2 mM	45	12	27	20	16	80	1	0	0	1	1	100
	3 mM	45	2	4	20	13	65	–	–	–	1	1	100
	4 mM	45	1	2	20	7	35	–	–	–	1	0	0
	5 mM	45	0	0	20	5	25	–	–	–	–	–	–
	6 mM	–	–	–	20	2	10	–	–	–	–	–	–
	7 mM	–	–	–	20	0	0	–	–	–	–	–	–
$K_2Cr_2O_7$	1 mM	45	6	13	20	11	55	1	0	0	1	0	0
	2mM	45	3	7	20	4	20	–	–	–	–	–	–
	3 mM	45	1	2	20	1	5	–	–	–	–	–	–
	4 mM	45	1	2	20	0	0	–	–	–	–	–	–
	5 mM	45	0	0	–	–	–	–	–	–	–	–	–

Conc. = Concentration, P = Planting, G = Growing, R % = Percentage of resistance, n = 3 (Replication number of experiment).

spp., (75, 55 and 5 %, respectively) strains except *Pseudomonas* spp. No resistance was specified against 5 mM nickel concentration (Table-4) in all isolates.

The highest resistances against all concentration of cadmium were determined in *Klebsiella* sp. (80 %) strains except *Pseudomonas* spp. No resistance was specified against 7 mM cadmium concentration (Table-4) in all isolates.

The highest resistances against all concentration of copper were stated in *Klebsiella* sp. strains (95 %) except *Citrobacter* and *Pseudomonas* spp. No resistance was determined against 7 mM copper concentration (Table-4) in all isolates.

The highest resistance against 1.2 and 3 mM concentration of chromium were designated in *Klebsiella* sp. strains (55, 20 and 5 %, respectively).

Nevertheless, the highest resistance to 4 mM chromium concentration was determined in *E. coli* (2 %). No resistance was specified against 7 mM chromium concentration (Table-4) in all isolates.

Different strains of isolated total 45 *E. coli* were resistant to Ni, Cd, Cu and Cr. These results are in agreement with Horitsu *et al.*[44]. Horitsu *et al.*[44] determined resistance to the Cd^{2+} in *E. coli* strains. Cu resistance in *E. coli* strains isolated from different environmental area depends on plasmid[39,45-47].

In present study, we specified that the different strains of isolated total 20 *Klebsiella* spp. were resistant against Ni, Cd, Cu and Cr heavy metal ions. Silver and Misra[48] explained that many species of bacteria have genes that control resistances to Cd^{2+}, CrO_4^{2-}, Cu^{2+}, Ni^{2+} and the others heavy metals[48,49].

The highest copper resistance was determined in *Klebsiella* spp., (6 mM) isolated from first station. We determined that, the first and fifth stations had highest contamination with copper (Table-5). Pennanen *et al.*[50] reported that the highest scores were found in the most polluted plots and the lowest scores were usually found in less polluted plots.

The highest nickel resistance was determined in *Klebsiella* sp., (4 mM) isolated from first station. This result is very interesting since this station was lesser contaminated with nickel among stations (Table-5).

The highest cadmium resistance was stated in *Klebsiella* sp., (6 mM) isolated from first station and in *E. coli* (6 mM) isolated from fourth station. But cadmium contamination of the water was not determined and no data is available about cadmium contamination level of Aksu river (Table-5).

The highest chromium resistance was observed in *E. coli* (4 mM) isolated from first station (Table-5). The findings of the highest resistant organisms is very surprising since the first station is near the city garbage dump.

Klebsiella sp. was determined to be highest resistant microorganism among isolates in all heavy metals and its various concentrations (except for chromium). This situation may be explained with its cell structure. Some *Klebsiella* spp., (*Klebsiella pneumonia*) have some barriers (capsid) and these barriers give resistance to bacteria against antimicrobial compounds. The proposed mechanism for resistance is that the glycocalyx may create a diffusion barrier to the antimicrobial agent[51-56]. Diffusion through a biofilm may be affected by charge (ionic) interactions between the glycocalyx and the antimicrobial agent, by an increase in the distance the agent which must diffuse, molecular sieving (size exclusion) and the viscosity of the glycocalyx. Some researchers suggest that the polyanionic nature of the glycocalyx creates a barrier (charge interactions) to the diffusion of cationic antimicrobial agents[57-59].

TABLE-5
HEAVY METALS RESISTANCE LEVELS IN THE ISOLATES
FROM THE DIFFERENT SAMPLE STATIONS

Bacterial genera		Bc N.	$NiCl_2 \cdot 6H_2O$ (mM)					$CdCl_2 \cdot H_2O$ (mM)						$CuSO_4 \cdot 5H_2O$ (mM)						$K_2Cr_2O_7$ (mM)				
			1	2	3	4	5	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5
1st station	EC	7	7	4	3	0	–	3	2	1	1	1	0	6	2	0	–	–	–	1	1	1	1	0
	KS	4	4	4	4	1	0	3	3	3	2	2	2	4	4	3	3	3	2	3	2	0	–	–
	Total	11	11	8	7	1	0	6	5	4	3	3	2	10	6	3	3	3	2	4	3	1	1	0
2nd station	EC	9	9	5	3	0	–	3	1	0	–	–	–	8	3	0	–	–	–	1	1	0	–	–
	KS	5	5	4	3	0	–	5	2	1	1	1	0	5	4	3	1	1	0	4	1	1	0	–
	Total	14	14	9	6	0	–	8	3	1	1	1	0	13	7	3	1	1	0	5	2	1	0	–
3rd station	EC	11	11	6	1	0	–	5	3	1	1	1	0	11	3	0	–	–	–	1	1	0	–	–
	KS	4	4	3	2	0	–	3	1	1	1	1	0	4	3	3	1	0	–	2	0	–	–	–
	PS	1	1	1	1	0	–	1	0	–	–	–	–	1	1	1	0	–	–	0	–	–	–	–
	Total	16	16	10	4	0	–	9	4	2	2	2	0	16	7	4	1	0	–	3	1	0	–	–
4th station	EC	13	13	7	4	0	–	9	6	4	3	3	2	11	4	2	1	0	–	2	0	–	–	–
	KS	1	1	1	1	0	–	1	0	–	–	–	–	1	1	1	0	–	–	0	–	–	–	–
	CS	1	1	0	–	–	–	0	–	–	–	–	–	1	0	–	–	–	–	0	–	–	–	–
	Total	15	15	8	5	0	–	10	6	4	3	3	2	13	5	3	1	0	–	2	0	–	–	–
5th station	EC	5	4	1	0		–	4	2	1	1	1	1	2	0	–	–	–	–	1	0	–	–	–
	KS	6	5	3	1	0	–	4	3	3	2	1	0	5	4	3	2	1	0	2	1	0	–	–
	Total	11	9	4	1	0	–	8	5	4	3	2	1	7	4	3	2	1	0	3	1	0	–	–

Bc. N. = Number of bacteria, n = 3 (Replication number of experiment)
EC = *E. coli*, KS = *Klebsiella spp.*, PS = *Pseudomonas spp.*, CS = *Citrobacter spp.*

Enzyme-mediated resistance mechanisms include heavy metal resistance and formaldehyde resistance. Resistance to heavy metals includes resistance to the following: mercury, antimony, nickel, cadmium, arsenate, cobalt, zinc, lead, telluride, copper, chromate and silver. Detoxification is usually made by enzymatic reduction of the cation to the metal, whereas some heavy metal resistance genes are carried on plasmids, whilst others are chromosomal. The resistant phenotype is usually inducible by the presence of the heavy metal. Some heavy metals induce resistance to a broader spectrum of heavy metals. Arsenate, arsenite and antimony, for example, induce resistance to each other in *E. coli*[60].

The results of this study show that Aksu river has a low level heavy metal contamination and a low level heavy metal resistance in isolated bacteria.

Contamination of groundwater with toxic and carcinogenic compounds is a serious concern for public health and environmental quality. This problem is generally showed as a pollutant migrating in the direction of groundwater flow from a point resource, which harms in our environment substantially affecting all strive health and welfare.

The results of this study show that Agar dilution method has proved useful in qualitative studies of morphogenetic effects of heavy metals, on bacteria that isolated from different polluted water samples. The metal resistance was increased because of the effluence of waste water without any purification treatment.

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