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Antibiotic and Heavy Metal Resistance of *Escherichia coli* Strains Isolated from the Seve Dam, and Konak Pond, Kilis, Turkey

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Research Article

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Abstract

Pollution of surface waters with coliform bacteria having antibiotic and heavy metal resistance has become an increasing public health risk. For this reason, antibiotic and heavy metal resistance profiles of fecal microorganisms in surface water samples collected from two different points (Seve Dam and Konak Pond, Kilis, Turkey) were analyzed in this study. The various physicochemical parameters such as pH, Temperature, Total Dissolved Solids (TDS), Electrical Conductivity (EC) of samples were also examined. The total coliform load in each sample was >1100 (MPN)/100 mL. As fecal indicator microorganisms, 21 *Escherichia coli* strains were isolated from sampling points. Despite the high coliform contamination, the multiple antibiotic resistance (MAR) index of *E. coli* was recorded as 0.187. The multiple heavy metal resistance (MHMR) index (0.202) was remarkably higher than the MAR value. Most isolates showed minimal inhibition concentrations (MIC) in the range of 25-1.600 µg/mL based on concentrations of heavy metals. These results indicate that surface waters may be discharge points of industrial and domestic wastewaters

Keywords: Antibiotic, Heavy metal resistance, Coliform pollution, Water quality.

Konak Göleti ve Seve Barajı'ndan, (Kilis, Türkiye) izole edilen *Escherichia coli* suşlarının antibiyotik ve ağır metal dirençliliği

Özet

Antibiyotik ve ağır metal dirençliliği gösteren koliform bakterilerle yüzeysel suların kirliliği artan bir halk sağlığı riski oluşturmaktadır. Bu nedenle, çalışmamızda Kilis İlinde iki farklı bölgeden (Seve Barajı ve Konak Göleti) toplanan yüzey suyu örneklerindeki fekal bakterilerin antibiyotik ve ağır metal dirençlilik profilleri araştırılmıştır. Örneklerin pH, sıcaklık, toplam çözünmüş katı madde (TDS) ve elektrik iletkenlik (EC) gibi çeşitli fizikokimyasal değişkenleri incelenmiştir. Her örnekteki total koliform sayısı >1100 (EMS)/100 mL'dir. Örnek noktalarında fekal kirliliğin indikatörü olarak 21 *Escherichia coli* suşu izole edilmiştir. Yüksek koliform kirliliğine karşın, *E. coli* izolatlarının çoklu antibiyotik direnç (ÇAD) indeksi 0.187 olarak kaydedilmiştir. Çoklu ağır metal direnç (ÇAMD) indeksi (0.202) ise çoklu antibiyotik direncinden yüksek bulunmuştur. Çoğu izolatın 25-1.600 µg/mL aralığında minimum inhibibe edici konsantrasyon gösterdiği belirlenmiştir. Bu sonuçlar yüzey sularının endüstriyel ve evsel atıksuların etkisi altında olabileceğini göstermektedir.

Anahtar Kelimeler: Antibiyotik, Ağır metal direnç, Koliform kirliliği, Su kalitesi

INTRODUCTION

Increased interaction between human and domestic animals adversely affects wildlife and ecosystem such as soil and surface waters (Hacioglu et al., 2015). Especially surface waters often are polluted with waste products of industrial, hospitals, and pharmaceutical companies. This is caused that surface waters are suitable environmental media for the growth of fecal coliform bacteria. The occurrence of coliform bacteria (*Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp. and *Serratia* spp.) in the water system such as brackish and fresh waters is indicated to contamination originated by human and animal feces (Dobrijević et al., 2017; Akkan and Topkaraoğlu, 2019).

As well as fecal contamination in water, the antibiotic resistance of bacterial pathogens as a factor of disease emergence is a serious and growing problem around the world (World Health Organization, [WHO] 2018; Eduardo-Correia et al., 2020). Antibiotics have been spread to the environment and pose a threat to the human society and ecosystems due to their large-scale usage in human healthcare, livestock management, and aquaculture (Li et al., 2020). However, most of the antibiotics performed to target organisms are poorly absorbed and released via feces or urine in the form of metabolites (Heuer et al., 2008). Though some of the excreted antibiotics and their metabolites can be degraded by abiotic and biotic pathways, the continuous-release may be caused to pseudo persistent antibiotics in the aquatic environments. (Wang et al., 2018). The untreated antibiotic wastes in the aquatic environments are contributed to the proliferation of bacteria having multiple antibiotic resistances increased virulence (Matyar et al., 2014; Akkan, 2017).

Metal contamination of aquatic environments often originates from mining activities, wood processing, shipping, dredging, urbanization, agrochemicals, and industrial processes (Jacquiod et al., 2018). Heavy metals toxicity and accumulation in surface waters are constituted a serious ecological hazard on wildlife and public health (Jie et al., 2016; Uncumusaoğlu et al., 2016). Akkan et al., (2011) had shown that an important proportion of gram-negative bacteria isolated from seawater contaminated with hospital waste discharge was resistant to cephalosporins and this situation carries a potential risk for public health. Tee and Najjah (2011) indicated that overdosing on antibiotics and chemical agents in prophylaxis may also be induced by antibiotic-heavy metal resistant bacteria. Similarly, George and Wan (2019) reported that antibiotic resistance linked to mobile genetics elements is co-occurred with heavy metal resistance in many bacteria. Bacterial metal resistance is associated with the precipitation of metals, presence of negatively charged groups in extracellular polymeric substances (EPSs), energy-dependent metal efflux systems, and metal volatilization via methyl or ethyl group addition (Matyar et al., 2014; Le and Yang, 2019).

This study is aimed to investigate physicochemical properties, the level of coliform pollution, the presence of *Escherichia coli*, antibiotic and heavy metal resistance of water-borne *E. coli* in surface waters obtained from the Seve Dam and Konak Pond, Kilis, Turkey.

MATERIAL AND METHODS

Collecting surface water samples

Surface waters were sampled in June 2020 from the Seve Dam and Konak Pond in Kilis. The geographic coordinates of the sampling sites were 37°14'46.1"E, 36°45'01.0"N (Site 1) and 37°13'30.2"E, 36°46'24.2"N (Site 2) (Fig. 1). Surface samples were collected by using 250-mL sterile bottles in accordance with APHA 2005. All samples were transported to the laboratory in an icebox and processed within 2-4 h.

Figure 1. Sampling location

Physicochemical properties of samples

Physicochemical properties such as the total dissolved solids (TDS), electrical conductivity (EC), pH and temperature of the surface water samples taken from 2 different sites were determined using HI 9812-5 Portable pH/EC/TDS/°C meter.

Bacteriological analyses

The level of coliform pollution of water samples was analyzed by the most probable number (MPN) method. Serial dilution of samples was inoculated including Lauryl Sulfate Broth (LST, Merck) in tubes and incubated at 37°C for 24-48 h. Following incubation, bacterial proliferation and gas bubbles collected in the inverted Durham tube present in tubes were examined. The presence of gas in tubes is showed to use the lactose in the medium by coliforms. In water samples, the number of total coliforms was calculated by counting the tubes giving positive reaction and comparing with standard statistical tables. For screening fecal coliform, 100 µL of positive tubes was transferred into the fresh LST and incubated at 44.5°C for 24-48 h. The gas formation in Durham tubes after incubation is revealed to the presence *E. coli* in surface water samples.

Endo C agar for isolation *E. coli* strains was used. 100 µL of the fecal coliform positive cultures was inoculated on Endo C agar and incubated at 37°C for 24-48 h. Subsequently, colony colors of *E. coli* were observed. On Endo C if *E. coli* is grown, a distinctive metallic green sheen will have occurred. Isolated colonies were identified by applying morphological (Gram staining and cell morphology) and standard microbiological (indole, methyl red, voges proskauer, citrate, and MUG (4-Methylumbelliferyl β-D-Glucuronide) agar test systems) procedures.

Antibiotic and Heavy Metal Resistance

Antibiotic resistance test was carried out on Mueller Hinton Agar (MHA) by Kirby-Bauer disc diffusion assay according to the Clinical Laboratory Standard Institute (CLSI) guidelines (Bauer et al., 1966). 16 different commercial antibiotic including Ampicillin (AMP; 10 µg), Cefazolin (CZ; 30 µg), Cefepime (FEB; 30 µg), Cefixime (CFM; 5 µg), Cefoperazone (CEP; 75 µg), Ceftizoxime (ZOX; 30 µg), Chloramphenicol (C; 30 µg), Clindamycin (CD; 2 µg), Erythromycin (E; 15 µg), Gentamicin (GEN; 10 µg), Imipenem (IMP; 10 µg), Meropenem (MRP; 10 µg), Metronidazole (MT; 5 µg), Streptomycin (S; 10 mcg), Tetracycline (TE; 30 mcg) and Trimethoprim (TR; 5 µg) were tested for all strains. The turbidity of the overnight bacterial culture was adjusted to 0.5 McFarland standard reference range. Following the inoculation, the plates were incubated at 37ºC for 12-24 h. Then, the diameter of the inhibition zone was measured and the results were evaluated as susceptible, intermediate resistant, or resistant by comparing to CLSI standard results (Table 1).

Table 1. Zone diameter interpretive criteria for testing antibiotic resistance (in terms of mm)

Standard antibiotics	Susceptible (S)	Intermediate (I)	Resistant (R)
Ampicillin	≥17	14-16	≤13
Cefazolin	≥23	20-22	≤19
Cefepime	≥18	15-17	≤14
Cefixime	≥19	16-18	≤15
Cefaperazone	≥21	16-20	≤15
Ceftizoxime	≥25	22-24	≤21
Chloramphenicol	≥18	13-17	≤12
Clindamycin	≥21	15-20	≤14
Erythromycin	≥23	14-22	≤13
Gentamicin	≥15	13-14	≤12
Imipenem	≥23	20-22	≤19
Meropenem	≥23	20-22	≤19
Metronidazole	≥21	16-21	≤16
Streptomycin	≥15	12-14	≤11
Tetracycline	≥15	12-14	≤11
Trimethporim	≥16	11-15	≤10

E. coli ATCC 25922 was used as the control for antibiotic and heavy metal resistance tests. The multiple antibiotic resistances (MAR) index of *E. coli* strains was calculated based on method declared by Krumperman (1983). The following formula was used:

MAR index = X/(YxZ), where X is the total of antibiotic resistance cases, Y is the total antibiotic tested and Z is total isolates. A MAR index value ≤0.20 is observed that antibiotics are seldom or never used, whereas value >0.2 is indicated that bacteria isolates are exposed to the antibiotics (Hacioglu et al. 2015).

The minimal inhibitory concentration (MIC) for four heavy metals against *E .coli* isolates was tested. Heavy metal resistance of *E. coli* strains was detected by using Nutrient Agar (Merck) supplemented with concentrations ranging from 25 to 3.200 µg/mL of Cd^{+2}, Cu^{+2}, Mn^{+2} and Pb^{+2}. The metals were used as $CdCl_2$, $CuSO_4.5H_2O$, $MnCl_2.4H_2O$ and $(CH_3COO)_2Pb.3H_2O$. Multiple heavy metal resistance index (MHMR) was calculated as defined above MAR index formula.

RESULTS and DISCUSSION

Physicochemical parameters of Seve Dam and Konak Pond surface waters are shown in Table 2. The pH values of the present study were within the standard limit (6.5-8.5) of Quality Criteria of Continental Surface Water Resources, Turkey.

Table 2. Physicochemical parameters of surface water samples

	Site 1	**Site 2**	**Standard Limit** (25730 Regulations, 2015 and WHO, 2008).
pH	8.2	8.2	6.5-8.5
Temperature	25°C	25°C	≤25°C
TDS (mg/L)	190	170	max. 500
EC (µS/cm)	390	350	<400

These values of samples are revealed to the surface waters of Kilis were alkaline. This may be explained to discharge the industrial and agricultural effluents including alkali into the water sources. The alteration of water temperature such as increase, decrease or fluctuation can be caused to the slowdown, speedup, and/or stop together of metabolic activities. Because of this, water temperature is an important factor that affected the biological activity of aquatic organisms and the variety of aquatic life (Jannat et al., 2019). The temperature of water samples was determined as 25ºC (Table 2) and this was an acceptable limit (≤25) according to surface water quality criteria of Turkey. TDS concentrations of the water samples were in the range of 170 to 190 mg/L, within the maximum allowable value (500 mg/L) of the World Health Organization (WHO 2008). Electrical conductivity (EC) related to the concentration of ions in the water is a measure of passing electrical flow. EC values of water samples were lower than 400 µS/cm which was the standard limit (<400) quality criteria of Turkey. Bulbul and Camur Elipek (2017) revealed the water temperature, pH values, and electrical conductivity of Meric River (Edirne, Turkey) were ranged between 4.5-22ºC; 7.4-8.4; 172-798 µS/cm, respectively. Similar results were acquired by Hulyar and Altug (2020) who stated that pH values and temperature of Cırpıcı River (Istanbul, Turkey) and its flowing areas recorded between 6.0-8.38 and 10.3-29.5ºC. The other study in Pindare River (Brazil) predicated that pH, temperature, TDS, and EC values in the three sampling points in the dry season were 7.06-8.13; 24-27°C; 140.66-185 mg/L and 213-363.33 µS/cm, respectively (Muniz et al., 2020).

The counts of total coliform bacteria in the water samples taken from the Seve Dam and Konak Pond were >1100 MPN per 100 mL of sample. *E. coli* strains isolated from tubes giving positive reaction at 44.5°C for 24-48 h. All isolates showing Gram-negative bacilli morphological character were identified by biochemical test (indole, methyl red, voges proskauer, citrate, and MUG test systems). These isolates giving positive reaction were evaluated as *E. coli*. A total of 21 *E. coli* isolates were obtained from Site 1 (14 strains) and Site 2 (7 strains), respectively. Based on the WHO (2017) and TS 266 (2005) guidelines, water resources used for drinking water and water intended for human consumption should not contain total and fecal coliform bacteria (0/100 mL). Bacteriological findings in this study are clearly emphasized to discharge of household and industrial wastewater systems into the dam without control. This anthropogenic activities may affect the metabolic activities in aquatic ecosystems and the biodiversity of aquatic life.

In many surface water studies, it is stated to domestic and industrial originated bacterial pollution (Kayis et al., 2017; Hulyar and Altug, 2020). Similarly, total coliform levels in samples obtained from three ponds of Nigeria were declared to range from 120 to 1200 MPN per 100 mL and the presence of fecal coliform was reported (Douglas and Isor, 2015). For Karasu River (Sinop, Turkey), the maximum total and fecal coliform level was reported as 240 MPN/100 mL in June (Avsar, 2018).

In addition to bacteriological load, several papers screened the antibiotic resistance potentials of bacteria isolated from surface waters (Ozgumus et al., 2007; Toroglu and Toroglu, 2009; Akturk et al., 2012). As shown in Figure 2, the results of the antibiotic susceptibility test indicated that 100% of the *E. coli* strains isolated were resistant to clindamycin, erythromycin, and metronidazole. However, none of the isolates developed any resistance to 56.25% of standard antibiotics (against cefepime, cefixime, cefoperazone, ceftizoxime, chloramphenicol, gentamicin, imipenem, meropenem, and trimethoprim).

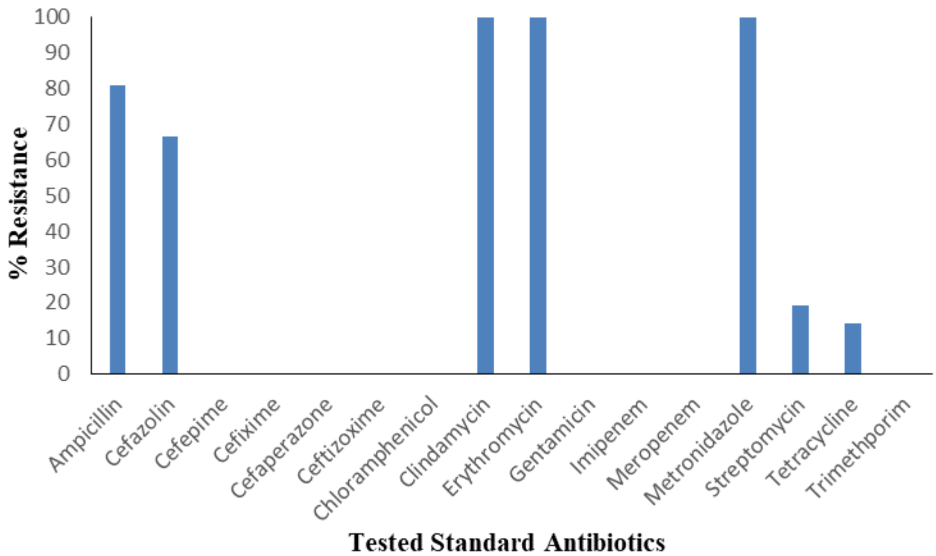

Figure 2 Antibiotic susceptibility profiles of *E. coli* isolates

Among screening 16 standard antibiotics, isolates were determined to be intermediate resistance against ampicillin (80.95%), cefazolin (66.67%), streptomycin (19.04%), and tetracycline (14.28%). *E. coli* ATTC 25922 showed only resistance to clindamycin and metronidazole antibiotics, while it had the intermediate resistance to erythromycin, ampicillin, and cefazolin. Kurekci et al., (2017) found that *E. coli* strains recovered from the Asi River (Turkey) showed resistance against extended-spectrum β-lactam antibiotics. In coliforms isolated from ten rivers in the northern region of Turkey, Ozgumus et al., (2009) notified resistance to ampicillin, streptomycin, trimethoprim, tetracycline, and chloramphenicol in contrast to our susceptibility results. A previous study reported the high coliform pollution (>1100 MPN/100 mL) in Aksu River, (Turkey) noted that 42.2% of *E. coli* strains were resistant against β-lactam antibiotics (Toroglu et al., 2005). Also, Akkan et al., (2019) reported that TC, FC, and FS rates were determined to 45%, 71.66%, and 56.66%, respectively, in the sixty surface water samples collected from Yağlıdere Stream, thus this situation indicating bacteriological pollution. The study on the Gram-negative bacteria was isolated from the seawater of Iskenderun Bay showed that antibiotic resistances of 161 isolates were at high percentages for erythromycin: 94.4%, ampicillin: 72.7%, streptomycin: 68.3%, cefazolin:64.6%, and carboksipeneme: 57.1% (Akkan et al., 2011).

This revealed to be the antibiotic resistance 18.75% or 63 cases and intermediary antibiotic sensitive case (11.30% or 38 cases) of 336 total cases. The recorded MAR index value was 0.187. Similar to this study result, the low MAR index for *E. coli* isolates in Kucukcekmece Lagoon (Istanbul, Turkey) was determined as 0.14 (Kimiran-Erdem et al., 2015). A recent study carried out in Karasu River; MAR index values of isolated *E. coli* strains were in range from 0.4-0.7 (Avsar, 2018) which these values higher than the result of this study. In a study conducted on the *Enterobacteriaceae* isolated from the Giresun coast, the researchers found that 91% of all isolates' multiple antibiotic resistance (MAR) index values were higher than 0.2 (Akkan and Mutlu, 2016). Matyar et al. (2014) reported that MAR index ranged from 0.2 to 0.81 for Gram-negative bacteria isolated Seyhan River and Seyhan Lake Dam.

All *E. coli* isolates showed high resistance to lead and manganese heavy metal. Tolerance to the highest MIC of this heavy metal was found as 1600 μg/mL. This value of copper was 400 μg/mL. All isolates were observed to sensitive against cadmium heavy metal. MHMR index of *E. coli* strains was calculated as 0.202. For *E. coli* ATCC 25922, it was observed to the different tolerance against Pb^{+2} (25-1600 μg/mL), Cu^{+2} (25-200 μg/mL) and Mn^{+2} (25-1600 μg/mL). Trends in heavy metal resistance of *E .coli* strains were Pb=Mn>Cu depending on concentrations. Similarly, Icgen and Yılmaz (2014) expressed the resistance to heavy metals including Cu^{+2}, $Mn^{+2,}$ and Pb^{+2} in Kızılırmak River isolates. Matyar et al. (2008) found that the heavy metal resistance of İskenderun Bay (Turkey) isolates was in the order of Cd>Cu>Pb>Cr>Mn. Similarly, another study of Matyar et al., (2009) showed that the heavy metal resistance of fish samples collected from Iskenderun Bay were; for gill Cd>Cu>Mn>Cr= Pb, and for intestine: Cd>Cu>Cr>Mn=Pb. Sipahi et al., (2013) reported that 134 isolates of the *Enterobacteriace* were resistant to copper while manganese 61.94% and lead 46.27% were resistant.

Ciftci Turetken et al. (2019) informed the frequency of bacterial antibiotics and heavy metal resistance and bioindicator bacteria in the water samples taken from Sapanca Lake. They notified that the highest bacterial resistance was recorded as 88.10% against ampicillin in a total of resistant Gram-negative strains. The heavy metal resistance against Cu and Cd was detected as 52.38% and 26.19%, respectively. This result showed that the lowest resistance property was observed against cadmium. Benhalima et al. (2020) investigated the determination of copper and cadmium concentrations and heavy metal-resistant bacteria identification in water samples obtained from Seybouse River, Algeria. The pH of the water samples was alkaline. The MIC value of *E. coli* KZ1 isolate was determined as 400 μg/mL and 200 μg/mL for copper and cadmium, respectively. Their results are similar to the results of this study especially in terms of copper value. Maal-Bared et al. (2013) noted that the distribution of antibiotic-resistant *Escherichia coli* and *E. coli* O157 isolated from water in an intensive agricultural watershed in Elk Creek, British Columbia. Both *E. coli* and *E. coli* O157 isolates showed the highest frequency of resistance to tetracycline, ampicillin, streptomycin, respectively. Their results are similar to the results of this study in terms of ampicillin value. On the other hand, tetracycline (14.28%) and streptomycin (19.04%) values are not similar. In contrast to the highest resistance values for tetracycline and streptomycin, the results showed that sensitive values for this study.

Even though isolated *E. coli* strains had a low MAR index (0.187), the study findings indicated the presence of high coliform contamination and fecal pollution in the Seve Dam and Konak Pond water samples. Multiple antibiotic resistance (MAR) analysis is a practice method performed to differentiate sources of fecal pollution. In brief, MAR patterns of coliform and/or fecal coliform strains may be informed of the types (human or animal origin) of pollution in waters. Kimiran Erdem et al. (2015) reported that the low MAR index was observed in *E. coli* of animal origin. According to this literature, it is clear that *E .coli* contamination in Seve Dam and Konak Pond originated waste of animals on which antibiotics were seldom or never used. The MHMR index (0.202) of *E. coli* strains was remarkably higher than the MAR index value. This MHMR value is indicated that marine environments such as Seve Dam and Konak Pond may be the discharge point with the heavy metal load from originated from various industrial facilities. This heavy metal tolerance level of *E. coli* isolates is showed that they could be used in bioremediation research of metal-contaminated aquatic sites.

CONCLUSION

In a conclusion, a high level of MHMR index and high counts of coliform bacteria is indicated that the Seve Dam and Konak Pond under the influence of human-sourced pollution as a potential reservoir of animal and industrial wastes. Therefore, the uncontrolled discharges and overloaded into Pond and Dam should be prevented to provide sustainable usage of these water sources in Kilis, Turkey.

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