

Research Article

Antibiotic and Heavy Metal Resistance Patterns of Indicator Bacteria in Surface Water Bodies of Kilis

Hatice Aysun Mercimek Takcı^{1,*} , Sevil Toplar² ¹ Molecular Biology and Genetics Department, Faculty of Sciences, Kilis 7 Aralık University, Kilis, Turkey² Institute of Science and Technology, Kilis 7 Aralık University, Kilis, Turkey

* Corresponding author: Hatice Aysun Mercimek Takcı

* E-mail: mersimek@hotmail.com/aysunmercimek@kilis.edu.tr

Received 03.04.2023

Accepted 11.09.2023

How to cite: Mercimek Takcı, and Toplar (2023). Antibiotic and Heavy Metal Resistance Patterns of Indicator Bacteria in Surface Water Bodies of Kilis. *International Journal of Environment and Geoinformatics (IJECEO)*, 10(3): 132-138. doi.10.30897/ijegeo.1276211

Abstract

The surface waters contaminated with coliform bacteria having antibiotic and heavy metal resistance have become an increasing public health risk. For this reason, it is aimed to detect the bacterial quality, the frequency of antibiotics, heavy metal resistance, and bioindicator bacteria in surface water sources taken from Kilis. The resistance profile of sixteen bacteria species belonging to class Gammaproteobacteria to standard antibiotics and heavy metal salts was investigated using Kirby-Bauer disc diffusion techniques. The various physicochemical parameters such as total dissolved solids (TDS), electrical conductivity (EC), pH, temperature, dissolved oxygen amount, and biochemical oxygen demand (BOD) of samples were also examined. The total coliform load was recorded as >1100 (MPN)/100 mL and calculated comparatively lower values (53(MPN)/100 mL) of fecal contamination for both stations. A high level of resistance to clindamycin in a total of 16 strains was observed. Ampicillin (56.25%), cefotaxime (37.5%), and ceftazidime (31.25%) followed them. The trends in heavy metal resistance of isolates increased in the order of Cd²⁺<Pb²⁺<Mn²⁺ for an average MIC of 50, 1600, and 3200 µg/mL, respectively. These results express higher anthropogenic activity such as discharge points of industrial and domestic wastewaters which can contribute to microbiological contamination in Kilis surface waters.

Keywords: Antibiotic resistance, Bacteriological contamination, *E. coli*, Heavy metal resistance, Surface water,

Introduction

Penicillin as the first antibiotic was discovered by Sir Alexander Fleming in 1928 and the innovative research and development of antibiotics have been continued until today (Yang et al. 2018). Antibiotics have been widely overused in medical treatments and veterinary and agricultural processes. This is caused by the rapid and global spread of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) in natural environments, especially aquatic areas (Eckert et al. 2018).

Some aquatic environments connected terrestrial ecosystems such as lakes and rivers having different hydraulic characteristics show different behavior. In rivers, a load of pollutants in sediments gradually reduces due to downstream of the water source. This property induces that contaminants (i.e. antibiotics, antibiotic resistance genes (ARGs), heavy metals, and biocides) in the inflowing rivers are transferred through water flows to lakes. However, lakes show a greater potential than rivers to accumulate antibiotics and antibiotic resistance genes (ARGs) because of the long retention times of pollutants from discharges in lakes (Yang et al. 2018). Lakes provide significant water sources including nearly 90% of the world's surface fresh water in contrast, rivers contain only 2% (McConnell and Abel 2013). These phenomena may lead to being natural reservoirs for ARBs and ARGs of

lakes showing high ecological risk for the ecosystem and global public health.

The European Centre for Disease Prevention and Control reported that the proportion of antibiotics consumed by humans in society was rather than in healthcare. Clinical data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) emphasized that antibiotic-resistant among Gram-negative bacteria increased and ARGs spread into the environment (Khan et al. 2019). The distribution of ARBs and ARGs in the lake system is easily impacted by discharging untreated wastewater from different sources such as hospitals, municipal inhabitants, livestock waste, and pharmaceutical industries (Ciftci Turetken et al. 2019; Liang et al. 2020; Nappier et al. 2020). Thus, the lake systems have deteriorated due to these human and industrial activities. Resulting in ecological stress turning these water bodies into reservoirs of antibiotic and heavy metal-resistant bacteria (Sultan et al. 2020). The other pollutants including heavy metals, biocides, and chemical preservatives in aquatic ecosystems may exert co-selection on the propagation of ARGs in lakes (Yang et al. 2018; Haberecht et al. 2019). Several literature studies revealed that heavy metals are the main course of ARG contamination in aquatic sediments and affect ARG distribution at sub-lethal levels (Czekalski et al. 2014; Devarajan et al. 2015; Xu et al. 2017). Moreover, the dissemination of ARGs among susceptible bacterial

strains may lead to the emergence of a new population of ARB (Parvez and Khan 2018).

Therefore, the emergence and distribution of antibiotic and heavy metal resistance Gram-negative bacteria in water bodies were investigated by using culture-dependent and culture-independent-based approaches in the past years (Avsar et al. 2018; Ciftci Turetken et al. 2019). In the



Figure 1 Sampling stations (Google Earth)

Materials and Methods

Physicochemical characteristics and sampling of water sources

Freshwater samples were collected from two different sites in Kilis, Turkey, on 21 September 2020. A map of study locations is shown in Fig 1. The geographic coordinates of the sampling sites were Station 1 (St1) and Station 2 (St2). All water samples were collected by using sterile bacteriological sample bottles (250 mL), according to APHA, (1998); WHO, (2006). Following collection, all samples were immediately brought to the laboratory in an icebox and stored at +4°C in a light-sensitive container until microbiological analyses. Physicochemical analyses (total dissolved solids (TDS), electrical conductivity (EC), pH, temperature, dissolved oxygen amount, and biochemical oxygen demand (BOD)) of samples were performed using HI 9812-5 Portable pH/EC/TDS/°C meter and Oxi 3205 SET 3 2BA103.

Microbiological analyses

The levels of fecal and coliform indicator bacteria in samples

The most probable number method (MPN) illustrated by Prescott et al. (1996) was used to determine the counts of coliform indicator bacteria in the water samples collected from each of the sites. Samples were inoculated into 9 tubes consisting of 3 tubes for each of the three dilution factors (0.1 ml, 1 ml, and 10 ml). The dilution tubes including Lauryl Sulfate Tryptose Broth (LST, Merck) were incubated at 37°C for 24-48 h for total coliform. Following incubation, each tube containing an inverted Durham tube was visually investigated for bacterial growth and noted gas production (bubbles). After that, 100 µL from each positive LST broth tube was transferred to an EC (*Escherichia coli*) medium broth tube including a Durham tube, and incubated at 44.5°C for 24-48 h for screening fecal coliform. The number of indicator bacteria was calculated by counting the tubes giving positive reactions and comparing them with standard statistical tables.

present study, the levels of indicator bacteria targeting the potential human pathogen by cultivation-based analysis and the frequency of antibiotic and heavy metal resistance of Enterobacteriaceae species as representative Gram-negative bacteria were investigated in the surface water samples taken from Kilis aquatic sources, in 2020, September.

Gram-negative bacteria isolation

MacConkey agar differentiated them based on lactose fermentation used for gram-negative bacteria from water samples. The serial dilutions were prepared by using 1 mL of water samples. 100 µL from five and eight-fold dilutions were spread on MacConkey agar. And plates were incubated for 24-48 h at 37°C. Subsequently, gram-negative bacteria were selected on agar. 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. Isolates grown in Tryptic Soy Broth (TSB) at 37°C for 24 h were stored at -20°C by adding glycerol to a final concentration of 30%.

Antibiotic and Heavy Metal Susceptibility Testing

The isolates were tested for susceptibility to the 8 standard antibiotics by disk diffusion method (Bauer et al., 1966) on Mueller-Hinton agar (Oxoid, UK) plate according to the guidelines and recommendations of the Clinical & Laboratory Standards Institute (CLSI).

The following 8 antibiotics that are important for the human healthcare system were Ampicillin (AMP; 10 µg), Ceftazidime (CAZ; 30 µg), Cefotaxime (CTX; 30 µg), Chloramphenicol (C; 30 µg), Clindamycin (DA; 2 µg), Imipenem (IMP; 10 µg), Streptomycin (S; 10 µg) and Tetracycline (TE; 30 µg) (HiMedia, India). The susceptibility of strains was interpreted by the CLSI standard results. *E. coli* ATCC 25922 was used for quality control. The multiple antibiotic resistance (MAR) indexes of strains were calculated based on the formula declared by Krumperman (1983). Heavy metal resistance (HMR) of strains was detected by using Nutrient Agar (Merck) supplemented with concentrations ranging from 25 to 3.200 µg/mL of Cd²⁺, Mn²⁺, and Pb²⁺. The metals were used as CdCl₂, MnCl₂.4H₂O, and (CH₃COO)₂Pb.3H₂O. *E.*

coli K12 was used as the control for heavy metal resistance tests. Resistance results were analyzed by using the software SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA)

Bacteria identification

DNA of the isolate showing the highest antibiotic and heavy metal resistance was extracted by using GF-1 Bacterial DNA Extraction Kit (Vivantis) following the manufacturer's standard protocol. The concentration and quality of DNA were detected by using a Nanodrop Multiskan G0 quantification spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA), obtaining 75.92 µg/µL and 1.81. PCR process was performed (initial denaturation at 94°C for 5 min; denaturation at 94°C for 35 s, primer annealing at 56°C for 35 s and extension at 72°C for 45 s of 45 cycles; final extension at 72°C for 5 min.) using *uidA* gene-specific primers (forward: 5'-TGG TAA TTA CCG ACG AAA ACG GC-3' and reverse: 5'-ACG CGT GGT TAC AGT CTT GCG-3', Bej et al. 1991). The amplified products were verified by running on agarose gel (1%). The nucleotide sequence of the *uidA* gene by using both primers was analyzed at the BM-Labosis (Ankara, Turkey). The gene sequence of strain was compared automatically using the Blast software (BLAST), against the sequences of bacteria available in databanks (<http://www.ncbi.nlm.nih.gov/>). The phylogenetic analysis was constructed using a neighbor-joining algorithm.

Results and Discussion

In-situ parameters such as pH, electrical conductivity (EC), total dissolved solids (TDS), dissolved oxygen amount (DO), biochemical oxygen demand (BOD), and temperature are presented in Table 1. According to the WHO guidelines (2008) and quality criteria of continental surface water resources of Turkey (2015), the Table 1. In situ parameters of water bodies from, Kilis

physicochemical properties of surface waters were characterized.

Hydrogen ion concentration and temperature of surface waters are important factors that affected the biological activity of aquatic organisms and the variety of aquatic life (Jannat et al. 2019). The alteration of temperatures such as increase, decrease, or fluctuation can be caused to the slowdown, speedup, and/or stop together of metabolic activities. The pH and temperature values of samples were appropriate for permissible limits of water quality parameters. Ions (i.e. sodium, chloride, calcium, and magnesium), salt and carbonate reservoirs, mineral water sources, and wastewater discharges in the basin are the most important reasons for TDS in surface waters (Gunes 2019). TDS concentrations of samples were in the range of 270 to 340 ppm, within the maximum allowable value (500 mg/L) of the World Health Organization (WHO 2008). EC values, the measure of passing electrical flow, was higher than that of the standard limit (400 µS/cm). A higher conductivity value shows the dissolution of more chemicals in the water and these dissolved salts and the other inorganic chemicals conduct electrical current resulting in a conductivity increase. Similar to EC values, the dissolved oxygen amount of samples was found to be greater than the standard criteria range. This is explained why the dissolved oxygen concentration is high in winter and early spring when the water temperature is low. Biological oxygen demand (BOD) generally expresses how much oxygen amount is needed to break down organic matter in water sources under aerobic conditions. In brief, it is the amount of oxygen consumed by bacteria and other microorganisms. BOD amount in water samples was calculated as 4±0.0 mg/L. Low BOD indicates that less oxygen is being removed from the water, signifying purer. Physico-chemical parameters from sampling sites the northern coastal line of Gökova Bay reported by Ozdemir et al. (2022) is similar to our results.

| | St1 | St2 | Standard Limit (25730 Regulations, 2015 and WHO, 2008) |
|------------------|---------|-----------|---|
| pH | 8.3±0.0 | 7.8±0.0 | 6.5-8.5 |
| Temperature (°C) | 25 ±0.0 | 25.33±0.2 | ≤25°C |
| TDS (mg/L) | 270±0.5 | 340±0.7 | max. 500 |
| EC (µS/cm) | 550±0.1 | 680±0.4 | <400 |
| DO (mg/L) | 33±0.2 | 27±0.3 | 8-13 |
| BOD (mg/L) | 4±0.0 | 4±0.0 | <4->20 |

The bacteriological status of Kilis water bodies follows the trends shown by that of limno-chemistry. Herein, the water of St1 and St2 exhibited high levels of total coliforms, >1100 MPN/100 mL. Both stations maintained comparatively lower values of fecal coliforms, 53 MPN/100 mL. Fecal coliforms have long been used as an indicator of pollution in water due to the potential for the introduction of pathogens and other pollutants along with these bacteria (Dangi et al. 2017). WHO (2017) and TS 266 (2005) guidelines predict that surface water sources overused 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 surface water without control. Further, the main source of contamination is the high loading of domestic sewage and solid wastes from surrounding densely populated areas. These anthropogenic factors may affect the metabolic activities in aquatic ecosystems and the biodiversity of aquatic life. The presence of fecal coliforms in aquatic environments indicates the contamination of waters with the fecal material of man or other animals. Several previous studies have also demonstrated a higher concentration of total and fecal coliforms in different water bodies in Turkey (Buyukkaya Kayis et al. 2017; Avsar 2018; Hulyar and Altug 2020; Mercimek Takcı et al. 2021). Our another a study performed for different

locations at same time in Kilis observed fecal streptococ contamination as 11 EMS/100 mL (Mercimek Takci and Toplar 2023). Although our fecal coliform contamination rates were lower than those shown by Yalim et al. (2020) and Akkan et al. (2019), our study clearly emphasized the discharge of household and industrial wastewater systems into the surface water without control.

Also, our bacteriological and antibiotic resistance findings obtained for Kilis province declared that the main source of contamination the high loading of domestic and animals sewage from surrounding densely populated areas.

Sixteen bacterial isolates recovered on MacConkey plates from the 2 stations were selected for the further antibiotic susceptible study. While the low frequency of resistance to imipenem (12.5%) was determined, these culturable bacteria showed high levels of resistance to streptomycin (25%), ceftazidime (31.25%), and cefotaxime (37.5%), followed by ampicillin (56.25%) and clindamycin (100%) (Table 2).

Table 2 Percentage of total isolates with antibiotic resistance

| | Pattern | Resistance (%) |
|------------------------|---------|----------------|
| Imipenem | R | 12.5±0.5* |
| Streptomycin | R | 25±0.6* |
| Ceftazidime | R | 31.25±0.25* |
| Cefotaxime | R | 37.5±0.5* |
| Ampicillin | R | 56.25±0.25* |
| Clindamycin | R | 100±0.0* |
| Chloramphenicol | S | 0±0.0* |
| Tetracycline | S | 0±0.0* |

* $P < 0.05$ compared with the results.

The levels of resistance to streptomycin (25%), imipenem (12.5%), and clindamycin (100%) were constant in 2 stations. By contrast, the obvious spatial heterogeneity was exhibited in resistance to ceftazidime, ampicillin, and cefotaxime, ranging from 0% to 62.5%, 37.5 to 75%, and 25 to 50%, respectively. In this present investigation, 50.0% Gr negative bacteria isolated from St1 were resistant to ceftazidime, cefotaxime, clindamycin, and ampicillin, whereas only %25 of the isolates from St2 showed clindamycin and cefotaxime resistance. Although the prevalence of ARB in many freshwater sources of Turkey has been previously reported (Ozgumus et al. 2009; Akkan et al. 2011), this is the first study that indicated the high frequency of ampicillin and clindamycin resistance in surface waters of Kilis. Buyukkaya Kayis (2022) noted that *E. coli* isolated from 5 different stations in Ataturk Dam in Adiyaman was resistant to chloramphenicol, and tetracycline antibiotics contrary to our findings. Mercimek Takci et al. (2023) pointed to the high-frequency resistance against cefotaxime (62.5%±0.25), ceftazidime (50%±0.5), tetracycline (100%±0.00) and streptomycin (50%±0.45) antibiotics for *E. coli* strains obtained from Alleben pond (Gaziantep) by contrast with our results. Similarly, Altug et al. (2020) reported the dissemination of high antibiotic resistance in surface water samples, Gulluk Bay.

The MAR index ranged from 0.125-0.625 for Gr-negative bacteria isolated from Kilis water sources. %81.25 of isolates showed a MAR index greater than 0.200. A MAR index of 0.200 is represented the difference between low- and high-risk degrees. Those multidrug-resistant bacteria having MAR indices >0.200 indicates the exposure of sewage effluents to high-risk contamination for water bodies with the increased distribution of possibly fecal microorganisms harmful to humans. Similar to our MAR data, Isik and Akkan (2021) indicated that 80,5% of isolates are the multiple antibiotic resistance levels (MAR) in the study conducted at Gelevera Creek (Giresun). In another study, similar results were acquired by Mercimek Takci et al., (2023) who stated that MAR index of Gr negative strains from the Gaziantep water source ranged from 0.33-0.67.

Water isolates exhibited high resistance for their heavy metal with MIC ranging from 25 to 3.200 µg/mL. The trends in heavy metal resistance of isolates increased in the order of Cd²⁺< Pb²⁺< Mn²⁺ corresponding to an average MIC of 50, 1600, and 3200 µg/mL, respectively. Isolates are resistant to higher concentrations of manganese than cadmium and lead. It is interesting to note that fourteen isolates (87.5%) were multi-resistant to heavy metals at different concentrations. While 87.5% of bacterial strains at A sites tolerated more than 1600 µg/mL of MnCl₂·4H₂O, this frequency for B sites was 37.5%. This points out that St1 is suffering more ecological risk than the other station. The distribution pattern of heavy metal-resistant bacteria is related to the unique terrain conditions of this sampling site and the migration of heavy metals. In most of the studies, heavy metal resistance of bacterial strains isolated from surface waters has been reported (Matyar et al. 2009; Sipahi et al. 2013; Icen and Yilmaz 2014). Mercimek Takci et al., (2021) indicated to the high MHMR index of Gr negative strains isolated from Seve Dam and Konak Pond, Kilis. In another study, Ciftci Turekten et al. (2019) showed the occurrence of high heavy metal and antibiotic resistance in Gammaproteobacteria strains isolated from Sapanca Lake, similar to our study.

PCR product with 133 base pairs was sequenced. And it was revealed that the Gr negative strain showing the highest antibiotic and heavy metal resistance has 100% identical to the *Escherichia coli* (Figure 2). The GenBank accession number allotted for uidA β-D-glucuronidase gene sequence of the isolate is OK057113 by using the NCBI BLAST database

In this study, a high level of heavy metals resistance is associated with multiple antibiotic resistance of bacteria collected from surface waters. Most literature results revealed that heavy metal and antibiotic resistance capacities of water isolates are often closely associated (Yamina et al. 2012; Nguyen et al. 2019). This interrelationship indicates that bacteria under metal stress adapt to environmental conditions faster by the spread of resistant factors than by mutation and natural selection. In brief, the prevalence of heavy metals as co-selecting agents in the aquatic environment can increase resistance

to antibiotics and heavy metals through the activation of co-resistance and cross-resistance mechanisms.

Additionally, the availability of heavy metals to bacterial pathogens can be affected by abiotic factors such as pH.



Figure 2. Maximum likelihood phylogenetic tree based on uidA gene sequences from *Escherichia coli*

Conclusion

Our results show the presence of Gr-negative bacteria having different antibiotic and heavy metal resistance which can spread resistance genes to the other susceptible aquatic microorganisms in Kilis surface waters. High microbiological contamination of these samples points out the influences of anthropogenic pollution as a potential reservoir of animal and industrial wastes. Moreover, the prevalence, concentration, and location of AMR and HMR bacteria play a crucial role in possible public health risks.

Acknowledgments

The authors thank Kilis 7 Aralık University, Turkey for help during the laboratory part of the study.

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