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Research Article

Investigation of bacterial pollution in Ceyhan River (Turkey) and the resistance levels of Gram (+) and Gram (-) bacteria to antibiotics

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Keywords

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ABSTRACT

In this study, bacteriological pollution of Ceyhan River flowing into Iskenderun Bay (Northeast Mediterranean) and antibiotic resistance of Gram (+) and Gram (-) bacteria were investigated. Water samples were collected from May 2014 to April 2015 in monthly periods and 222 Gram (-) and 74 Gram (+) bacteria were isolated from collected water samples. The isolates were contained 8 different species (*Acinetobacter baumannii, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis*) which have been identified with Vitek II automated culture system. Microorganism susceptibility tests were performed in accordance with CLSI (Clinical and Laboratory Standards Institute, 2015) criteria. Resistance of the isolates to 15 different antibiotics (Amikacin, Meropenem, Levofloxacin, Imipenem, Piperacillin, Gentamycin, Cefepime, Ceftazidime Penicillin, Oxacillin, Clindamycin, Erythromycin, Ciprofloxacin, Vancomycin, Rifampin) was investigated. The highest antibiotic resistance was found in *E. faecalis* with 37% against Penicillin antibiotics. No resistance to vancomycin antibiotics has been observed. It was concluded that the Ceyhan River was exposed to fecal bacterial contamination, and it was revealed that this situation would adversely affect both the ecosystem and human health. Measures to protect and improve the ecological and microbiological qualities of rivers and lakes are key to preserving the quality and quantity of water resources for the future.

Introduction

Streams are ecosystems that are primarily affected by environmental pollution. Pollutants originating from agricultural, domestic and industrial activities are first introduced into surface waters. When human populations were low, waste materials mixed with streams could be diluted and disintegrated naturally in a short distance. However, with the industrialization and rapid increase in the population that came with development, industrial and domestic wastes also increased and rivers became unable to clean themselves. Quality of surface waters such as lakes, streams, dams and agricultural waters is very important for the continuity of the aquatic ecosystem and agricultural activities as well as public health (Noori et al., 2018; Gümüş et al., 2021). Water resources used for drinking and household usage should be adequate amount with appropriate chemical and bacteriological characteristics (Gümüş et al., 2013).

A bacteriological analysis is based on the presence of coliform bacteria. For example, *E. coli*, a coliform bacterium, is used as an indicator in the bacteriological analysis. Although *E. coli* is not a pathogenic microorganism, its presence is important for health as it may represent presence of fecal or orally transmitted disease agents. If sewage water is dumped into lakes or rivers without any prior treatment, it causes the

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transmission of pathogenic microbes to humans (Unat, 1997).

According to the reports from relevant organizations such as the United Nations and UNESCO, approximately eighty countries, including forty percent of the world's population, are already suffering from water shortages. For this reason, it is vital to evaluate the water resources very well, to prevent the pollution of the river resources by wastewater, and conscious water management and precautions to be taken without disturbing the quality of life.

The Ceyhan River is an important stream that is exposed to wastes from industrial activities, septic tanks from rural areas, domestic wastewaters from settlements, pesticides and artificial fertilizers used in agricultural areas, leachate from solid waste storage facilities, wastes from cattle, and ovine livestock. Wastewater from the settlements is mostly collected by the sewerage system and discharged without adequate treatment. In this study, bacterial species causing microbiological pollution (along with antibiotic resistance of these bacteria) were investigated.

Material and Methods

Ceyhan River is one of the largest rivers of Turkey. The first source of Ceyhan River is in the mountains surrounding the Elbistan lowland. The length of the Ceyhan River is 509 km and the rainfall area is 20,000 km^2. The river comes out under the name Söğütlü Creek, grows on various sources, and is named as Ceyhan River with joining the Göksun and Hurman streams. After passing through the straits in the Ahır and Engizek Mountains, it enters the northeastern part of the Çukurova plain, after crossing the Misis Mountains, it flows in its wide delta and flows into the Gulf of İskenderun (Figure 1).

During the bacterial isolation and identification phase, water samples were brought to the Bacteriology Laboratory of the Department of Microbiology under the cold chain by taking 100 ml sterile containers from the water surface monthly. Samples were planted on Blood Agar for Gram (+) bacteria isolation and on EMB (Eosin Methylene Blue) agar for Gram (-) bacteria isolation. For transplantation, 1 ml of water sample was placed in plates and spread with a sterile drumstick. In the incubator, the Petri plates were incubated in a flat position for 1 hour for the plates to absorb the liquid material. Then, the incubation phase was started for at least 72 hours. At the same time, water samples were planted in Mueller-Hinton Broth medium as 10% of the medium and left to incubate at 37°C for 72 hours with agar smears. Microbial isolations were performed by making bacterial passages from Mueller-Hinton broth into solid media when it is needed. The bacterial identification stage was started by applying Gram staining, catalase test, plasma coagulase test, oxidase test, sugar tests and advanced biochemical tests from the microorganism colonies that appeared at the end of the incubation. The gram staining method is used in the first step for the identification of microorganisms grown on solid media. A clean slide of microorganism colonies produced in blood and EMB agar was suspended with 1 drop of saline solution and the bacterial colony spread was homogenized with the help of a loop and then allowed to dry in air. The preparations dried in the air were treated with Crystal Violet (2 min), Lugol (2 min) after the flame fixation process, followed by 1 % aqueous acid fuchsin (30 sec) after the alcohol decolorization process and dyeing process was carried out. Then, the preparations were examined with immersion oil used with the 100x objective and Gram (+) Gram (-) bacteria distinction was made, which is the first step of the identification. The antimicrobial susceptibilities of the microorganisms identified with conventional methods were also examined by verifying their species identification with automated systems. The identified isolates were stored at -70°C in storage media containing 20% glycerol until the study was completed.

Bacterial identification (with bacteria identification kits; Biomerieux, France) was performed by microbiological analysis of water samples. Gram(-) and Gram (+) antibiogram susceptibility tests were performed according to the type of bacteria identified. In the study, for Gram (-) bacterial origins, Amikacin, Levofloxacin, Gentamicin, Cefepime, Meropenem, Ciprofloxacin, Imipenem, Piperacillin, Ceftazidim susceptibility with Gram (+) cocci (staphylococci), Oxacillin, Clindamycin,

Figure 1. A view from Ceyhan River (original)

Ciprofloxacin, Penicillin, Erythromycin, susceptibility were investigated. Microbial identifications were evaluated simultaneously with conventional methods and automated culture systems. Verification of microorganisms identified by the conventional method was also performed by automated culture systems. Antimicrobial susceptibility tests were performed with the help of Vitek II (Biomerieux, France) automated culture system. Microorganism susceptibility tests were performed according to CLSI (Clinical delta and flows into the Gulf of İskenderun (Figure 1).

In this study, water samples were taken from the sampling location (37°01.696'N-35°48.669'E), which is under intense pollution. The study was conducted between May 2014 and April 2015. Water samples were taken in monthly periods and studied in 3 parallel (Figure 2).

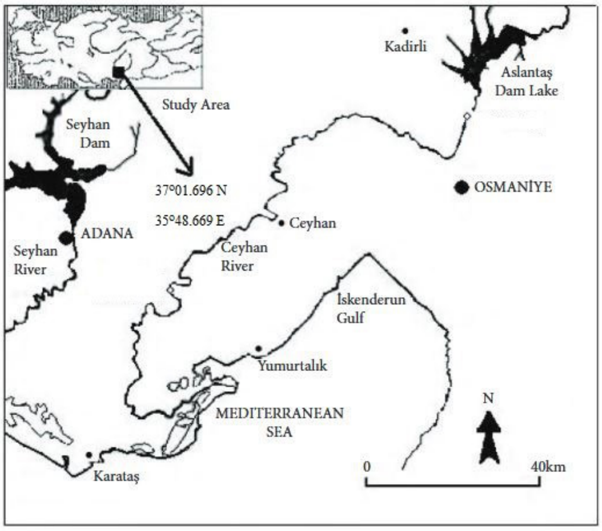

Figure 2. Study area (modified from Kurutaş Belge et al., 2009)

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Table 1. Bacterial species isolated from Ceyhan River *(Jan: January, Feb: February, Mar: March, Apr: April, Jun: June, Jul: July, Aug: August, Sep: September, Oct: October, Nov: November, Dec: December, A.B.: Acinetobacter baumannii, E.C.: Escherichia coli, E.F.: Enterococcus faecalis, K.P.: Klebsiella pneumoniae, P.A.: Pseudomonas aeruginosa, P.V.: Proteus vulgaris, S.A.: Staphylococcus aureus, S.E.: Staphylococcus epidermidis*

MONTHS	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Ceyhan River	A.B. P.A. S.E. E.F. K.P. P.V. E.C.	A.B. P.A. S.E. E.F. K.P. P.V. E.C.	A.B. P.A. S.E. E.F. K.P. P.V. E.C.	A.B. P.A. S.A. E.F. K.P. P.V. E.C.	A.B. P.A. E.F. K.P. P.V. E.C.	A.B. P.A. S.A. E.F. K.P. P.V. E.C.	A.B. P.A. E.F. K.P. P.V. E.C.	A.B. P.A. S.E. E.F. K.P. P.V. E.C.	A.B. P.A. S.E. E.F. K.P. P.V. E.C	A.B. P.A. S.E. E.F. K.P. P.V. E.C	A.B. E.F. K.P. P.V. E.C	A.B. E.F. K.P. P.V. E.C

Results

Studies on microbiological contamination in aquatic environments have always been important research issues of scientists. Also, most of the studies on antibiotic resistance in aquatic environments deal with fecal-derived bacteria, which are both indicators of pollution and may related to infectious diseases. Recently, the emergence of antibiotic resistance of pathogenic bacteria in clinical environments has caused serious problems in all over the world.

When all water samples in our study were examined, pathogenic microorganisms for human health were found (*S. aureus, S. epidermidis, P. aeruginosa*). In addition, intestinal bacteria (*E. coli, K. pneumoniae, A. baumannii, E. faecalis, P. vulgaris*) was detected.

Looking at the months; *A. baumannii, E. coli, P. aeruginosa, K. pneumoniae, E. faecalis,* and *P. vulgaris* species were detected in all months of the year; while, *S. aureus* was detected in April, June, and *S.epidermidis* was detected in all months except May, July, November, December. When the species are considered as variety, high diversity in the Ceyhan River was observed. The reason for this is that the Ceyhan River is exposed to a wide range of wastes such as domestic, industrial, slaughterhouse wastes, and the wastewater supplied to the river is mostly collected by the sewage system and discharged without adequate treatment.

Microorganisms, which are pathogenic for human health, were found in all reproductive samples. Antimicrobial susceptibility tests are applied to determine the in-vitro activity of an antimicrobial agent against a particular bacterial species. In this study, resistance to Penicillin, Oxacillin, Clindamycin, Erythromycin, Ciprofloxacin, Vancomycin and Rifampin antibiotics were evaluated with antibiogram kits (Biomerieux, France) in gram-positive bacteria isolated from water samples. In the study, the lowest resistance rate was found to be 7% against Oxacillin followed by 10% Rifampin (Figure 3).

In the study, susceptibility to Amikacin, Meropenem, Ciprofloxacin, Levofloxacin, Imipenem, Piperacillin, Gentamicin, Cefepime and Ceftazidime was investigated with antibiogram kits (Biomerieux, France) in Gram-negative bacterial strains. The lowest resistance rate was found as Amikacin with 2.85%, followed by Meropenem with 3.09% and Imipenem with 4.12% (Figure 4).

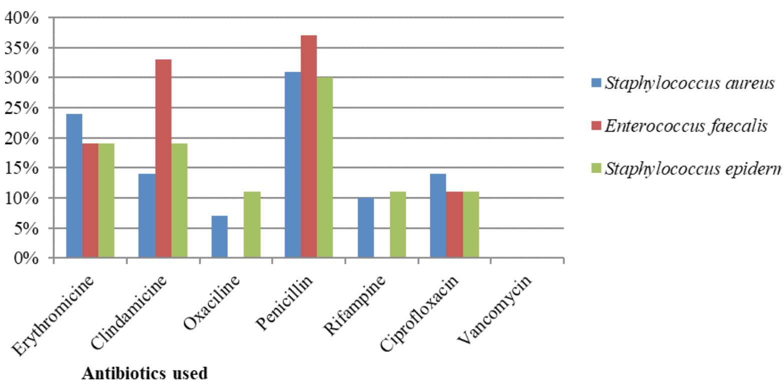

Figure 3. Antibiotic resistance rates of Gram (+) bacteria

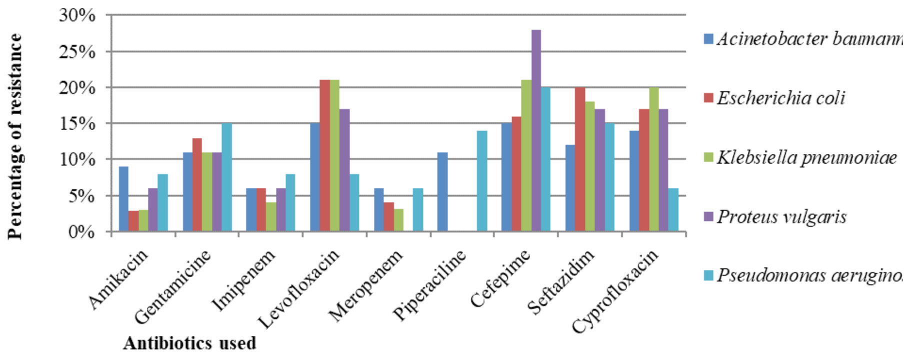

Figure 4. Antibiotic resistance rates of Gram (-) bacteria

Discussion

Eight different types of bacteria have been identified after the identification of the isolates isolated from the Ceyhan River which was pour its water into the Iskenderun Bay. When the identification results are examined, it is seen that 70.7% of the total isolates were *E. coli*. It is known as fecal pollution indicator in aquatic environments, and high abundance (70.7%) of *E. coli* indicates disposal of large amounts of contaminated sewage wastes directly or indirectly in water environments (Toroğlu et al., 2008). In the study conducted by Böger et al. (2021), they detected antibiotic-resistant bacteria in all water samples and found that *E. coli* and *Enterococcus* species of these bacteria were resistant to Cyprofloxacin in some rivers in Brazil. According to this study, they concluded that antibiotics are likely to come from household waste and may contribute to the spread of bacterial resistance. In our study, it was found that *E. coli* and *Enterococcus* species are resistant to Cyprofloxacin. Yerlikaya et al. (2021) found that *E. coli* strains were resistant to Amikacin 2.3% and Meropenem 4.6%. In our study, it was found that *E. coli* was 2.8% resistant to Amikacin and 4% Meropenem. Tahri et al. (2021) tested the resistance of *E. coli* strains to 16 antibiotics in Moroccan groundwater and found the highest resistance to Ceftazidime. In our study, *E. coli* was found resistant to Ceftazidime. Most of the genus *Pseudomonas* bacteria, which account for 56% of the isolates, are found extensively in nature, soil and water. It has a diseasing feature for some of the plants, animals, and humans. Since it is a bacteria that can be found widely in nature, it can survive in organic substances and water for a long time. The presence of this bacteria in aquatic environments has been reported to pose a risk to human health (Mena and Gerba, 2009). In our study, *P. aeruginosa* was highly isolated (approximately 56%), which is important in terms of the ability of bacteria to spread between aquatic ecosystems and humans and animals and to have high antibiotic resistance potential.

K. pneumoniae is commonly found bacteria in nature. This bacterium is found in the upper respiratory tract and fecal flora of humans, and become an opportunistic pathogen in the face of unfavorable conditions. Therefore, it is responsible for hospital infections (Çetinkaya et al., 2005). The isolation of *K. pneumoniae* from the studied water sources is important, because it represents that sewage and hospital waste water are mixed discharged in water resources. In the study, *K. pneumoniae* showed high resistance to Cefepime and Cyprofloxacin antibiotics. Similarly, in the study conducted by Bircan Yıldırım and Vurmay, in 2017, it was reported that *K. pneumoniae* showed high resistance to Cefepime (52.9%) and Ciprofloxacine (47.1%). *Acinetobacter* species, which are opportunistic pathogens, can settle in the hospital environment and cause serious nosocomial infections in hospitalized patients and patients with the suppressed immune system. *A. baumannii* is the most isolated species from clinical samples, especially in hospital-acquired infections (Koneman et al., 1992). In the study, *A. baumannii* was found in most isolates. *P. vulgaris*, one of the bacterial species isolated in the study, is found in human feces as a normal flora element. For this reason, it is often found in sewage waters. When find suitable conditions in humans, it causes infections. It is especially found in wound infections and urinary tract, which are hospital infections. *E. faecalis* is also isolated in our study which is found at a higher rate in the stool compared to other enterococci species. Morinigo et al. (1990) studied the isolation of *E. faecalis* in water samples taken from seashore and river settlements. As a result of their study, they isolated *E. faecalis* at a level that could pose a risk to human health, especially in areas with a risk of contamination with faecal wastes. Although *S. epidermidis*, one of the bacteria isolated in our study, is not usually a pathogen, it is a big risk for patients with an insufficient immune system and patients with permanent catheters. Since it is part of the normal flora of humans, it has developed resistance to many common antibiotics such as methicillin, novobiocin, clindamycin and benzyl penicillin (Nilsson et al., 1998). *S. auerus*, one of the bacteria isolated in our study, is densely found in the feces of humans and animals. *S. auerus* is a highly virulent microorganism that is common in humans as a disease agent. In the 1950s, it gained resistance to many antibiotics, and in 1961, the problem of multiple antibiotic resistance emerged in methicillin-resistant *S. auerus* strains and staphylococci became one of the "problematic" microorganisms (Tambic et al., 1997; Günaydın et al., 2002). Penicillin resistance has gradually increased in staphylococci since 1944. In addition to penicilin, resistance to antibiotics such as erythromycin, tetracycline, and streptomycin was also developed in the 1950s (Haznedaroğlu, 2008). Yerlikaya et al. (2021) found erythromycin resistance as 23.3% and the highest antibiotic resistance 98.5% against penicillin in the study of *S. auerus* strains. Similarly, in our study, it was found that *S. aureus* was resistant to erythromycin by 24% and showed the highest antibiotic resistance to penicillin (31%). In many studies, vancomycin resistance was not found as in our study (Yerlikaya et al., 2021; Sümer et al., 2001; Zer et al., 2002; Yurtsever et al., 2009).

Differences in the pollution between the months were considered as total coliforms related since due to variation human-animal wastes and contamination. Spot pollution sources disrupt the ecological balance of the environment due to continuous waste inputs and therefore constantly change the environment of competition between microorganisms. Therefore, there are no expected changes from environmental factors in the areas where there are point sources of pollution (Clark, 1989).

Resistance percentages of *E. coli* bacteria were found to be between 0-39% against Amikacin and 1-54% against Gentamicin (Can et al., 2005; Çiçek, et. al., 2006). The percentages of resistance in our study also fall within this range. In Yugoslavia, Mirovic et al. (2000) found that penicillin resistance rate was 0.9% in *E. faecalis* strains; Toutouza et al. (2001) found that penicillin resistance rate was 75.8% in *E. faecalis* strains isolated.

In recent years, the problem of increasing resistance to antibiotics has become a threat to the whole world (Akçam et al., 2004). Antibiotic resistance rates in this study are from the years of our study and are likely to change in the following years.

Antibiotics have a high water solubility and are mixed with aquatic environments following human activities through sewer systems and as a result of a farm, slaughterhouse, and land uplifting studies (Daughton and Ternes, 1999). Domestic wastes contain bacteria carrying R-plasmids, most of them originating from the human intestinal flora. Since R-plasmids, which give resistance to antibiotics, are widespread in these bacteria, the discharge of wastewater into environmental waters causes the spread of such resistant bacteria to the environment (Karayakar et al., 2004). Recent studies have shown that non-therapeutic antibiotic use plays an important role in increasing antibiotic resistance in surface waters (Kümmerer, 2009). Since domestic wastewater is the most important source of antibiotic resistance, excessive and misuse of antibiotics should be avoided by following the rational antibiotic usage policy. It can be thought that the emergence of resistance is due to the intensive and unconscious use of antibiotics and the addition of resistant bacteria in waste to water environments without being purified from city sewers.Antibiotic use is a common practice in animal nutrition. Resistant bacteria, which are transferred to the receiving environment (as fertilizer) by animal faeces, often cause epidemic infections in humans through the food chain and ultimately contribute to the spread of antibiotic resistance.

Conclusion

When the results of the study are examined, it is concluded of the river water flowing into Iskenderun Bay has a potential risk in terms of public health. It will be appropriate to prevent sewage and other domestic and similar wastes flowing into these waters, to ensure the control and operation of the treatment system, and to examine the parts where rivers pass through cities in terms of microbiological and physicochemical aspects. Suitability of the water quality is important for the use of water for a specific purpose. It is important to speed up water quality monitoring and evaluation studies in order to improve water resources polluted by industrial and agricultural reasons and to protect our natural resources.

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COMPLIANCE WITH ETHICAL STANDARDS

Authors' Contributions

Authors contributed equally to this paper.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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