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Gram Negative Bacterial Diversity and Antimicrobial Resistance Profiles in Heavily Polluted Environmental Samples around The Sır Dam Lake (Turkey)

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Authors' contributions

This work was carried out in collaboration between both authors. Author EBB designed the study, performed the molecular analyses, analyzed the results, drafted and revised the manuscript. Author ST collected the samples, performed the other laboratory studies. Both authors read and approved the final manuscript.

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ABSTRACT

Background: The Sır Dam Lake is located on the Ceyhan River and provides a major water-supply to agricultural fields in Southern Turkey. However, it has so far received various industrial and household wastes in excessive amounts.
Aims: This study aims to analyze Gram negative bacterial diversity and antimicrobial resistance profiles in polluted environmental samples (soil, sludge, water) collected around the Dam Lake.
Study Design: Gram negative bacteria were isolated from 15 polluted environmental samples and identified using both phenotypic and molecular methods. In addition, antimicrobial resistance profiles of the strains were determined by disk diffusion method.
Place and Duration of Study: Environmental samples were collected from 15 different locations in Avsar region located around the Sır Dam Lake in March 2014.

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Methodology: Gram negative bacterial strains were identified based on biochemical tests. Biochemical identification of the bacterial isolates was further verified using CHROMagar ECC (France). A fragment corresponding to 16S rRNA gene was amplified using universal primers. The sequence analyses of amplified fragments were used for molecular identification of unidentified isolates by biochemical tests. Antimicrobial susceptibilities of the isolates were determined with disk diffusion method.

Results: A total of 17 identified strains were grouped as *Escherichia coli* (n=8), non-fermenters (n=5) and *Enterobacteriaceae* (n=4). Only two strains (*Klebsiella oxytoca* and *Enterobacter* spp.) were identified from *Enterobacteriaceae*. *Acidovorax temperans* emerged as the second predominant bacteria following to *E. coli* and did not display significant resistance to the tested antimicrobials. However, most of *E. coli* isolates as well as a single *K. oxytoca* and a single *Acinetobacter* spp. isolates displayed higher multiple antimicrobial resistance (MAR) index values that should be considered seriously for local public health.

Conclusion: Gram negative bacterial distribution remarked the presence of fecal contamination. In addition, the presence of *A. temperans* might indicate a presence of different types of pollution, and this organism might be useful in biodegradation processes after its metabolic capability is defined.

Keywords: Gram negative bacteria; Enterobacteriaceae; non-fermenters; Acidovorax temperans; antimicrobial resistance profiles.

1. INTRODUCTION

The Sır Dam Lake is located in the Southeastern Turkey and mainly fed by the Ceyhan and Aksu Rivers. The Sır Dam Lake provides an important water-supply to several cities, including Kahramanmaras, Osmaniye and Adana for irrigation of agricultural fields. Although the dam is 35 km away from Kahramanmaras, Sır Dam Lake is closer to Kahramanmaras (5 km). The lake has a length of 50 km with a surface area of 47.5 km^2 and a water capacity of 1120 hm^3. The essential purpose of the Sır Dam was electricity generation; however, the dam lake has so far been an important irrigation source for agricultural fields for approximately 100.000 ha by Iskenderun Bay. Besides, annual fish production in the lake was estimated at approximately 86 tons in 1997. Apart from agricultural production, many other industrial facilities were established around the lake. Therefore, the Sır Dam Lake has received various wastes in recent years. Among these wastes, textile wastes were predominant. Other wastes include paper, leather, metal, ice-cream, slaughterhouses and households [1]. Although the dam lake is exposed to apparent pollution due to the rapid development of local society and economy, only few studies have addressed the pollution. Heavy metal pollution of the lower Ceyhan River basin (Adana-Turkey) was studied [2]. Heavy metal contamination caused by substances such as nickel (Ni), cadmium (Cd), copper (Cu) and chromium (Cr) was also pointed out based on water samples from the Sır Dam Lake [3]. In addition to heavy metal pollution, surface water pollution in the upper and middle basin of the Ceyhan River was demonstrated by an analysis of 13 physico-chemical parameters on the surface water samples collected from 31 stations around the Sır Dam Lake. The results of that study indicated that particular samples from three stations (Sır 2, Sır 3, and Aksu 4) near the city of Kahramanmaras were extremely polluted by nutrients, Cl^- and Na^+ due to industrial and domestic wastewaters [1]. Microbial contamination was also proved through high fecal coliform counts in water samples collected from the Aksu River [4]. Although Turkey has around 26 river basins, most remain unstudied [5]. Another study addressed the occurrence of the multi-drug resistant Enterobacteria from rivers in the northern Turkey and concluded that studied rivers could serve as reservoirs for the antimicrobial resistance determinants in the environment [6].

Continuous environmental contamination by various pollutants affects native microbiota inhabiting certain environments [7]. Although this negatively influences native inhabitants, it might be advantageous for the adaptation of suitable organisms possessing favorable metabolic features. In fact, such organisms can be useful for bioremediation purposes. Therefore, the identification of bacterial distribution in polluted environments might be a crucial step for selection of bacteria with biodegradation abilities. Although specific metabolic activities of those bacteria is of vital importance for biodegradation purposes, other features such as low antibiotic resistance rates might be significant concerns for

public health since antibiotic resistant bacteria can act as a transmission tool for the emergence of new antibiotic resistant bacteria [8]. Therefore, antibiotic resistance along with the bacterial diversity from polluted environments must be monitored to determine the risk status for transmission of resistance determinants from environment to humans and animals. To this end, the present study aims to analyze Gram negative bacterial diversity and their antimicrobial susceptibility profiles in heavily polluted environmental samples (soil, sludge, water) around the Sır Dam Lake in Kahramanmaras, Turkey.

2. MATERIALS AND METHODS

2.1 Sample Collection

In March 2014, heavily polluted environmental samples (soil, sludge, water) were collected with a sterile cotton swab from 15 different locations (37°34 ′47.09″N–36°48 ′03.84″E; 37°35 ′05.19″N–36°47 ′02.98″E) in Avsar Region around the Sır Dam Lake which was approximately 1 km away from the Kahramanmaras Sutcu Imam University (Fig. 1). Subsequently, they were transferred into the sterile disposable culture tube containing 1 mL of sterile 1X Phosphate Buffered Saline (PBS) at pH 7.4. Buffer contained 137 mmol of NaCl, 2.7 mmol g of KCl, 10 mmol of $Na_2HPO_4.2H_2O$ and 2mmol of KH_2PO_4 per L. The collected samples were immediately taken to the laboratory for processing and bacteriological analysis.

2.2 Bacteriological Analyses

Bacterial inoculation was initially performed on Eosin-Methylene Blue (EMB) agar. Afterward, inoculated plates were incubated at 35℃ for 24 hours. Following the incubation, the plates were analyzed for selection of colonies with different morphological appearances from each sample. Then, selected colonies were re-streaked on fresh EMB agar for purity, and Gram-staining was performed. Gram negative pure bacterial isolates were then transferred into Tryptic Soy Broth (TSB) and grown at 35℃ for 24 hours. Those cultures were then used to prepare long-term bacterial stocks containing 15% of sterile glycerol at the final volume [9].

Fig. 1. The location of the Sır Dam Lake in Turkey is marked with square symbol. Sampling area was circled on the closer view of the map. GPS coordinates of the sampling area are 37°34 ′47.09″N–36°48 ′03.84″E; 37°35 ′05.19″N–36°47 ′02.98″E. (Satellite imagery: Google/Google Earth)

2.3 Phenotypic Identification of Gram Negative Bacterial Strains

Gram negative bacterial strains were identified based on biochemical tests including oxidase, triple sugar iron (TSI), indole, methyl red, Voges-Proskauer and citrate [10]. Biochemical identification of the bacterial isolates was further verified using CHROMagar ECC (France).

2.4 Molecular Identification of Isolates

Whole cellular DNA was prepared for PCR amplifications in accordance with the previously described procedure [11]. A fragment corresponding to 16S rRNA gene was amplified using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') universal primers [12] in accordance with the previously outlined procedure [9]. The PCR products were then cleaned and sequenced by the Refgen Company (Turkey). Sequences were stored in the NCBI database under accession numbers from KY029029 through KY029035. Nucleotide sequences were compared with those in the GenBank database using the BLAST search tool. Sequence identities of ≥98% and ≥92% were used as criteria for species or genus identification, respectively.

2.5 Phylogenetic Analysis

All 16S rDNA sequences including isolates and their closest relatives were aligned with Clustal W. Distance matrices were calculated with the Kimura-2-parameter algorithm [13]. A phylogenetic tree was constructed by the neighbor-joining method and evaluated by bootstrap sampling for 1.000 replicates using the MEGA 6.06 program [14]. *Staphylococcus aureus* (L37597) was used as an outgroup.

2.6 Antimicrobial Susceptibility Test

Antimicrobial susceptibilities of the isolates were determined with Kirby-Bauer Disk Diffusion Susceptibility Test protocol as described by Clinical and Laboratory Standards Institute (CLSI, 2013) [15]. Thirteen different antimicrobial containing disks (Oxoid, UK) were used for the testing susceptibilities of isolates. Antimicrobial agent concentrations on each disk were as follows: amoxicillin-clavulanic acid (30 µg), piperacillin-tazobactam (110 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefpodoxime (10 µg), ceftriaxone (30 µg), ertapenem (10 µg), imipenem (10 µg), meropenem (10 µg), aztreonam (30 µg), ciprofloxacin (5 µg), amikacin (30 µg) and trimethoprim-sulphamethoxazole (25 µg). *E. coli* ATCC 25922 was used as quality control strain. The results were evaluated in accordance with CLSI (2013) criteria [15].

2.6.1 Multiple antibiotic resistance index

The multiple antibiotic resistance (MAR) index is defined as a/b where a represents the number of antibiotics to which the strain is resistant, and b represents the number of antibiotics to which the strain is exposed [16].

2.7 Double Disk Synergy Test

ESBL producing isolates were detected with double disk synergy test using disks of following antimicrobial agents: amoxicillin/clavulanic acid (30 µg), aztreonam (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg) and cefpodoxime (10 µg) (Oxoid, UK) [15]. Briefly, inoculums from each isolate were prepared in sterile saline solutions and adjusted to a turbidity value of 0.5 McFarland. Then, the cultures were spread onto Mueller Hinton Agar (Merck) by a sterile cotton swab. Following to inoculation, amoxicillin/clavulanic acid disk was placed at the center and the others around it to make them 24 mm far from each other. Subsequently, they were incubated at 37ºC for 18-20 hours. A clear extension or protrusion (synergistic effect) in the edge of the inhibition zone of any of the antimicrobial disk towards the disk with amoxicillin/clavulanic acid was evaluated as positive for ESBL production. Three clinical isolates of ESBL-producing *E. coli*, harboring bla_{TEM}, bla_{SHV} and bla_{CTX-M} beta-lactamase genes, were used as control strains [17].

3. RESULTS AND DISCUSSION

3.1 Bacterial Diversity in Environmental Samples

A total of 17 Gram negative bacterial strains were isolated from 15 heavily polluted environmental samples in the present study. Based on the phenotypical identification results and color of colonies on CHROMagar ECC (France), the strains were grouped as *Escherichia coli* (n=8), non-fermenters (n=5) and *Enterobacteriaceae* (n=4). Phenotypical identification did not allow identification of isolates at either genus or species level in the non-fermenters and *Enterobacteriaceae* groups. However, molecular identification based on the analysis of 16S rRNA gene sequences with known sequences in the GenBank was found to

be more powerful in identification of most strains in those groups as shown in Table 1. Molecular identification resulted in more than 98% identity for all identified strains, except for the isolate (T1-4) that had only 92% identity to homologous sequences in the GenBank. Most of the matched sequences for this isolate were composed of *Enterobacter* spp. isolates, while only a small portion of them was composed of isolates of other genera such as *Raoultella* spp., *Lelliottia* spp. and *Pantoea* spp. Therefore, T1-4 isolate was identified as *Enterobacter* spp. This isolate was clustered with the single *Klebsiella oxytoca* isolate (YK2-4) in the phylogenetic tree that was constructed for all identified isolates (Fig. 2). Considering taxonomical relationships of *Klebsiella* and *Enterobacter* species based on the biochemical, physiological, and morphological characters as well as 16S rDNA and *rpoB* sequence analyses [18,19], their close placements in the phylogenetic tree were expected.

According to both phenotypic and molecular identification results, *E. coli* appeared as the most encountered species among all isolates in the present study, indicating a fecal bacterial contamination. It was detected from almost all types of environmental samples including three water, four soil and one sludge samples. This result overlapped the results of the previous studies conducted on water samples both from the Aksu River, which is the second main river feeding the Sır Dam Lake and different geographic area [4,20]. The detection of coliform bacteria >1100 PMN/100 mL in the water samples demonstrated the presence of fecal contamination in the Aksu River [4].

Following to *E. coli*, *Acidovorax temperans* was found as the second dominating species and was mainly isolated from sludge and soil samples in the present study. This result might suggest the potential role of *A. temperans* as an indication of pollution. The other Gram negative bacteria identified in low frequencies from the samples around the the Sır Dam Lake were as follow: *Acinetobacter* spp., *Enterobacter* spp. and *Klebsiella oxytoca*. No previous report is available on the identification of *Acidovorax temperans*, *Acinetobacter* spp. and *Enterobacter* spp. from the Dam Lake, which makes this result significant as the first one.

The *Acidovorax* genus is originally described as a new genus for three species*; Acidovorax facilis (*formerly *Pseudomonas facilis)*, *Acidovorax delafeldii (*for the former *Pseudomonas delufeldii*), and *Acidovorax temperans* (for several former *Pseudomonas* and *Alcaligenes* strains) [21]. Members of *Acidovorax* genus may contain commensal pathogen and plant pathogen species in addition to other species possessing diverse metabolic activities suitable for biodegradation process due to their ability to adapt to polluted environments. *A. temperans* detected in the present study was not tested for biodegradation potential; However, the emergence of this species in the present study might indicate the presence of preferable features for biodegradation purposes as predicted by earlier reports. In one of those reports, *Acidovorax* was detected as one of the dominant genus along with *Pseudomonas*, *Acinetobacter*, *Sphingomonas*, and *Thiobacillus* in mineral oil hydrocarbon contaminated soils [22]. In another study, *Acidovorax avenae* as well as other species such as *Acinetobacter radioresistence*, *Rhodococcus fasciens* and *Pseudomonas putida* were reported from soil samples adjacent to oil pomp. In addition, strong chemotaxis responses of most strains including *A. avenae* to various pollutants including crude oil, standard oil, quinoline, phenol and n-hexadecane were reported, suggesting their possible potential for biodegradation processes [23]. *A. avenae* was also isolated from a polluted aquifer and was shown to utilize 1,2-dichlorobenzene as the sole carbon source under aerobic conditions [24]. In addition to those studies, some other studies offered direct evidences to the potential roles of *Acidovorax* spp. in biodegradadion of aromatic hydrocarbons. Moreover, sequences of *Acidovorax* genus were primarily found in ^{13}C-labeled DNA fractions in a bioreactor for the treatment of polycyclic aromatic hydrocarbon (PAH)-contaminated soil following to the incubations of slurry with [U-^{13}C] phenanthrene [25]. Later, a phenanthrene-degrading *Acidovorax* sp. strain NA3 was further studied for the characterization of a polycyclic aromatic hydrocarbon degradation gene cluster [26]. Although those studies underlined the importance of *Acidovorax* spp. for biodegradation purposes, some others indicated that *A. temperans* and *A. delafieldii* isolated from shower heads in residences from a Korean city led to the deterioration of tap water quality in the household water supply system [27]. Moreover, *A. temperans* was reported to adhere readily to surfaces forming biofilms [28]. Bacterial biofilms were known to be associated with antimicrobial resistance by different mechanisms including poor antibiotic penetration, nutrient limitation,

slow growth and adaptive stress responses. Traditional resistance mechanisms such as chromosomal β-lactamase, upregulated efflux pumps and mutations in antibiotic target molecules in bacteria are also effective in biofilms [29].

Table 1. Molecular identification of isolates based on 16S rRNA gene sequence analysis

Isolates	Phylogenetic affiliation	Similarity%	GenBank accession numbers
Non-fermenters			
S2-4	*Acidovorax temperans*	99	KY029029
SC4-1	*Acidovorax temperans*	99	KY029030
YK2-3	*Acidovorax temperans*	99	KY029031
YK3-2	*Acidovorax temperans*	99	KY029032
H4-4	*Acinetobacter* spp.	98	KY029034
Enterobacteriaceae			
T1-4	*Enterobacter* spp.	92	KY029033
YK2-4	*Klebsiella oxytoca*	98	KY029035
H3-4	Unidentified	-	
S1-5	Unidentified	-	

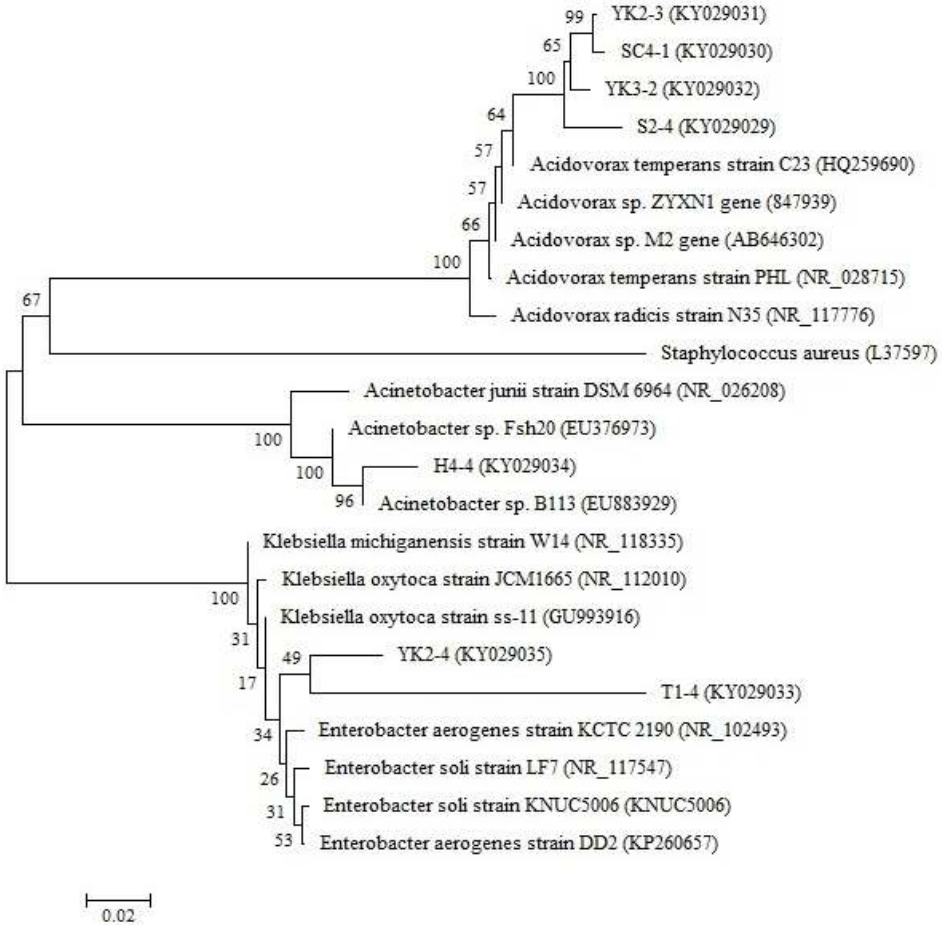

Fig. 2. Phylogenetic tree showing relative positions of environmental isolates YK2-4, S2-4, SC4-1, YK2-3, YK3-2, T1-4 as inferred by the neighbor-joining method of partial 16S rDNA sequences

Accession numbers of closely related species are given in parentheses. Staphylococcus aureus was used as an outgroup. Bootstrap values for a total of 1.000 replicates are indicated at the nodes. The scale bar indicates a inferred nucleic acid change of 2% per position

K. oxytoca is another identified strain in the present study. Similar to other *Klebsiella* spp., *K. oxytoca* can be found in a wide range of environments and causes hospital-acquired infections as an opportunistic pathogen [30]. The presence of multiple antibiotic resistance feature of this species was also indicated in many studies [31,32,33]. It was also significant in geochemistry and industrial microbiology for biomineralization of iron salt solutions with the production of ferrihydrite nanoparticles [34]. The ferrihydrite nanoparticles produced by *K. oxytoca* were shown to possess two fractions of biogenic ferrihydrite with different levels of magnetic susceptibility. *K. oxytoca* is also known to breakdown cyanide, a chemical extremely toxic to humans [35]. This species was also shown to be useful in removing cyanide existed in wastewater, thus offering a more affordable and non-hazardous alternative compared to current water treatment methods (e.g. alkaline chlorination and wet-air oxidation).

3.2 Antimicrobial Resistance Profiles of Environmental Isolates

Antibiotic resistance profiles of all isolates are given in Table 2. The results indicate that antibiotic resistance was more common among *E. coli* isolates compared to other identified strains. Moreover, amoxicillin-clavulanic acid resistant phenotype was commonly found (37.5%) in *E. coli* isolates, which overlaps the study conducted on *E. coli* strains isolated from municipal and hospital wastewaters in France [36]. A single isolate (SC4-2) displaying multiple antibiotic resistances was resistant to six antimicrobials, including amoxicillin-clavulanic acid, cefotaxime, ceftazidime, ceftriaxone, cefpodoxime and trimethoprim-sulphamethoxazole. This isolate was also phenotypically detected as Extended-Spectrum β-Lactamase (ESBL)-producer with double disk synergy test (Fig. 3), and no other organism was detected as ESBL-producer in the present study. Therefore, the detection rate of ESBL-producing *E. coli* was 12.5%, compared to a previous study which found remarkable β-lactamase production (42.2%) among *E. coli* strains isolated from Aksu River [4]. Three *E. coli* isolates that were susceptible to cephalosporins were resistant to several carbapenems, including imipenem, meropenem and ertapenem in the present study. Moreover, a single isolate was resistant to cefotaxime. In contrast to our results, none of the *E. coli* isolates recovered from Aksu River was resistant to cefotaxime [4]. Moreover, similar to the results of the study mentioned above, carbapenem and cefotaxime resistances did not co-exist in any of *E. coli* isolates in the present study. This result might be useful for controlling infections caused by carpapenem resistant bacteria alone or vice versa since carbapenems and cephalosporins are two main group of antimicrobial agents with interchangeable usages.

MAR index is a general criterion for determining the risk status of bacteria when more than three antibiotic resistances are apparent. MAR indices higher than 0.2 generally indicates high risk [37]. Considering the most resistant species observed in the two groups, namely non-fermenters (n=5) and *Enterobacteriaceae*, a single *Acinetobacter* spp. isolate and a single *Klebsiella oxytoca* had high MAR values (0.3 and 0.5, respectively). It must be noted that all *A. temperans* isolates were susceptible to all tested antibiotics (Table 2). In contrast to our findings, *A. avenae* strains isolated from soil sample nearby oil pump was resistant to aztreonam, cefoperazon, gentamicin and netilmicin [23]. MAR indices above 0.2 were detected at a rate of 63% among *E. coli* isolates in the present study, thus overlapping the results of the study in which *E. coli* was used as the fecal contamination indication in various water ecosystems [36,38].

Many studies demonstrated that using a consortium composed of several bacteria offered advantages compared to a single bacterium containing inoculum for the biodegradation processes of certain contaminants [39-41]. For instance, species from different genus such as *Stenotrophomonas*, *Bacterium*, *Klebsiella*, *Bacillus*, *gamma proteobacterium*, *Citrobacter* and *Raoultella* were shown to exist within PAH-degrading bacterial consortia [39] and among them, *Bacterium*, *Citrobacter* and *Bacillus* are well-known degraders of PAHs in soil. It is also evident that bacterial species near polluted sites must be identified for the most efficient degradation [39]. This might be attributed to the fact that bacterial diversity was highly affected by the type of contaminant. In this respect, bacterial species adjacent to polluted sites should be identified in the first place compared to the bacterial species obtained from unrelated environment to specific pollution sites. Therefore, *Acidovorax* strains identified in this study might be used as either a member of consortium or alone for biodegradation purposes after determining their metabolic potentials on certain types of pollutant. Moreover, their low antibiotic resistance to most β-lactams might provide an additional benefit for public health.

Table 2. Antimicrobial resistance profiles of isolates

Isolates	Sample	Antimicrobial[a] resistance phenoype	MAR[b] Index	ESBL[c]
E. coli				
S1-1	Water	AMC	0.1	-
S2-2	Water	-	0	-
S3-2	Water	AMC	0.1	-
SC4-2	Sludge	AMC, CTX, CAZ, CRO,CPD, SXT	0.5	+
YK1-3	Soil	ATM, CPD	0.2	-
YK2-2	Soil	IPM, MEM, ETP	0.2	-
YK3-3	Soil	IPM, MEM, ETP	0.2	-
H2-3	Soil	IPM, MEM, ETP	0.2	-
Non-Fermenters				-
S2-4	Water	-	0	-
SC4-1	Sludge	-	0	-
YK2-3	Soil	-	0	-
YK3-2	Soil	-	0	-
H4-4	Soil	CTX, CAZ, CRO, ETP	0.3	-
Enterobacteriaceae				-
T1-4	Soil	-	0	-
YK2-4	Soil	AMC, ATM, CTX, CAZ, CRO,CPD	0.5	-
H3-4	Soil	-	0	-
S1-5	Water	-	0	-

[a] *AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CAZ, ceftazidime; CTX, cefotaxime; CPD, cefpodoxime; CRO, ceftriaxone; ETP, ertapenem; IPM, imipenem; MEM, meropenem; ATM, aztreonam; CIP, ciprofloxacin; AK, amikacin; SXT, trimethoprim-sulphamethoxazole*

[b] *Values of Multiple Antibiotic Resistance (MAR) index*

[c] *Presence or absence of Extended-Spectrum β- Lactamase (ESBL)*

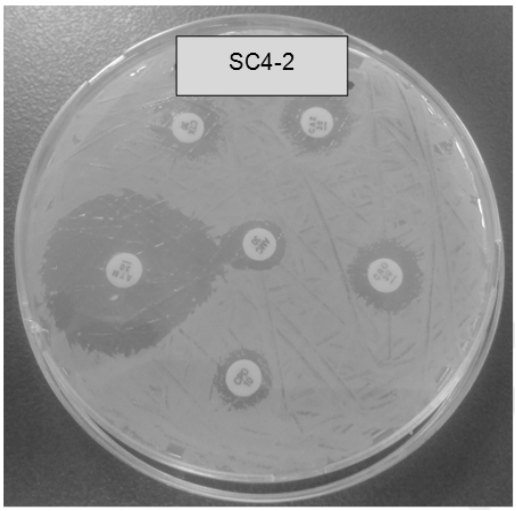

Fig. 3. ESBL producing isolate selected by double disk synergy test

4. CONCLUSION

E. coli was the most abundant organism detected in the present study, indicating the presence of fecal contamination in the Sır Dam Lake. In addition, most of *E. coli* isolates with a single *K. oxytoca* isolate and a single *Acinetobacter* spp. isolate displayed highest multiple antimicrobial resistance (MAR) index values that should be considered seriously for local public health. *A. temperans* occurred as the second predominant bacteria and did not have significant resistance to tested antimicrobials. Therefore, its presence might indicate presence of different types of pollution in heavily polluted environmental sites. Moreover, they could serve in biodegradation process after their metabolic capabilities are explored. At this point, the selection of bacterial species with low antibiotic resistance in addition to high metabolic capacities might be beneficial for protecting public health from transmission of antibiotic resistance during bioremediation processes.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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