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Original Article

Tracking of chloramphenicol, erythromycin, and sulfamethoxazole antibiotic-resistant bacteria from untreated wastewater effluents to receiving river

Rahim Aali[1,2] , Sepideh Baragh[3] , Esrafil Asgari[4] , Reza Fouladi Fard[2] , Hassan Izanloo[2] , Saeed Hosseinpoor[5], Jalhe Bagheri Hamzyan Olia[6] , Roya Naseri[3] , Mohsen Mehdipour Rabori[7*]

[1]Student Research Committee, Urmia University of Medical Sciences, Urmia, Iran

[2]Research Center for Environmental Pollutants, Qom University of Medical Sciences, Qom, Iran

[3]Environmental Health Engineering, Student Research Committee, Urmia University of Medical Sciences, Urmia, Iran

[4]Environmental Health Engineering, Department of Environmental Health, School of Health, Khoy University of Medical Sciences, Khoy, Iran

[5]Environmental Health Engineering, Department of Environmental Health, School of Health, Urmia University of Medical Sciences, Urmia, Iran

[6]Biochemistry Ph.D, Khoy University of Medical Sciences, Khoy, Iran

[7]Environmental Health Engineering Research Center, Kerman University of Medical Sciences, Kerman, Iran and Department of Environmental Health, School of Public Health, Kerman University of Medical Sciences, Kerman, Iran

Abstract

Background: The aim of this study was to investigate the frequency and type of bacteria resistant to chloramphenicol (CHL), erythromycin (E), and sulfamethoxazole (SXT) antibiotics from untreated wastewater effluents to receiving river.

Methods: In total, 32 samples were taken from eight sites located in the raw wastewater to the downstream of the receiving Ghotor river in Khoy city. Resistant microorganisms were studied through modified HPC method and CLSI standards. Different and specific colonies were selected and re-cultured in R2A culture medium and enough colonies were used for DNA extraction. Bacterial 16sr RNA target gene was amplified and sequenced. *Pseudomonas sp.*, *Comamonas sp.*, and *Thiobacillus sp.* were predominant bacterial species identified in water samples. Also, antibiotic-resistant genes (ARGs) including *sul*1, *erm*B, and *cml*A1 were tracked by PCR.

Results: The average total number of antibiotic-resistant bacteria (ARB) increased in the downstream (2.4×10^2 to 2.6×10^4 CFU/100 mL). The comparison of the results related to the river's upstream and downstream also indicated a significant difference between ARB and ARGs contents ($P < 0.05$). The average number of bacteria resistant to SXT, E, and CHL antibiotics was obtained to be 2.3×10^4, 2.3×10^4, and 3.4×10^4 CFU/mL, respectively. Finally, the evaluation of water revealed that only the aeration lagoon was able to decline the number of CHL-resistant bacteria (88.9%) while this process increased the number of SXT- (51%) and E- (16%) resistant bacteria. The lowest and highest percentages of the identified genes were related to *erm*B (12.5%) and *sul*1 (81.25%), respectively.

Conclusion: According to the results, wastewaters play an important role in releasing ARB and their antibiotic resistance genes to downstream of Ghotor rivers in Khoy city.

Keywords: Heterotrophic bacteria, Sulfamethoxazole, Chloramphenicol, Erythromycin, Polymerase chain reaction, Wastewater, Rivers

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*Correspondence to:
Mohsen Mehdipour Rabori
Email: mmehdipoor@yahoo.com

Introduction

Resistance to all antibiotic groups have been increasingly reported in the environment (1). Studies have shown that the high levels of antibiotic resistance in environmental microorganisms are due to human activities. This phenomenon resulted in the release of the antibiotic resistance genes (ARGs) into the water resources, such as rivers and lakes, and also soil resources (2).

Antibiotic-resistant microorganisms transmit a wide spectrum of coding genes to the native microbial'

population via their genetic components including plasmids, integrons, and transposons (3).

The horizontal transmission of antibiotic resistance genes is the main way to gene transmission in aquatic systems and most of the microorganisms in water resources acquire multiple resistance (3).

Municipal wastewater is one of the important sources of antibiotic-resistant bacteria (ARB) and their ARGs. Investigations have demonstrated that wastewaters are the most frequent source of disseminating such contaminants, even after wastewater treatment. Moreover, some studies have shown that wastewater treatment plants increase the level of these contaminants. Some researchers have attributed the observed increase to the promotion of resistance factor and high microbial and nutrition contents in wastewaters (4,5).

This issue is very important since the effluents of wastewater treatment plants, directly or indirectly, are used for irrigation. Therefore, there is a great concern about the release of ARB to farmlands and pollution of the soils, as well as the emission of ARB and ARGs to drinking waters. The presence of ARB and ARGs in drinking water and water distribution systems have been confirmed. It is suggested that water treatment processes and water distribution systems can increase the number of ARB and ARGs. Although there is a serious concern about antibiotic resistance in the environment, many aspects of this issue need to be more studied and discussed in details, including the dissemination of these contaminants from wastewater to water resources, the behavior of different microorganisms, various ARBs and encoding ARGs, and the effect of environmental parameters (6).

Antibiotic consumption in Iran is higher than the global standard levels, subsequently, there is a high level of antibiotic resistance in Iran (4). In this country, many of hospitals lack appropriate wastewater treatment units and hospital sewage directly enter the municipal network. This situation intensify the presence of ARB and ARGs in the environmental sources (4).

The Iranian government has started using treated wastewaters for irrigating and feeding groundwater resources due to the severe water scarcity in Iran. This study was performed to investigate the ARB and ARGs from untreated municipal wastewater to wastewater effluent and entering the downstream of suburban Ghotor river in Khoy city. The antibiotics examined in this study include chloramphenicol (CHL), erythromycin (E), and sulfamethoxazole (SXT). In addition, this research is one of the first studies that evaluated the performance of partial aeration lagoons in removal/reduction of the bacteria and ARGs.

Materials and Methods

Selection of the sampling sites and points

To monitor ARB and ARGs, eight sampling points from the untreated wastewater site to the downstream of the receiving river were considered (Figure 1). The wastewater was disposed into the river after being treated in a partial aeration lagoon. The studied wastewater treatment plant is located at geographical location of 38°32'35.7"N 45°00'17.4"E, treating the wastewater produced by 400 000 people. Conventional wastewater parameters such as temperature (2550 A), pH (4500-H), TSS (2540 A), and BOD (5210 A) were evaluated based on the standard methods for the examination of water and wastewater (SMEWW, 22nd edition), respectively. The effluent of this wastewater treatment plant was disposed to a river at a 1 km distance. In downstream, water is used for farming and industrial activities. In total, 32 samples were taken and transferred to an environmental microbiology laboratory under the cold chain conservation.

Antibiotic resistance test

The data indicate 3 repeated experiments. The susceptibility was measured according to CLSI protocol (2016).

The antibiotic resistance of the samples was examined by a modified HPC method. In this method, R2A agar culture medium was used. The concentration of antibiotics applied on the plates was determined based on the CLSI standards (2007) as 30 and 15 μg/mL for CHL and ER, respectively. 50.4 μg/mL was used for SXT on the basis of previous study (7,8). At first, samples were well mixed, and then, the untreated wastewater samples were diluted to 10^{-3}. 0.1 mL of each diluted solution (3 diluted concentration for each sample) was transferred to the nutrient agar culture plates. The plates were incubated at 37°C for 2 days, and then, for 5 days. Afterwards, the grown ARB colonies were counted by a colony counter and their average numbers were calculated in CFU/100 mL.

Isolation and identification of resistant bacteria

Specific and different colonies were selected and re-cultured on R2A agar culture medium. Enough pure

Figure 1. Location of study area and sampling points (Wastewater of Khoy city in west Azerbaijan and Ghotor river).

colonies were used for DNA extraction (9). Genomic DNA was extracted, according to the manufacturer's instructions of the extraction kit (Bioneer, Korea). In summary, a definite volume of the main sample (50 mL) was centrifuged at 6000 rpm for 10 minutes. The supernatant was removed, and 300 µL distilled water was added to the precipitate. Then, DNA extraction was conducted on the Column kit protocol. In the first step, lysis component was added. Protein precipitation was obtained in the second step, and in the third step, DNA was precipitated and extracted. Concentration of the extracted DNA was determined by a spectrophotometer (NanoDrop, Thermo Scientific, USA). Bacterial 16s rRNA target gene was amplified by the universal primer pairs (27F (forward): AGAGTTTGATCCTGGCTCAG, (reverse) 1492R1: TACGGYTACCTTGTTACGACTT) (9). PCR products (30 µL) were sequenced and compared using the Ribosomal Database Project (RDP), GenBank, and NCBI database. Sequence similarities above 80%-100% were identified for the species. Analysis of sequences and drawing the phylogenetic tree were performed in Chromas and Mega 7 software.

Detection of ARGs

To distinguish the encoding ARGs, several specific primers were employed (10). The utilized primer pairs are reported in Table 1. The PCR programs of initial denaturation (94°C, 10 minutes), denaturation (94°C, 45 seconds), annealing (varied temperature, 30 seconds), extension (72°C, 5 seconds), and final extension (72°C, 10 minutes) were utilized. Denatuartion to extention steps was repeated for 30 cycles. The PCR products were investigated by an electrophoresis system with horizontal tank and a transilluminator.

Results

Untreated municipal wastewater is one of the most important sources of transmission of ARB and ARG to the environment. Aeration lagoons has been introduced as one of the effective methods for removal/reduction of pollutants in municipal wastewaters. In this study, the samples were taken from a site located before the treatment plant, different units of the treatment plant, disposal site of the treated wastewater to Ghotor river, and the downstream of the Ghotor river stream. The average of temperature, pH, TSS, and BOD of the wastewater was

obtained to be 23°C, 7.9, 313, and 375 mg/L, respectively. The results showed a high level of antibiotic resistance in the samples with a mean value of 6.8×10^3 CFU/100 mL. More specifically, the observed antibiotic resistance was detected as 2.4×10^2 to 2.6×10^4 CFU/100 mL.

Therefore, it is indicated that antibiotic resistance against different groups of antibiotics did not follow the same pattern. Antibiotic resistance pattern is illustrated in Figure 2.

As shown in Figure 2, antibiotic resistance increased from the first sampling point towards the last point of the treatment plant, and then, reduced slightly towards the downstream of river. This trend is not followed by SXT. In the case of SXT, antibiotic resistance improved after the treatment plant at the disposal site. Furthermore, the
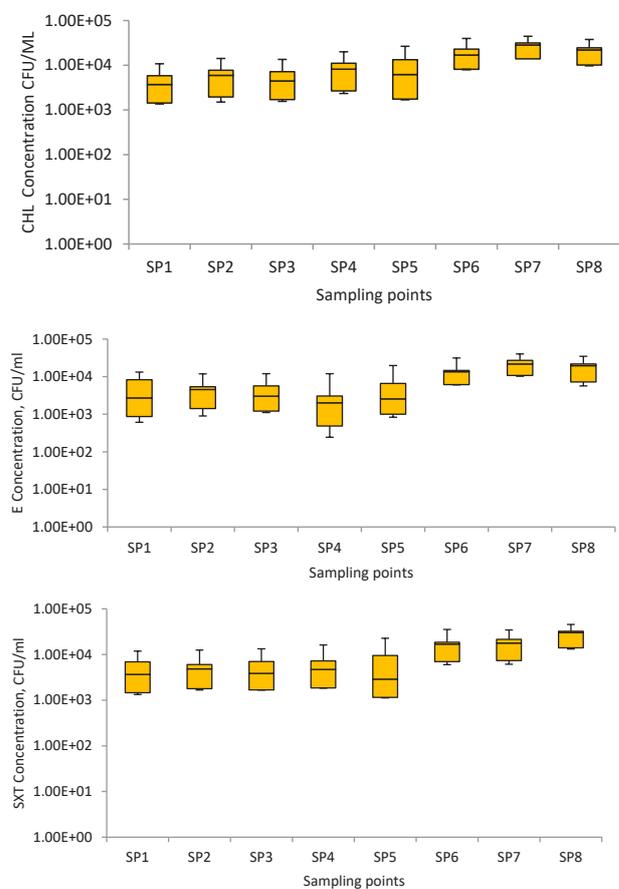
Figure 2. Average Log of ARB related to HPC, SXT, E, and CHL; SXT= sulfamethoxazole, E= erythromycin, CHL= chloramphenicol.

Table 1. Primer sequences and target genes

Genes	Sequences	Annealing Temperature	Amplified Size (bp)	Ref.
Sul1	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	55.9	163	(8,11,12)
ErmB	AAAACTTACCCGCCATACCA TTTGGCGTGTTTCATTGCTT	60	193	(13,14)
CmlA1	TAGTTGGCGGTACTCCCTTG GAATTGTGCTCGCTGTCGTA	61	137	(14,15)

resistance against the three tested antibacterial agents enhanced after the treatment lagoon and the river's downstream. The identified ARGs are depicted in Figure 3. The lowest percentage of the identified genes is related to the SP8 treatment unit and the highest levels were related to the SP1, SP4, and SP6 units (Figure 4). The lowest and highest average percentages of the identified genes were related to *erm*B (12.5%) and *sul*1 (81.25%), respectively. The cumulative percentages of the ARGs are presented in Figure 4.

Diversity of ARB to different antibiotics is shown in Table 2. As shown in this table, the resistance bacteria were different against antibiotics. On the basis of the phylogenetic relationships of the total bacterial species in Figure 5, *Comamonas sp., Pseudacidovorax sp., Pseudomonas sp., Brevundimonas sp., Thiobacillus sp.*, and *Collimonas sp.* were dominant in the isolated colony resistant to the selected antibiotics (Table 2).

Discussion

The dissemination of ARB and ARGs to the environmental resources has turned to a serious global challenge in recent years. Different studies have reported different results about the emission of such contaminants into the environment. However, the common issue reported by all studies is that the emission of ARB and ARGs into the environment can pose serious negative impacts on the environment and human health.

In this study, the analysis of ARB and ARGs that are resistant against three antibacterial groups of E, SXT, and CHL showed that ARB existed in the untreated wastewater, effluent of the wastewater treatment plant, and the downstream of the river (the average ARB in river downstream was 6.8×10^3 CFU/100 mL). This finding is consistent with the results of other studies (8,16-18). The ARB variations ranged from 2.4×10^2 to 2.6×10^4 CFU/100 mL, which is consistent with the results of other studies by Munir et al (8) and Aali et al (4). The average ARB reported by these studies for untreated and treated (effluent) wastewaters has been about $10^{3.9}$ to $10^{5.96}$ and $10^{1.16}$ to $10^{3.14}$ CFU/100 mL, respectively (8,19). On the other hand, the obtained range of ARB in this study (Figure 2) is lower than what reported by some other studies such as the study of Aali et al, which was conducted in Isfahan, Iran. The higher level of ARB in this study, compared with the results of the study of Aali et al, can be due to the differences in the type and quality of the wastewater studied, texture of the regions, and the treatment processes. Particularly, the wastewater treatment plant of north Isfahan is located in a region that is involved with considerable agricultural and animal husbandry activities and contains various workshops. Consequently, regional factors might affect the ARB dissemination (20).

The findings of this study revealed that not only the wastewater treatment procedure could not reduce the level of ARB, but also it increased the ARB content. Specifically, after the SP6 disinfection stage, ARB was significantly increased. Additionally, after this stage, a significant level of ARB was detected in the wastewater treatment effluent of SP7. Such observations have also been reported by other studies (8,21,22). The Pearson correlation coefficient also confirmed a significant difference in the ARB content of the SP1 and SP6 wastewater effluents.

Lin et al investigated chlorination for disinfection of wastewater and reported its variable effects on the removal/decrease of ARB and ARGs (21), whereas a study by Munir et al showed that disinfection by chlorination can have an insignificant impact on the ARB reduction (8). On the other hand, Al-Jassim et al demonstrated that the pattern and bacterial diversity alters after disinfection (22). They reported that *Pseudomonas aeruginosa* was abundant in the tested untreated wastewater, which was replaced by other *Pseudomonas* species after the disinfection unit. For this reason, some researchers believe that the assessment
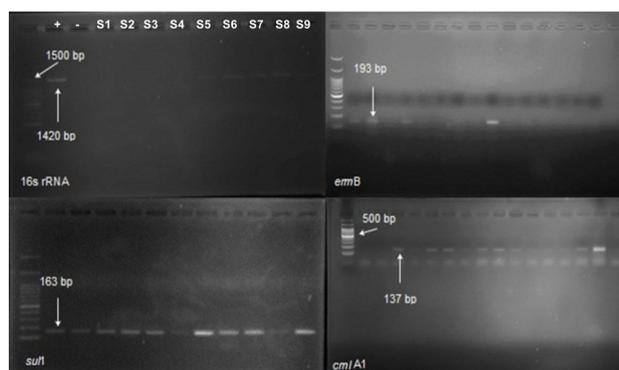

Figure 3. Agarose gel electrophoresis of the PCR results.

Table 2. Type and feature of the isolated colony, dominant bacteria, similarity and accession number of ARB in this study

| Resistant to Selected Antibiotics	Type of Colony	Bacteria Name	Query Cover (%)	Identification (%)	Accession Number
Sulfamethoxazole	Uniform mucoid, whitish	*Comamonas sp.*	88	91.89	NR_148635.1
	Mucoid and small	*Pseudacidovorax sp.*	88	92.42	NR_044241.1
Erythromycin	Circular and Simi yellow	*Pseudomonas sp.*	94	95.6	AF331664.1
	Circular, translucent and whitish	*Brevundimonas sp.*	94	95.42	JX196601.1
Chloramphenicol	Conventional small shape and clear edge	*Thiobacillus sp.*	81	72.05	NR_117560.1
	Flat and whitish	*Collimonas sp.*	88	71.9	NR_042824.1
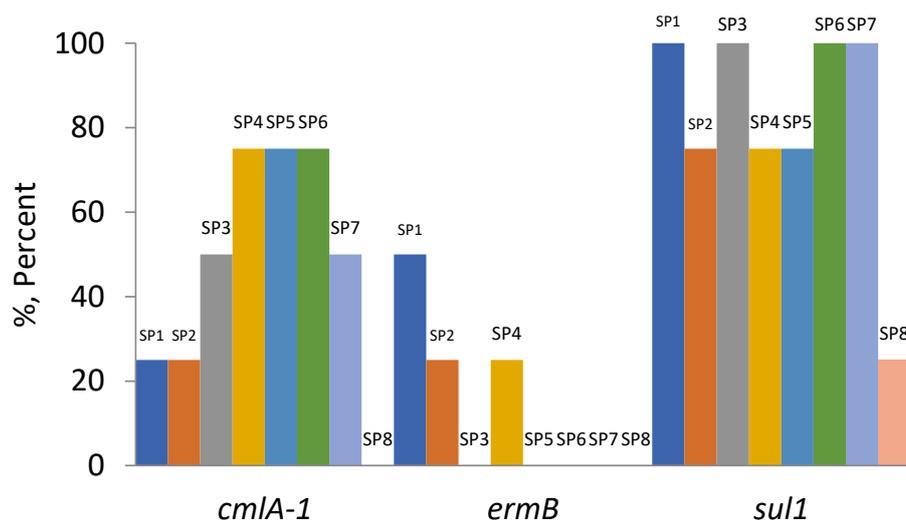
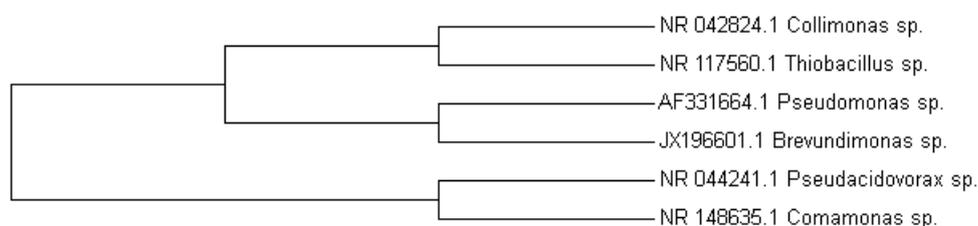**Figure 4.** The ARGs cumulative percentage.

Figure 5. Type of bacteria and its similarity detected in the studied river by neighbor-joining phylogenetic tree. It shows the 16S rRNA gene sequences of bacteria isolated from the ARB colonies.

of the extent of disinfection influence should be revised technically and scientifically. Indeed, the application of chlorination alone does not seem sufficient for controlling or eliminating ARB. Therefore, it is suggested to use complementary, combinatorial, and efficiency promoting processes (23).

Besides, Iranian researchers have investigated the effect of chlorination unit on ARB and reported an increase in the ARB content of chlorinated wastewaters (4,24). This finding is consistent with the results of other studies that identified wastewater treatment plants as one of the most important point sources for the release of antibiotics, ARB, and ARGs into the environment (25-29). In addition, the analysis of the wastewater treatment effluents demonstrated that although the treatment processes up to the SP6 unit could slightly influence ARB, but the aeration wastewater treatment could not significantly influence the ARB removal.

Some studies have shown that wastewater treatment procedures increase ARB and ARGs through applying selective pressure on the available microorganisms (30). The present study confirms that ARB can increase in wastewater treatment process (Figure 2). However, the increase cannot be generalized to all wastewater treatment

processes, and the type of involved treatment, unit design and operation can be very effective on the resultant ARB behavior (16,31). Consequently, the appropriate management of wastewater treatment processes is crucial for the control and removal of ARB, and the processes should be devised so that it is compatible with such purposes. Otherwise, the treatment processes would lead to sustainability and ARB increase, and may develop resistance in opportunistic pathogens (8). Due to the importance and hazardous effects of ARB on the environment and human health, researchers have suggested to consider environmental ARB as an environmental contaminant (32).

In this study, the trend of changes in the downstream showed that ARB varied after wastewater disposal into the river. Thus, the resistance against CHL decreased while resistance against ER and SMX increased at the downstream. Meanwhile, the highest level of resistance in the untreated wastewater was observed in CHL. Similarly, Akiyama and Savin reported a change in resistance at 240 to 2000 m distance from the studied river downstream, and attributed the observed variation to the existing chemicals compounds (33). They also reported that the ecological characteristics of the river can alter resistance

considerably, in addition to the chemical properties of the river and wastewater. Similarly, Marti et al investigated the impact of the effluent of Ripoll wastewater treatment plant on the transfer of ARB and ARGs to the Ter river and reported a significant increase in ARB and ARGs in the downstream (34).

The noticeable point is that the hazard of ARB contaminant is not limited to rivers, and they can contaminate the farmlands that are irrigated by the contaminated water. In this regard, Sun et al reported contamination of farmlands by ARGs, e.g. *sul*1. Specifically, the presence of ARGs is more hazardous for fruits (35). The reason is that the detected level of ARB in farmlands is higher than that in the receiving water resources (8). Therefore, the entrance of high concentrations of ARB and different types of ARB such as *Comamonas sp.*, *Pseudacidovorax sp.*, *Pseudomonas sp.*, *Brevundimonas sp.*, *Thiobacillus sp.*, and *Collimonas sp.* (Table 2 and Figure 5) to the environment can put pressure on water resources and natural ecosystems (36). Shakibaie et al isolated *Pseudomonas aeruginosa* and *E. coli* from lake waters and reported successful transfer of plasmid from ARGs from the effluent of factory to plasmid less antibiotic sensitive bacteria isolated from lake (3). Unfortunately, some studies have reported that ARB enters water treatment plants via water resources, and then, diffuses into the distribution network of drinking water due to the inefficiency of water treatment processes. Hence, some studies have exhibited the abundances of ARB in drinking water (6,37).

Among the ARGs studied in this research, *sul*1 had the highest percentage of coding ARGs (58%). According to the results of this study, the *sul*1 gene was more abundant in the influent and effluent wastewater of the wastewater treatment plant and the downstream. Consistent with the results of this study, Munir et al (8) and Aali et al (4) also identified the *sul*1 gene as the most abundant gene in wastewater treatment plants.

The *sul*1 gene can be found in many environmental bacteria (38,39). This gene is one of the segments of class 1 integrons and can be transited horizontally among different bacterial species in wastewater and other aquatic environments (40,41). The abundance of this gene can be due to high abundance of genetic elements in wastewater and the specific role of class 1 integrons.

In this study, the case wastewater treatment method was aeration lagoon. Some researchers have ascribed the decrease of ARB and ARGs due to solar radiation during this process. An evaluation by Sinton et al on the effect of solar radiation on the antibiotic resistance in gastric bacteria showed that solar-radiated in aeration lagoons may increase the studied ARB colonies (42), which is consistent with the results of a study by Mezrioui and Echab (43). In another study, Rizzo et al attributed the increased number of *E. coli* strains in aerated lagoons to suitability of aeration treatments for their colonization (23). Moreover, they found an increase in the ARB and ARGs and reported that the fluctuations in the quantity of ARB and ARGs could not follow a similar pattern. They attributed the reason to the diversity of antibiotic-resistant microorganisms and their different gene carriers, transmission and dissemination pathways. Besides, they reported that wastewater complexity and environmental factors influence ARB and ARG trends (5).

Conclusion
The ARB and ARGs release from effluent municipal wastewaters treatment plants to receiving water resources. In fact, water resources, e.g. rivers, deliver an important portion of ARB and ARGs and support their development. Since the downstream of contaminated rivers are employed for irrigation and drinking, therefore, it is more likely that ARB and ARGs be transferred to drinking water resources.

Ethical issues
The authors certify that all data collected during the study is presented in this manuscript, and no data from the study has been or will be published separately.

Competing interests
The authors have declared that they have no conflict of interests.

Authors' contribution
All authors contributed in the study design, data collection, analysis, and interpretation. All authors reviewed and approved the manuscript.

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