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Tracking antibiotic resistance genes and class 1 integrons in *Escherichia coli* isolates from wastewater and agricultural fields

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ABSTRACT

Considering high concentrations of multidrug-resistant bacteria and antibiotic resistance genes (ARGs) in wastewater, agricultural reuse of treated wastewater may be a public health threat due to ARG dissemination in different environmental compartments, including soil and edible parts of crops. We investigated the presence of antibiotic-resistant *Escherichia coli* as an indicator bacterium from secondary treated wastewater (STWW), water- or wastewater-irrigated soil and crop samples. ARGs including $bla_{CTX\text{-}m\text{-}32}$, $bla_{OXA\text{-}23}$, *tet*-W, *sul*1, *cml*-A, *erm*-B, along with *intI*1 gene in *E. coli* isolates were detected via molecular methods. The most prevalent ARGs in 78 *E. coli* isolates were *sul*1 (42%), followed by $bla_{CTX\text{-}m\text{-}32}$ (19%), and *erm*-B (17%). *IntI*1 as a class 1 integrons gene was detected in 46% of the isolates. *Cml*-A was detected in STWW isolates but no *E. coli* isolate from wastewater-irrigated soil and crop samples contained this gene. The results also showed no detection of *E. coli* in water-irrigated soil and crop samples. Statistical analysis showed a correlation between *sul*1 and *cml*-A with *intI*1. The results suggest that agricultural reuse of wastewater may contribute to the transmission of antibiotic-resistant bacteria to soil and crop. Further research is needed to determine the potential risk of ARB associated with the consumption of wastewater-irrigated crops.

Key words: antibiotic resistance, *Escherichia coli*, integrons, wastewater irrigation

HIGHLIGHTS

- Antibiotic-resistant *E. coli* presented different abundance in STWW, irrigated soil and crops.
- Antibiotic resistance genes were detected in high numbers of *E. coli* isolates.
- *sul*1 was the most abundant ARG in the *E. coli* isolates.
- Wastewater irrigation could aggravate antibiotic resistance in soil and crops.

INTRODUCTION

In recent decades, the excessive use of antibiotics as therapeutic drugs in humans, animals, and plants has caused selective pressure on bacterial populations, leading to the dissemination of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the environment (Osińska *et al.* 2017; Yuan *et al.* 2020). As a result, World Health Organization (WHO) established antibiotic resistance (AR) as a critical global health concern of the 21st century (Campo *et al.* 2020; Yuan *et al.* 2020).

ARB and ARGs are mainly spread by various environmental compartments such as wastewater treatment plant (WWTP) effluents (Osińska *et al.* 2017), surface water (Koczura *et al.* 2012), livestock manure (Yuan *et al.* 2020), and agricultural environment (Bougnom *et al.* 2020). Wastewater as a nutrient-rich environment with a high microbial concentration and diversity provides favorite conditions for horizontal gene transfer (HGT) among bacteria (Osińska *et al.* 2017; Campo *et al.* 2020).

As communities are increasingly facing with water crisis, agricultural irrigation becomes a key point challenge, especially in arid and semi-arid areas. Therefore, wastewater reuse for agricultural activities is considered worldwide (Farhadkhani *et al.*

2018). Considering high concentrations of ARB and ARGs in the effluent of WWTPs, agricultural reuse of secondary treated wastewater (STWW) could considerably spread antibiotics, ARB and ARGs into the environment (Cerqueira *et al.* 2019a; Kumar *et al.* 2020). This may lead to an elevated resistance level in microbiome of soil and crops (Cerqueira*et al.* 2019a, 2019b), thus threatening human and animal health. In other words, these environments turn into hotspots for ARB, ARGs, and mobile genetic elements (MGEs) (Yuan *et al.* 2020).

The increase in AR is facilitated by the association of ARGs with a variety of MGEs (Osińska *et al.* 2017). Integrons, as one of the genetic elements involved in the environmental spread of AR are found in MGEs such as plasmids and transposons, which promote their spread within bacterial communities (Cerqueira *et al.* 2019a). The common types of integrons which confer resistance to antibiotics including β-lactam antibiotics, chloramphenicol, sulfonamides, aminoglycoside, spectinomycin and others (Su *et al.* 2012) are class 1 integrons. This type of integrons are widely distributed in ARB, especially in Gram-negative bacteria (Osińska *et al.* 2017).

In this context, the presence of Gram-negative bacteria including Enterobacteriaceae members such as *Escherichia coli* in the environment as important vectors of ARGs, may pose a threat to human health (Cerqueira *et al.* 2019a). *E. coli* is a commensal bacterium which lives in the gut of humans and warm-blooded animals and could widely spread in different natural environments via feces or treated wastewater (Osińska *et al.* 2017). Furthermore, some pathogenic strains of *E. coli* can cause infectious diseases in animals and humans, such as urinary tract infections, diarrhea, and septicemia (Araújo *et al.* 2017).

It seems that *E. coli*, as an important indicator of fecal contamination, has an important role in the transfer of ARGs to pathogens (Holvoet *et al.* 2013; Araújo *et al.* 2017; Osińska *et al.* 2017). Therefore, the frequency of antibiotic-resistant *E. coli* in the environment can be used as a good estimate of the prevalence of other resistant pathogenic bacteria such as *Salmonella* (Holvoet *et al.* 2013). Conversely, determination of the resistance level of indicator bacteria may be useful to monitor the changes in the AR of the intestinal microbiota of human populations (Paulshus *et al.* 2019).

We surveyed the presence of antibiotic-resistant *E. coli* in STWW, water- or wastewater-irrigated soil and crop samples. Antibiotic resistance was determined by detecting six ARGs, including $bla_{\text{CTX-m-32}}$, $bla_{\text{OXA-23}}$, *tet*-W, *sul*1, *cml*-A, *erm*-B as well as *intI*1 gene (a key component of class 1 integrons) as a marker of anthropogenic pollution in *E. coli* isolates.

MATERIALS AND METHODS

Sample collection and preparation

This study was carried out in Isfahan, in the central part of Iran. In total, 51 samples including wastewater (18 samples); agricultural soil (18 samples) and crop (15 samples) were collected and analyzed for the presence of antibiotic-resistant *E. coli*.

Wastewater samples were collected from STWW of two WWTPs. Water- or wastewater-irrigated soil and crop samples were taken from agricultural fields. Soil samples were collected by an auger from the 0–20 cm topsoil layer. From each agricultural field a composite soil or crop sample consisting of three subsamples were analyzed. The edible parts of crop samples washed with tap water and 10^{-1} dilutions were prepared in sterile peptone water by homogenization for 3 minutes in a stomacher. A certain amount of each soil sample was also mixed with sterile peptone water (1:10 w/v) and then homogenized in a shaker incubator for 1 h.

All samples were taken in sterilized glass bottles or bags and were immediately transported to the laboratory for further processing.

E. coli isolation

For detection of *E. coli*, ten-fold serial dilutions (10^{-1}–10^{-6}) of samples were prepared. From all diluted samples, aliquots of 100 µL were plated onto lauryl sulphate MUG X-gal (LMX) agar in duplicate and incubated at 37 °C for 18–24 h. After incubation time the presence of *E. coli* in samples was determined by a light blue fluorescence of colonies under UV light (Farhadkhani *et al.* 2020). Finally, *E. coli* colonies were isolated using eosin methylene blue agar and incubated at 37 °C for 18–24 h.

Molecular detection of antibiotic resistance genes and class 1 integrons (*intI*1)

E. coli isolates were screened by PCR for detection of genes conferring resistance to β-lactam ($bla_{\text{CTX-m-32}}$, $bla_{\text{OXA-23}}$), tetracycline (*tet*-W), sulfonamide (*sul*1), chloramphenicol (*cml*-A), and macrolide (*erm*-B). The PCR amplification of *intI*1, was performed to evaluate the presence of class 1 integrons. PCR conditions and primer sets used for amplification of the genes are presented in Table 1.

Table 1 | Primers used in the study

Gene	Primer	Sequence (5′ → 3′)	Amplified fragment (bp)	Annealing temperature (°C)	References
tet-W	tet-W-F tet-W -R	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	168	64	Munir *et al.* (2011)
*sul*1	sul1-F sul1-R	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	163	55.9	Munir *et al.* (2011)
erm-B	erm-B-F erm-B-R	AAAACTTACCCGCCATACCA TTTGGCGTGTTTCATTGCTT	193	60	Knapp *et al.* (2010)
$bla_{\text{CTX-m-32}}$	CTX-m-32-F CTX-m-32-R	CGTCACGCTGTTGTTAGGAA CGCTCATCAGCACGATAAAG	156	60	Mirhoseini *et al.* (2016)
cml-A	cml-F cml-R	TAGTTGGCGGTACTCCCTTG GAATTGTGCTCGCTGTCGTA	137	60.4	Aali *et al.* (2014)
$bla_{\text{OXA-23}}$	OXA-23-F OXA-23-R	GATCGGATTGGAGAACCAGA ATTTCTGACCGCATTTCCAT	501	54	Mirhoseini *et al.* (2016)
*intI*1	intI1-F intI1-R	GCCTTGATGTTACCCGAGAG GATCGGTCGAATGCGTGT	196	60	Dungan *et al.* (2018)

For PCR detection of the genes, genomic DNA was extracted from the *E. coli* isolates using a boiling technique. The PCR assay was performed in a 25 μL volume, as described previously (Farhadkhani *et al.* 2019). The PCR cycling conditions consisted of 5 minutes denaturation at 95 °C followed by 35 cycles of 94 °C for 45 seconds, annealing at varied temperatures (Table 1) for 45 seconds, polymerization at 72 °C for 45 seconds, and a final extension at 72 °C for 5 minutes. Positive and negative controls were included in all PCR assays. Analysis of the PCR products was performed by electrophoresis in a 1.5% (w/v) agarose gel and visualizing of gels by ultraviolet (UV) transilluminator (UV Tech, France).

Statistical analysis

Spearman's correlation was performed to assess the relationship between the presence of ARGs at a significance level (*P*-value) of <0.01. Statistical analyses were carried out using the statistical package SPSS software and Microsoft Excel (version 2016).

RESULTS AND DISCUSSION

Detection of *E. coli*

AS a part of the normal gut flora in warm-blooded animals, *E. coli* is a good indicator of the fecal pollution of environmental samples (Osińska *et al.* 2017). *E. coli* was detected in almost all STWW samples and 20% (3 of 15) and 13% (2 of 15) of soil and crop samples, respectively. *E. coli* was not detected in soil or crop samples irrigated with other types of water (well or channel water) which indicates no fecal pollution of the samples. Low detection frequency of *E. coli* in STWW-irrigated soil and crop samples, could be related to the environmental condition of the region. Environmental conditions of arid and semi-arid areas including high temperature, sunlight intensity and low humidity affect the survival of microorganisms in soil and on crop samples (Farhadkhani *et al.* 2018). In consistent with our results, *E. coli* was detected with a low frequency in wastewater-irrigated soil and crop samples despite relatively high levels in wastewater (Gatta *et al.* 2016; Farhadkhani *et al.* 2018).

In this work, 78 *E. coli* isolates were identified, including 62 isolates from STWW, 12 from the soil, and four from the crop samples. Detection of *E. coli* as a fecal indicator bacterium in wastewater-irrigated soil and crop samples indicates that agricultural reuse of wastewater may be a potential route for the transmission of pathogenic microorganisms and ARB to humans through the food chain.

Identification of antibiotic resistance genes

E. coli has great genomic plasticity in the nutritional aspects as well as the acquisition of MGEs, which highlights its importance as a vector for ARGs dissemination in the environment (Aristizábal-Hoyos *et al.* 2019).

Analysis of ARGs showed the presence of *sul*1, *erm*-B, $bla_{\text{CTX-m-32}}$, *cml*-A, and *intI*1 genes in *E. coli* isolates with the highest frequency of detection for *sul*1. None of the isolates harbored *tet*-W and $bla_{\text{OXA-23}}$ resistance genes (Table 2).

In general, PCR assay revealed that 74% (58 of 78) of the *E. coli* isolates harbored at least one type of ARGs. *sul*1 which encodes the resistance to sulfonamide antibiotics, was the most abundant ARG (42%). The second most abundant gene was $bla_{\text{CTX-m-32}}$ (19%), followed by *erm*-B (17%) and *cml*-A (8%). *IntI*1, class 1 integron gene, was detected in 46% of the isolates (Table 2).

Detection of the *sul*1 gene as the most frequent ARG in *E. coli* isolates consistent with the results of another study which reported that the presence of the gene was correlated to the presence of Beta and Gammaproteobacteria phylum's (e.g. *Escherichia*). These bacterial phylum's with a relatively high AR are generally found in wastewater (Cerqueira *et al.* 2019b). Besides, a higher frequency of *sul*1 gene in STWW may be related to long-time use of sulfonamides in medicine in the past years (Kumar *et al.* 2020). Other researches also addressed the detection of sulfonamide ARGs at higher concentrations than quinolone (*qnr*), macrolide (*erm*-B), and tetracycline (*tet*-W) resistance genes in environmental samples (Dungan *et al.* 2018; Kumar *et al.* 2020).

The high frequency of detection of $bla_{\text{CTX-m-32}}$ gene in *E. coli* isolates is also consistent with other studies that found a great number of CTX-m-group resistance genes in environmental samples (Aristizábal-Hoyos *et al.* 2019). Yuan *et al.* (2020) reported that 97.35% of *Escherichia* spp. which isolated from livestock manure, hospital wastewater, and WWTPs carrying $bla_{\text{CTX-m}}$. CTX-m-32 is a β-lactamase-encoding gene which confers resistance to cephalosporins (one of the antibiotic groups which is commonly used in clinical practice) (Aristizábal-Hoyos *et al.* 2019). Although the presence of β-lactam-resistant bacteria has mainly been described in hospital environments (Mirhoseini *et al.* 2016; Yuan *et al.* 2020), their occurrence in other environments such as agricultural crops, municipal WWTPs, and different water bodies (Koczura *et al.* 2012; Osińska *et al.* 2017; Aristizábal-Hoyos *et al.* 2019) indicates that ARB have the ability to disseminate in other environments besides the hospital environment (Aristizábal-Hoyos *et al.* 2019; Yuan *et al.* 2020).

As STWW isolates, the *sul*1 gene was detected with the highest frequency in soil and crop isolates, whereas the *erm-B* gene was not detected in crop samples. *Cml*-A with low frequency (10%) in STWW isolates was not detected in soil and crop samples (Table 2). This trend may be related to the relative abundance of genes in STWW. Conversely, it is most likely a consequence of ARB die-off when transferred from STWW to the soil and crop (Bougnom *et al.* 2020). However, other factors such as the presence of antibiotics, heavy metals, and antimicrobial disinfectant residues in STWW may influence ARGs frequency in the irrigated soils and crops (Dungan *et al.* 2018; Yuan *et al.* 2020).

Another interesting observation related to gene occurrence was that all detected ARGs in the soil and crop isolates were found in the STWW isolates. Some studies indicated the impact of WWTPs effluents on ARB dissemination in the receiving environments (Broszat *et al.* 2014; Chen *et al.* 2014). Although WWTPs play an important role to remove or minimize a wide range of pollutants, they may act as the main entry pathways of ARGs and ARB into the environment (Kumar *et al.* 2020). In addition, the chlorine-based disinfection in STWW could induce the discharge of ARGs from damaged microbial cells (Campo *et al.* 2020). Based on the results, using STWW for agricultural irrigation might contribute to the spread of ARG and ARB to the environment. A previous study conducted in Beijing and Tianjin, China indicated that soils receiving municipal wastewater in agricultural settings are enriched in ARGs (Chen *et al.* 2014), which supports the results of our study. For example, comparing ARG levels in the soil samples in China revealed that the relative abundance of *sul*1, *sul* 3, *tet*-A, *tet*-C, *tet*-E, *tet*-G, and *tet*-S were significantly higher in wastewater-irrigated soils than in non-irrigated soils (Chen *et al.* 2014).

Table 2 | Detection frequency of ARGs in *E. coli* isolates

	***E. coli* isolates harboring ARG/total of *E. coli* isolates (%)**						
Sample type	***tet*-W**	***sul*1**	***erm*-B**	**$bla_{\text{CTX-m-32}}$**	***cml*-A**	**$bla_{\text{OXA-23}}$**	***intI*1**
STWW	ND	24/62 (39)	8/62 (13)	9/62 (14)	6/62 (10)	ND	35/62 (56)
Soil	ND	6/12 (50)	5/12 (42)	5/12 (42)	ND	ND	ND
Crop	ND	3/4 (75)	ND	1/4 (25)	ND	ND	1/4 (25)
Total	ND	33/78 (42)	13/78 (17)	15/78 (19)	6/78 (8)	ND	36/78 (46)

ND: not detected, STWW: secondary treated wastewater.

Detection of antibiotic-resistant *E. coli* in agricultural soils indicates that consumption of wastewater-irrigated crops may be a concern from a public health point of view (Bougnom *et al.* 2020).

Prevalence of multidrug resistance *E. coli*

Multidrug resistance (MDR) is defined as resistance to at least three different categories of antimicrobials agents (Osińska *et al.* 2017). High concentrations of MDR bacteria were detected in municipal wastewater, hospital wastewater, and livestock feeding drainage (Kumar *et al.* 2020). In our study, 9% of (7 of 78) *E. coli* isolates were multiresistant, 13% of the isolates harboring 2 ARGs, 33% of the isolates harboring 1 ARG, and 45% of them did not carry any resistance gene (Figure 1).

The results of this study revealed a small percentage of MDR (9%) *E. coli* compared with other studies (Osińska *et al.* 2017; Aristizábal-Hoyos *et al.* 2019). Osińska *et al.* (2017) reported that nearly 38% of *E. coli* strains isolated from river water and wastewater in Poland were MDR. Furthermore, 35% of *E. coli* strains isolated from treated wastewater were MDR, and 10% of isolates were resistant to one antibiotic. In addition, in Colombia 63.6% of *E. coli* isolates in the final WWTP were MDR (Aristizábal-Hoyos *et al.* 2019).

It is important to note that all the MDR isolates harbored *sul*1, *erm*-B, and $bla_{\text{CTX-m-32}}$ resistance genes. It has been reported that MDR genotypes of β-lactam-resistant Enterobacteriaceae which have mainly been identified for resistance to sulfamethoxazole, were shown to have resistance caused by $bla_{\text{CTX-m}}$ (Bajaj *et al.* 2016).

The highest percentage of MDR was detected in *E. coli* strains isolated from the soil (42%) samples, whereas MDR was not observed in *E. coli* isolates from the crop samples (Figure 1). A previous study by Broszat *et al.* (2014) indicated that MDR presence is more noticeable in wastewater-irrigated soils (25%) than in rain-irrigated soils (6%).

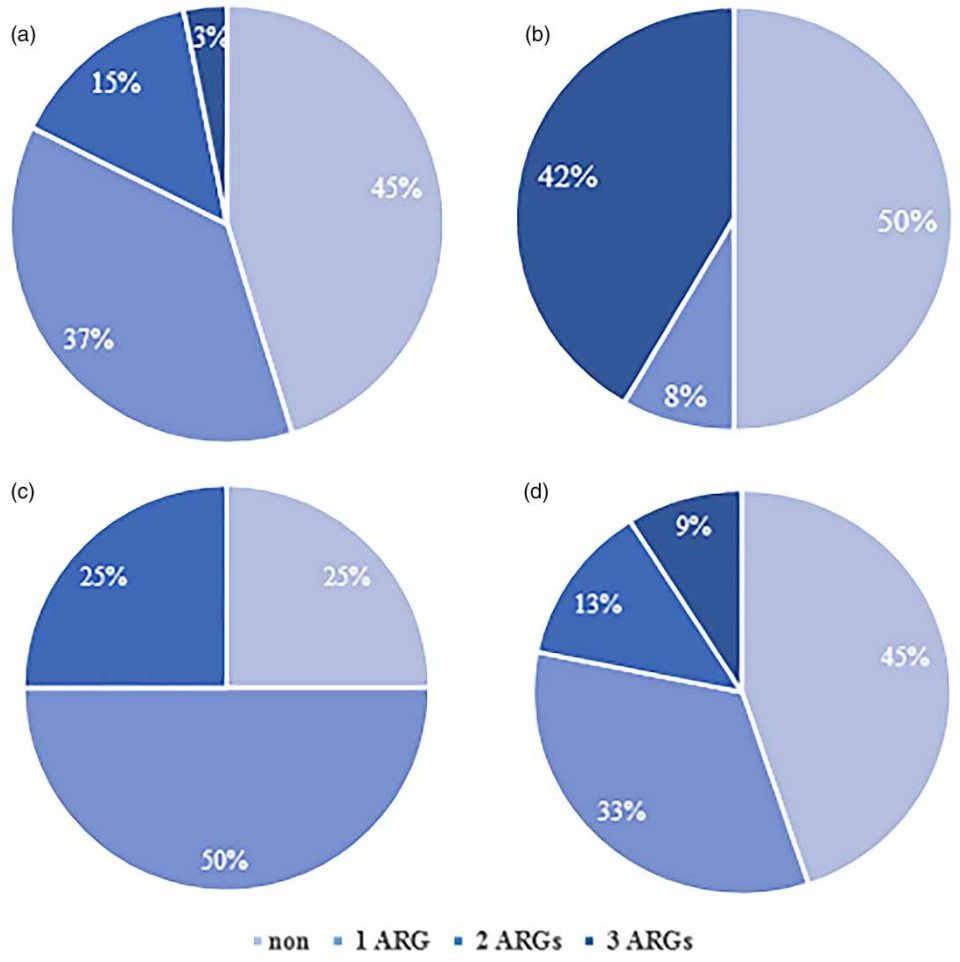

Figure 1 | Percentage of *E. coli* strains harboring ARGs: (a) STWW, (b) Soil, (c) Crop, (d) Total.

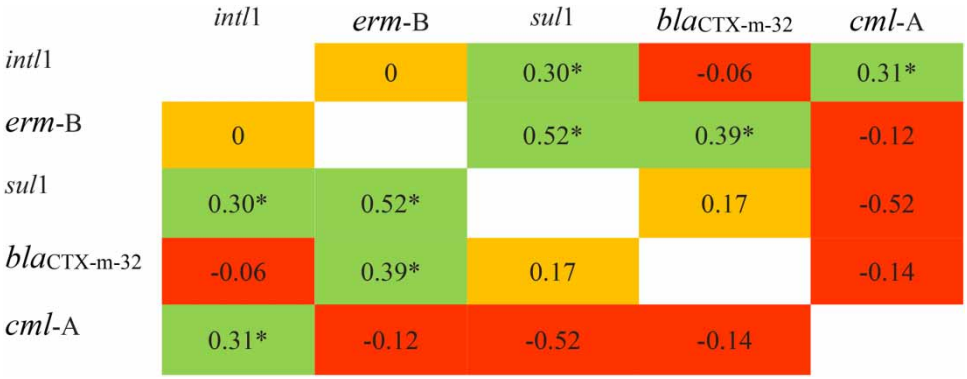

Figure 2 | Heat map of Spearman's correlation among ARGs and *intI*1. *Indicates the correlation is significant at the 0.01 level (2-tailed) according to Spearman's test.

Frequency of integron harboring *E. coli*

Integrons play a crucial role in the transfer and dissemination of antimicrobial resistance genes (Su *et al.* 2012). They can capture one or more gene cassettes and transfer them among different bacterial species, especially Gram-negative bacteria via the HGT mechanism (Su *et al.* 2012; Yuan *et al.* 2020). Our results showed the detection of *intI*1 in 46% (36 of 78) of *E. coli* isolates. The frequency of detection of *intI*1 in *E. coli* isolates is presented in Table 2. The highest level of detection was noted in STWW samples (35 of 62; 56%). A similar result was reported by Yuan *et al.* (2020) who found that 51.17% of *E. coli* strains isolated from municipal WWTPs harbored the *intI*1 gene. In contrast, Koczura *et al.* (2012) found that only 11% of *E. coli* strains which were recovered from WWTP harbored *intI*1.

Among the 36 isolates containing *intI*1, 75% (27 of 36) carried ARGs, indicating that 25% (9 of 36) of *intI*1 harboring isolates were free of ARG or carried other types of ARGs. Here, 25% of *E. coli* isolates recovered from the crop samples were integron positive whereas *intI*1 was completely absent in *E. coli* isolates of soil samples (Table 2). Furthermore, 6% (2 of 36) of integron harboring *E. coli* isolates were multiresistant. In the study carried out by Araújo *et al.* (2017) on irrigation of water and vegetables in household farms, 16% of *E. coli* isolates were found to be MDR among them, five isolates carried class 1 integrons.

As noted in previous studies, integrons are frequently found in livestock manure, urban wastewater, hospital wastewater (Yuan *et al.* 2020), agricultural ecosystems, and even in the water bodies not exposed to antibiotics (Araújo *et al.* 2017). The presence of *intI*1 imposes a higher potential for the dissemination of ARGs in the environment. Therefore, the consumption of crops contaminated with bacteria harboring *intI*1 may trigger a human health risk in terms of exposure to ARGs (Cerqueira *et al.* 2019a).

Spearman's correlation among ARGs and *intI*1 in the different sample types showed that the presence of *erm*-B gene was correlated with *sul*1 ($r = 0.52$) and $bla_{\text{CTX-m-32}}$ ($r = 0.39$) (Figure 2). As mentioned earlier, these three ARGs were detected in all MDR isolates which indicates that these ARGs may be present in the same MGE (Cerqueira *et al.* 2019b).

The results showed that *sul*1($r = 0.30$) and *cml*-A ($r = 0.31$) positively correlated with *intI*1. The correlation of *sul*1 with *intI*1 is related to the location of *sul*1 gene on 30-CS of the classic class 1 integrons (Su *et al.* 2012). *Cml*-A is one of the ARGs which confers resistance to chloramphenicol. The positive correlation between other chloramphenicol resistance genes (like *cat*) and *intI*1 was noted previously (Wu *et al.* 2011). The lack of correlation between *intI*1 and other AGRs may be attributed to the following reasons. Although the *intI*1 is known as an effective marker of antimicrobial resistance (Osińska *et al.* 2017), the occurrence of the gene is often associated with anthropogenic sources but not necessarily with the AR (Narciso-da-Rocha & Manaia 2017). Furthermore, integrons may be free of gene cassettes encoding AR. Recently, integrons without ARG cassettes were recovered from soils and sediments from the bacterial community (Zhang *et al.* 2009). However, the lack of significance may be related in part to the low numbers of *E. coli* isolates.

CONCLUSIONS

The results indicated the presence of *E. coli* isolates harboring ARGs and *intI*1 in the STWW, wastewater-irrigated soil and crop samples. Although the environmental conditions of arid and semi-arid areas may contribute to die-off of many ARB in

soil and on crop surfaces, STWW could be a major source for ARB dissemination in the environment. Therefore, agricultural reuse of wastewater may be a risk factor for human health. Further studies are needed to estimate the risk of ARGs associated with the consumption of wastewater-irrigated crops for human health.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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