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Antibiotic resistance, virulence factors, and phylogenetic groups of *Escherichia coli* isolated from hospital wastewater: A case study in the west of Iran

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Abstract

Background: The appropriate management of hospital wastewater is an essential process to prevent the spread of pathogenic strains of bacteria including *Escherichia coli* in this study, the antibiotic resistance, virulence characteristics, and phylogenetic diversity of *E. coli* isolated from the raw and treated hospital wastewater in a general hospital in the west of Iran were evaluated.

Methods: *E. coli* isolates were recovered and identified using culture and biochemical tests. Sixty isolates were used and antimicrobial resistance, virulence genes, antibiotic-resistant genes (ARGs), and phylogeny groups of isolates were determined using polymerase chain reaction (PCR) assay. The antibiotic resistance was tested using disk diffusion.

Results: The antibiotic susceptibility testing indicated that the resistance to co-trimoxazole was the most common, followed by ceftriaxone, amikacin, and gentamicin. Multi-drug resistance (MDR) was observed in 90% of raw and 96.66% of treated sewage isolates. The phylogeny groups B1 and A were the most common groups among isolates of raw and treated sewage, respectively. The most common virulence genes detected were *sfa*, *pap*C, and *fyu*A; while *pic* and *sep*A genes were not found in the isolates. The most common ARGs were bla_{TEM} (in 90% isolates of raw and 92.5% of treated sewage) and bla_{CTX-M} (in 60% isolates of raw and 77.5% of treated sewage). The bla_{SHV-5} gene was not detected among isolates.

Conclusion: The results highlight the potential of hospital wastewater as a source for spreading the virulent and multi drug-resistant strains of *E. coli*.

Keywords: *Escherichia coli*, Phylogeny, Drug resistance, Sewage

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Introduction

Escherichia coli is a gram-negative bacillus and a normal flora in the gastrointestinal tract of humans and various animals. However, it can cause severe diseases in the gastrointestinal and extraintestinal tissues, including the urinary tract, meninges, and blood (1). This wide range of *E. coli* pathogenesis is due to its capability to acquire virulence and the antibiotic-resistant genes (ARGs) from the genetic pool in its environment (2). Virulence genes encode proteins that contribute to the pathogenesis of *E. coli* strains and are located on either chromosome or more importantly, on extrachromosomal genetic elements such as plasmids, which can facilitate their transmission within bacterial communities (2).

Escherichia coli strains have been classified based on their phylogenic relationship from A to F and *Escherichia* clade I groups (3). Socioeconomic factors, geographic location, dietary habits, and the climate may play roles in the distribution of *E. coli* phylogenetic groups across the various regions (4). Researchers showed that the pathogenic extraintestinal strains of *E. coli* mainly belong to the B2 phylogroup, while the most commensal strains are in the phylogroups A and B1. In addition, the A and B1 phylogroups are highly adapted to humans and animal hosts, respectively (5).

As a serious challenge to public health, in recent years, *E. coli* strains have become dramatically resistant to conventional antibiotics that narrow down their

therapeutic choices (6). In gram-negative bacilli such as *E. coli*, the production of extended-spectrum beta-lactamases (ESBLs) is the major resistance mechanism to various antibiotics, including penicillins, broad-spectrum cephalosporins, and monobactams (7). ESBL-producing *E. coli* strains have been typically involved in both community-acquired and hospital-acquired infections. However, the rate of ESBL-producing and highly virulent *E. coli* in hospital-acquired is much higher than community-acquired infections (8).

Given the fact that hospital sewage has been recognized as a major source of spreading virulent and antibiotic-resistant strains of bacteria, the management of hospital wastewater in a safe and effective way has received increasing attention throughout the world (9-11). Few studies have evaluated the characterizations of *E. coli* strains such as virulence factors, antimicrobial susceptibility, and phylogenetic groups isolated from hospital wastewaters (12,13). The aim of this study was to determine phylogenetic diversity, antimicrobial susceptibility, and virulence characteristics of *E. coli* strains isolated from local hospital wastewater.

Materials and Methods

Collection of samples

Escherichia coli strains were isolated from hospital wastewater before and after sewage treatment in Imam Reza hospital, Kermanshah, Iran, between December 2017 and March 2018. The wastewater treatment plant in this hospital works basis on the extended aeration-activated sludge system. The sewage sampling was taken twenty times from the raw (before sewage treatment) and the treated (after sewage treatment) separately, with a few day intervals between each sampling time. The samples were put in an icebox and transported to the Laboratory of Microbiology Department in the Kermanshah University of Medical Sciences (14).

Bacterial isolation and identification

The samples were collected in 100 mL sterile bottles and transferred to the microbiology laboratory on ice and tested in less than two hours. Serial dilutions of each wastewater sample were made in saline solution (0.85% NaCl) and mixed well. Then, 200 µl of each dilution was cultured in plates containing EMB culture medium to isolate *E. coli* (15). Gram staining was done from the grown colonies. Then, different biochemical tests based on standard methods were used to identify *E. coli* isolates (16). Subsequently, two batches of growing colonies were kept at -70°C for the later examinations. Collected *E. coli* colonies were determined for the production of ESBL enzymes by combined disk test (17).

The average percent of three independent tests was used to calculate the percentage of ESBL-producing *E. coli*. Finally, 30 *E. coli* isolates from raw sewage (10 non-EBSL and 20 ESBL-producing isolates) and 30 *E. coli* isolates from treated sewage (10 non-EBSL and 20 ESBL-producing isolates) were arbitrarily selected for further testing.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the disc diffusion method basis on the Clinical and Laboratory Standards (CLSI) guideline (18). The used discs were fluoroquinolone (ciprofloxacin, 5 µg), sulfonamides (sulfamethoxazole-trimethoprim or Co-trimoxazole, 25 µg), aminoglycosides (amikacin, 30 µg and gentamicin,10 µg), cephalosporin (ceftriaxone, 30 µg), carbapenem (imipenem, 10 µg), and penicillin (piperacillin, 10 µg). The inhibition zones of colonies were measured and scored as sensitive, intermediate (with reduced susceptibility), or resistant. The *E. coli* ATCC 25922 was used as a reference strain for quality control. The isolates non-susceptible to ≥ 3 different antibiotic classes were considered as multi-drug resistant (MDR) (19).

Detection of virulence factor, antibiotic-resistant gene, and phylogenetic group

The whole genome of *E. coli* isolates was extracted by boiling method and used as DNA template for polymerase chain reaction (PCR). DNA quality and quantity were assessed using a NanoDrop spectrophotometer (Implen, Germany) at 260 nm. The PCR reactions were performed in 25 µL volume that contained 200 µM of deoxynucleotide triphosphates (dNTPs), 2.5 µL of 10X PCR buffer, 0.7 mg/µL $MgCl_2$, 0.6 units of Taq polymerase, 10 pmol of each primer, and 2 µL of isolate DNA. The PCR cycle was performed using Thermal Cycler (Eppendorf, Germany). The PCR products were determined on a 1.5% agarose gel electrophoresis. A 100 bp DNA marker (SinaClon, Iran) was used to estimate the size of PCR bands. Negative controls (samples without DNA templates) and positive controls (samples with DNA from our previously-reported positive strains) were included in all PCR assays. Genes involved in the resistance to fluoroquinolones (*qnrA*, *qnrB*, and *qnrS*) and β-lactams (bla_{TEM}, bla_{CTX-M}, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, bla_{SHV-5}, and bla_{SHV-12}) were determined by PCR (Table 1). The phylogenetic group of each strain was determined as previously described (3). The presence of 13 virulence-encoding genes, including 4 adhesions (*agg*R, *pap*C, *sfa*S, and *fim*H), 6 toxins (*hly*A, *sep*A, *sig*A, *pic*, *cnf1*, and *stx1*), 2 siderophores (*iut*A and *fyu*A), and 1 capsule synthesis gene (*kps*MT) was also investigated by PCR (Table 1).

Data analysis

The data were statistically analyzed using SPSS version 18. The chi-square and Fisher's exact tests were used to evaluate the relationship between variables. The $P < 0.05$ was considered statistically significant.

Table 1. Primers used in PCR reactions for the phylogenetic groups, virulence, and antibiotic-resistant genes

Gene Category	Genes' name	Primer Sequence (5' – 3')	Amplicon (bp)	Reference
Antibiotic-resistant				
Fluoroquinolones	*qnrA*	TCAGCAAGAGGATTTCTCA GGCAGCACTATTACTCCCA	516	(20)
	qnrB	GATCGTGAAAGCCAGAAAGG ACGATGCCTGGTAGTTGTCC	469	(20)
	qnrS	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	714	(20)
β-lactams	bla_{TEM}	AGTGCTGCCATAACCATGAGTG CTGACTCCCCGTCGTGTAGATA	431	(21)
	bla_{CTX-M}	TTTGCGATGTGCAGTACCAGTAA CGATATCGTTGGTGGTGCCAT	544	(22)
	$bla_{CTX-M-1}$	CGTCACGCTGTTGTTAGGAA ACGGCTTTCTGCCTTAGGTT	780	(23)
	$bla_{CTX-M-2}$	ATGATGACTCAGAGCATTCG TTATTGCATCAGAAACCGTG	884	(22)
	bla_{SHV-5}	TATCGGCCCTCACTCAAGGA TGCTCATCATGGGAAAGCGT	231	(24)
	bla_{SHV-12}	GCCGCGTAGGCATGATAGAA CGGCGTATCCCGCAGATAA	677	(24)
Virulence factors				
Adhesions	*papC*	GACGGCTGTACTGCAGGGTGTGGCG ATATCCTTTCTGCAGGGATGCAATA	328	(25)
	sfa	CTCCGGAGAACTGGGTGCATCTTAC CATCAAGCTGTTTGTTCGTCCGCCG	419	(25)
	fimH	AACAGCGATGATTTCCAGTTTGTGTG ATTGCGTACCAGCATTAGCAATGTCC	465	(26)
	aggR	GCAATCAGATTAARCAGCGATACA CATTCTTGATTGCATAAGGATCTGG	426	(27)
Toxins	*pic*	ACTGGATCTTAAGGCTCAGGAT GACTTAATGTCACTGTTCAGCG	572	(27)
	sepA	GCAGTGGAAATATGATGCGGC TTGTTCAGATCGGAGAAGAACG	794	(27)
	sigA	CCGACTTCTCACTTTCTCCC CCATCCAGCTGCATAGTGTTTG	430	(27)
	stx1	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	180	(28)
	cnf-1	AAGATGGAGTTTCCTATGCAGGAG CATTCAGAGTCCTGCCCTCATTATT	498	(29)
	hlyA	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	534	(30)
Siderophores	*fyuA*	TGATTAACCCCGCGACGGGAA CGCAGTAGGCACGATGTTGTA	880	(31)
	iutA	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG	302	(32)
Capsules	*kpsMT*	GCGCATTTGCTGATACTGTTG CATCCAGACGATAAGCATGAGC	269	(29)

Results

Frequency of ESBL-producing Escherichia coli strains

On average, the frequency of the phenotypically identified ESBL-producing *E. coli* in raw and treated sewage was 15% and 19%, respectively, although the rate of increase in the treated sewage was not statistically significant ($P = 0.367$).

Antibiotic resistance of isolates

The highest rate of resistance was reported to co-trimoxazole (86.7% in raw and 93.3% in treated sewage isolates) followed by ceftriaxone (80% in raw and 90% in treated sewage isolates) and piperacillin (83.3% in raw and 80% in treated sewage isolates). Although the rate of resistance to antibiotics was slightly higher in treated isolates for most antibiotics used, there was no statistically significant difference between antibiotic resistance of raw and treated isolates ($P > 0.05$) (Table 2). The rate of MDR was observed in 90% and 96.66% of raw and treated sewage isolates, respectively (Figure 1).

Generally, 8 out of 9 ARGs investigated, were detected

in one or more *E. coli* isolates, with higher frequencies in treated sewage isolates (Figure 2).

The most frequent ARGs were bla_{TEM} (90% in raw and 92.5% in treated sewage isolates) and bla_{CTX-M} (73.3% in raw and 73.3% in treated sewage isolates). The frequency of ARGs between raw and treated sewage isolates was not statistically significant, except for the $bla_{CTX-M-1}$ gene (P = 0.01), which was significantly higher in treated sewage isolates.

Of the 60 *E. coli* isolates, 39 (65%) isolates carried at

Table 2. Frequency of antimicrobial resistance in *E. coli* isolates in the raw and treated sewage of hospital

Antibiotic	Raw Sewage (n=30)			Treated Sewage (n=30)			*P* value
	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant	
Ciprofloxacin	6 (20)	3 (10)	21 (70)	5 (16.7)	2 (6.7)	23 (76.7)	0.83
Co-trimoxazole	3 (10)	1 (3.3)	26 (86.7)	2 (6.7)	0 (0.0)	28 (93.3)	0.67
Amikacin	17 (56.7)	4 (13.3)	9 (30)	14 (46.7)	10 (33.3)	6 (20)	0.17
Gentamicin	13 (43.3)	8 (26.7)	9 (30)	14 (46.7)	8 (26.7)	8 (26.7)	0.95
Ceftriaxone	4 (13.3)	2 (6.7)	24 (80)	2 (6.7)	1 (3.3)	27 (90)	0.56
Imipenem	13 (43.3)	4 (13.3)	13 (43.3)	12 (40)	8 (26.7)	10 (33.3)	0.41
Piperacillin	0 (0.0)	5 (16.7)	25 (83.3)	1 (3.3)	5 (16.7)	24 (80)	1.00

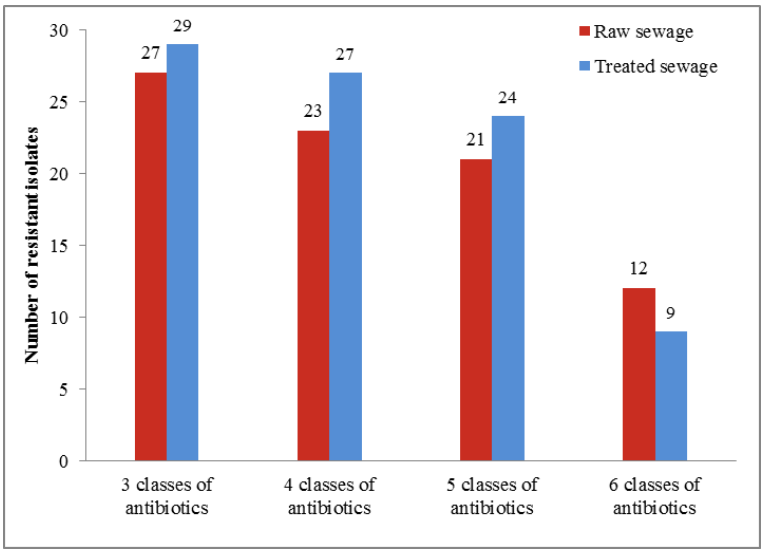

Figure 1. The frequency of multiple drug resistance (MDR) among *E. coli* isolates in the raw and treated sewage of the hospital wastewater

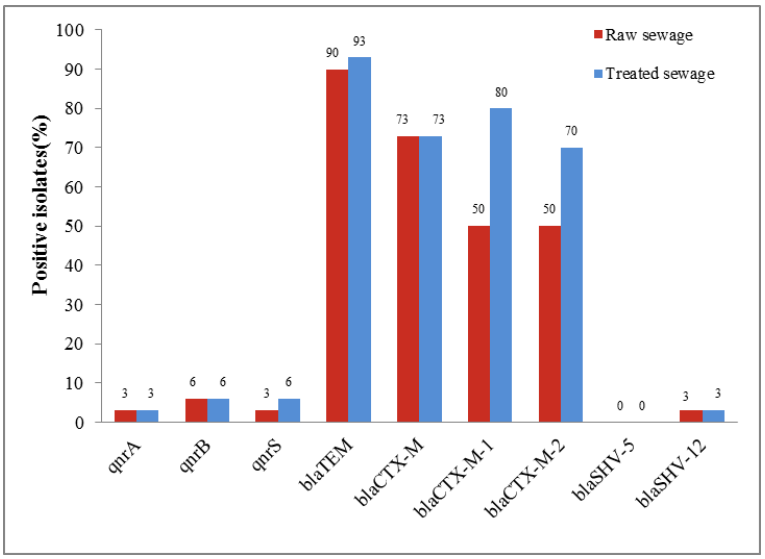

Figure 2. The frequency of antibiotic-resistant genes of *E. coli* isolates in the raw and treated sewage of the hospital wastewater. The difference in the frequency of antimicrobial-resistant genes between raw and treated sewage *E. coli* isolates was not statistically significant except for *blaCTX-M1*, *P*<0.05

least one virulence gene (35% in raw and 30% in treated sewage isolates). The frequencies of virulence genes were determined for *fyu*A (21.7%, n = 13), *papC* (21.7%, n = 13), *sfa* (20%, n = 12), *fimH* (15%, n = 9), *kps*MT (11.7%, n = 7), *hlyA* (10%, n = 6), *iutA* (1.3%, n = 5), *cnf-1* (6.7%, n = 4), *stx 1* (6.7%, n = 4), *aggR* (6.7%, n = 4), and *sigA* (3.3%, n = 2). *Pic* and *sepA* genes were not found in any isolates. In general, the frequency of the most virulence genes was lower after sewage treatment; however, a few genes showed no changes or a little increase in their frequency rates (Figure 3).

The *hlyA*, *cnf-1*, *sigA*, and *stx1* genes were found only in the raw sewage isolates. The *aggR* gene was found only in the treated sewage isolates. Regarding the frequency of isolates with multiple virulence genes, only two raw sewage isolates (6.6%) contained five virulence genes. However, the number of isolates with 4 or 3 virulence genes was equal in both raw and treated sewage isolates (data not shown).

Phylogenetic analysis of *E. coli* isolates showed that they mainly belonged to phylogroup B1 (28 isolates; 46.7%), followed by A (14 isolates, 23.3%), D (8 isolates, 13.3%), E (5 isolates, 8.3%), B2 (3 isolates, 5%), and F (2 isolates, 3.3%). The difference in frequencies of phylogenetic groups of raw and treated sewage isolates was not significant (Figure 4). The rate of virulence genes was higher in B1 and D compared to other phylogenetic groups (Table 3).

Discussion

The release of hospital wastewater into the environment is one of the sources of pathogenic *E. coli* dissemination, which can potentially cause infection in susceptible people (9). The antibiotic-resistant and virulent *E. coli* strains are serious therapeutic and epidemiological concerns. In the present study, the proportion of ESBL-producing *E. coli* in both raw and treated sewage isolates was considerable, highlighting the importance of hospital wastewater in the pathogen dissemination. The proportion of ESBL-producing *E. coli* in treated municipal wastewater was estimated to be 5.3% in a study in Japan (33), while it was reported to be 26% in another study in Slovakia (34). This variation in the prevalence of ESBL-producing isolates can be explained by the difference in sample

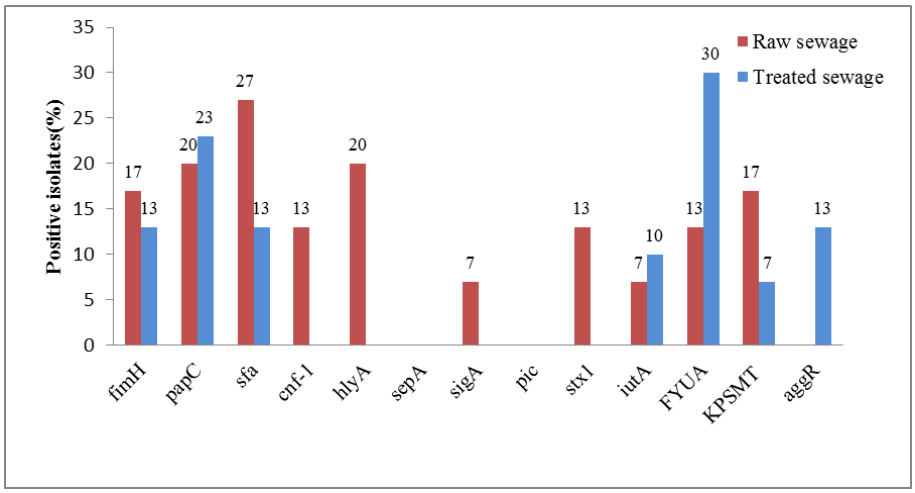

Figure 3. The frequency of virulence genes among *E. coli* isolates. The difference in the frequency of *virulence* genes between raw and treated sewage *E. coli* isolates was statistically significant for *cnf-1*, *hly A*, *stx1*, and *aggR* (P<0.05)

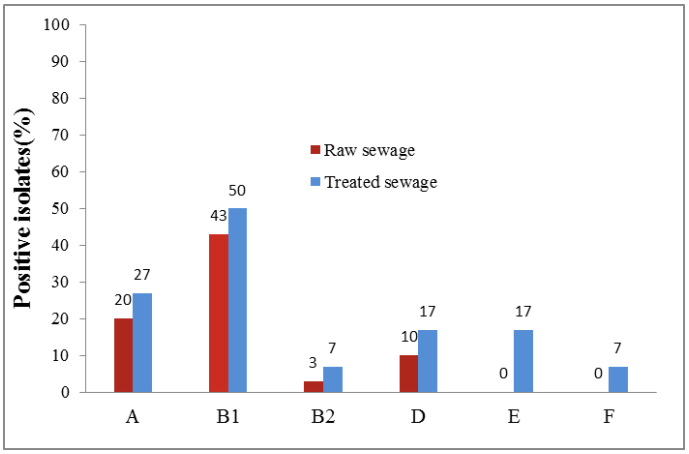

Figure 4. The rate of phylogenetic groups of *E. coli* in the raw and treated sewage of hospital

Table 3. Association between phylogenic groups and the virulence factors in *E. coli* isolates from the raw and treated sewage of hospital

Virulence genes	Isolates contained virulence genes (number)	Phylogenic groups, No. (%)					
		A (n =14)	B1 (n= 28)	B2 (n=3)	D (n =8)	F (n =2)	E (n=5)
hlyA	6	1(7.14)	4 (14.28)	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)
cnf	4	0 (0.0)	2 (7.14)	0 (0.0)	1 (12.5)	1 (50)	0 (0.0)
sigA	2	0 (0.0)	2 (7.14)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
pic	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
sepA	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
stx1	4	0 (0.0)	3 (10.71)	1(33.33)	0 (0.0)	0 (0.0)	0 (0.0)
papC	13	2 (14.28)	6 (21.42)	0 (0.0)	2 (25)	1 (50)	2 (40)
sfa	12	2 (14.28)	6 (21.42)	0 (0.0)	2 (25)	1 (50)	1(20)
fimH	9	1 (7.14)	4 (14.28)	0 (0.0)	3 (37.5)	1 (50)	0 (0.0)
ituA	5	2 (14.28)	1 (3.57)	0 (0.0)	2 (25)	0 (0.0)	0 (0.0)
fyuA	13	3 (21.43)	3 (10.71)	1(33.33)	6 (75)	0 (0.0)	0 (0.0)
kpsMT	7	0 (0.0)	1 (3.57)	2 (66.66)	0 (0.0)	2 (100)	2 (40)
aggR	4	1 (7.14)	3 (10.71)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

sources, geographical regions, and methods of wastewater treatment. Most ESBL genes are located on mobile genetic elements such as conjugated plasmids, which can be easily transmitted to other bacteria (35). Research showed that in comparison to the city wastewater, *E. coli* isolates from hospital wastewater possess much higher rates of conjugated plasmids containing multi-drug-resistant genes (36).

As a consequence of the widespread prescription of antibiotics, resistance in *E. coli* isolates has been dramatically increased globally (37). The majority of the isolates studied in the present study were resistant to ciprofloxacin, ceftriaxone, co-trimoxazole, and piperacillin, which can reflect this issue. On the other hand, aminoglycosides seem to be effective as therapeutic options in cases of waterborne *E. coli* infections in our region.

Moreover, the majority of *E. coli* isolates were remarkably resistant to three or more antibiotic classes, defined as MDR, highlighting the concern for health systems in our region. In comparison to studies on surface water and wastewater *E. coli* isolates from other countries, the rate of antibiotic resistance in the present study was higher (38), which can be attributed to the different sources of the isolates (the hospital versus municipal wastewaters).

Beta-lactam antibiotics especially the third-generation cephalosporins are widely used for the treatment of infections caused by Enterobacteriaceae (39). Consequently, resistance to this group of antibiotics has increased worldwide. In this study, the bla_{TEM} and $bla_{CTX\text{-}M}$ genes were the most prevalent in isolates of raw and treated sewage samples. These findings are consistent with the results reported by a study on isolates of treated wastewater effluents in South Africa (40). These genes are likely to be the most common in ESBL-producing *E. coli* isolates from various clinical samples in many regions (41). The *qnr* genes encode proteins capable of protecting DNA gyrase and topoisomerase IV from quinolones (42). In this study, 60 *E. coli* isolates were analyzed for the presence of *qnrA*, *qnrB*, and *qnrS* genes but their frequencies were very low. Similarly, in a study from South China, quinolone-resistant genes were negative in both influent and effluent wastewater (43).

The frequency of virulence genes among *E. coli* isolates in the present study was higher than the rate reported from the Netherlands (17%) and Canada (14%) (38,44), but it was lower than the rate reported from *E. coli* strains isolated from a wastewater treatment plant in Poland (45). The result of this study showed that adhesions-related genes of *sfa*, *pap*, and siderophore gene encoding yersiniabactin receptor (*fyu*A) are quite frequent among *E. coli* strains of hospital wastewater. This is important since the expression of different types of adhesions is required for the development of infection.

The iron-uptake system of highly pathogenic strains is mediated via yersiniabactin, which is encoded by *fyu*A and is associated with strain virulence. This could be the reason for the high abundance of *fyu*A in *E. coli* isolated from hospital wastewater (46).

The presence of the *stx1* gene, which is specific for the enterotoxigenic *E. coli* (ETEC), was detected in small proportion of isolates. Diarrhea is an important health problem in particular for children, and ETEC accounts for about 9% of all diarrheal cases in the Iranian population (47). Although we did not attempt to isolate or identify Shiga toxin-producing *E. coli*)STEC(or EPEC, the detection of *stx1* gene in *E. coli* isolates from hospital wastewater in Kermanshah raises health concerns due to the possible environmental spreading of these strains.

The heat-stable enterotoxin 1 (EAST1) and *agg*R are found in enteroaggregative *E. coli* (EAEC), which causes persistent diarrhea in children and adults (48). The *agg*R

gene was detected in a small percentage of isolates from treated sewage, indicating the low prevalence of this pathotype. This result is consistent with the results of a study from Dutch wastewater for the low frequency of *agg*R gene in ESBL-producing *E. coli* (38). In the present study, the frequency rate of the majority of virulence genes tested was lower in the treated sewage isolates in comparison to the raw sewage isolates. Nevertheless, the detection of virulence genes in the isolates of treated wastewater indicates the potential pathogenicity of isolates.

The hospital wastewater contains both commensal strains from healthy carriers and pathogenic *E. coli* strains, which can affect the phylogenetic distribution. There is a positive relationship between the phylogroup distribution of *E. coli* isolates in wastewater and human hosts (4). In this study, phylogenetic analysis of 60 *E. coli* isolates from hospital wastewater showed that the B1 and A groups comprised 41 (68.3%) of the total *E. coli* isolates, which is similar to the results reported by previous research on strains isolated from water (49). Research has also shown that group B2 was significantly more common among the clinical isolates of ESBL-producing *E. coli* ($P<0.01$) than wastewater and environmental water isolates (50).

Conclusion

In this study, the remarkable percentages of MDR among *E. coli* isolates in hospital wastewater were reported. There is no significant decline in the rate of antibiotic resistance as well as ARGs after sewage treatment. The results also indicated the presence of virulence genes in the treated wastewater isolates of *E. coli*. Although a relatively small number of isolates were used in this study, the results suggest that the presence of these antibiotic-resistant and virulent strains in the treated hospital wastewater could be a source for the spreading of virulent and ARGs. Further studies with a larger sample size are recommended to characterize isolates of local hospital wastewater.

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Supervision: Alisha Akya.
Validation: Arezoo Bozorgomid.
Visualization: Alisha Akya.
Writing–original draft: Arezoo Bozorgomid, Roya Chegene Lorestani.
Writing–review & editing: Alisha Akya, Mosayeb Rostamian, Arezoo Bozorgomid.

Competing interests

The authors declare no competing interests to disclose.

Ethical issues

This study was approved by the Ethics Committee of Kermanshah University of Medical Sciences (Ethical code: IR.KUMS.REC.1397.333).

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