The Antibiotic Resistance Profiles of *Escherichia Coli* Isolated from Pre and Post Treated Hospital Sewage

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Abstract

Background: The dissemination of resistant strains of bacteria into the environment through hospital sewage has been recognized as a public health concern. We investigated the antibiotic resistance profile of *E. coli* isolated from hospital sewage.

Methods: *E. coli* strains were isolated from the hospital sewage system from both pre and post treatment phases in a general hospital of Kermanshah city (west of Iran). Resistance to antibiotics (clindamycin, ceftriaxone, Co-trimoxazole, penicillin, ciprofloxacin, amikacin, gentamicin, imipenem, and piperacillin) was determined by disc diffusion. Isolates were screened phenotypically for extended-spectrum beta-lactamases (ESBL) production. The frequency of common antibiotic resistance genes (*bla* CTX-M, *bla* TEM, *bla* SHV, and *qnr*) were detected by PCR and data was statistically analyzed.

Results: Sixty *E. coli* strains (30 for pre treatment and 30 for post treatment sewage) were randomly selected from isolates. All ESBL-producing isolates showed resistance to three antibiotic classes and were MDR. For non-ESBL isolates, 70 and 90 percent were MDR for pre and post treatment sewage, respectively. Of isolates tested, 100% had at least one of resistance genes. The frequency of *bla* CTX-M-1 gene was significantly higher in isolates of post treatment sewage. The *bla* TEM gene was more common than other genes in ESBL-producing isolates.

Conclusion: The high rate of antibiotic resistance and resistance genes in *E. coli* isolates of hospital sewage, especially in post treatment is alarming. These data suggest that despite the widespread use of active sludge system to treat hospital sewage, they may not capable to adequately eliminate or reduce the antibiotic resistance strains of *E.coli*.

Background

Hospital sewage is one of the most spreading sources for the resistant strains of bacteria into environment. Bacteria that enter into hospital sewages have been exposed to a wide range of antimicrobial agents in hospital environments (1). By killing and preventing the growth of sensitive bacteria, antibiotics can select the strains with resistance genes and, in particular, new mutations. The resistance genes can be propagated and disseminated by the horizontal gene transfer between strains or different species of bacteria (2). *Escherichia coli* is one of the common causes of nosocomial infections such as urinary tract, gastrointestinal and blood infections. The resistant strains of this bacterium can survive in the hospital sewage and enter into environment, consequently, disseminate the resistant strains contained antibiotic resistance genes (3). The *E. coli* strains resistant to various antibiotics have been isolated from hospital sewages (4).

The most common antibiotics for the treatment of *E. coli* infections include the broad spectrum cephalosporins, carbapenems and fluoroquinolones (5). In recent years, resistance to these antibiotics has dramatically increased. Acquired resistance to beta-lactams is mainly attained by the Extended-spectrum beta-lactamases (ESBL) in *E.coli* (6). The *bla* CTX-M, *bla* TEM, and *bla* SHV are the most common types of ESBLs that have been reported in bacteria isolated from hospital sewage (7). The TEM and SHV beta-lactamases, by hydrolysis the molecules of beta-lactam antibiotics, make them inactive. These types of enzymes tend to have preferred substrates, such as third and fourth cephalosporin generations (8). ESBL genes are often located on plasmids, and this phenomenon can cause the rapid spread of these genes among strains and even different species of bacteria, in particular inside hospital sewage (4). Due to the rapid spread, the plasmid-mediated quinolone resistance has played an important role in the quinolone resistance of enterobacteriaceae in recent years (9, 10). The most common genes of this group are *qnr*A, *qnr*B and *qnr*S. The *qnr* genes code proteins that protect DNA by preventing the binding of quinolones to the DNA gyrase and tropoiosomerase IV enzymes (11).

Since the hospital sewage is an important source of spreading the resistant strains of *E. coli* to environment, the effective treatment of hospital sewage is important to eliminate these resistant strains (7). The aim of this study was to evaluate the antibiotic resistance and some common resistance genes of *E. coli* isolated from hospital sewage before and after treatment.

Methods

Bacteria isolation, identification, and Antibiotic susceptibility testing

This study was done on the isolates from hospital sewage treated by an activated sludge system in Imam Reza Hospital in Kermanshah city (West of Iran). The proposal of this study was approved by the Kermanshah University Ethical Committee. During 2 months, twenty times samplings were done to collect hospital sewage in sterile tubes (20 mL). Two sets of samples were collected simultaneously each time as pre and post treatment hospital sewage to find out the effect of sewage treatment process on antibiotic resistance profiles of *E.coli* strains. Samples were transferred to the microbiological laboratory of Infectious Diseases Research Center and Microbiology Department of Medical faculty.

The *E.coli* isolates were identified using bacteriological culture methods according to the standard procedures (12). The Eosin methylene blue (EMB) culture medium was used to grow both ESBL and non-ESBLs *E. coli* isolates. After confirming *E. coli* colonies, a sub-cultivation was performed on the Blood agar medium and two batches of growing colonies were stored at -70 °C for the rest of subsequent examinations. To determine the percentage of ESBL producing *E. coli* among total *E. coli* strains, 100 colonies of *E.coli* isolates grown and approved on antibiotic-free EMB were carefully picked up by toothpicks and cultured on EMB medium contained 1 mg/liter of each ceftazidime and cefotaxime (13). The ESBL producing *E. coli* colonies were subsequently confirmed by combined disk test as described (14). The average of three independent experiments was used to determine the percentage of ESBL producing *E.coli*.

Of the identified *E.coli* strains, 40 ESBL producing (20 for pre and 20 for post treatment) and 20 not ESBL producing isolates (10 for pre and 10 for post treatment) were randomly selected from all samples for further examinations. Antimicrobial susceptibility testing was done by the disc diffusion according to the standard method for a number of selected antibiotics classes, including Fluoroquinolone (ciprofloxacin), Clindamycin (clindamycin), Sulfonamides (Co-trimoxazole), Aminoglycosides (amikacin, gentamicin), Cephalosporin (ceftriaxone), Carbapenem (imipenem) and Penicillin (penicillin, piperacillin) (MAST, England)(14).

Identification of antibiotic resistance genes

The whole genome of ESBL-producing isolates was extracted by boiling method and used as DNA template for polymerase chain reaction (PCR). Specific primers were used to identify antibiotic resistance genes using PCR (Table 1). The PCR products were characterized on a 1% agarose gel electrophoresis contained gel-red stain along with a 100 bp DNA marker (SinaClon, Iran). The known strains of gram-negative bacteria from our Laboratory collection contained ESBL and *qnr* genes were used as controls (15, 16).

Table 1
The primers used.

Genes	Primer sequence (5' – 3')	Amplicon (bp)	Annealing Temperature (°C)	Reference
*qnr*A	TCAGCAAGAGGATTTCTCA	516	55	9
	GGCAGCACTATTACTCCCA			
*qnr*B	GATCGTGAAAGCCAGAAAGG	469	53	9
	ACGATGCCTGGTAGTTGTCC			
*qnr*S	ACGACATTCGTCAACTGCAA	714	53	9
	TAAATTGGCACCCTGTAGGC			
*bla*TEM	AGTGCTGCCATAACCATGAGTG	431	52	13
	CTGACTCCCCGTCGTGTAGATA			
*bla*CTX-M	TTTGCGATGTGCAGTACCAGTAA	544	58	13
	CGATATCGTTGGTGGTGCCAT			
*bla*CTX-M-1	CGTCACGCTGTTGTTAGGAA	780	55	5
	ACGGCTTTCTGCCTTAGGTT			
*bla*CTX-M-2	ATGATGACTCAGAGCATTCG	884	55	5
	TTATTGCATCAGAAACCGTG			
*bla*SHV-5	TATCGGCCCTCACTCAAGGA	231	58	3
	TGCTCATCATGGGAAAGCGT			
*bla*SHV-12	GCCGCGTAGGCATGATAGAA	677	59	3
	CGGCGTATCCCGCAGATAAA			

Statistical analysis

Antibiotic resistance patterns and the frequency of antibiotic resistance genes of isolates from pre and post sewage treatment samples were analyzed by Pearson Chi-Square test. The SPSS software version 20 with the significant level of $p < 0.05$ was used.

Results

ESBL production and Antibiotic susceptibility of isolates

On average, 15% percent of *E. coli* strains isolated from pre treatment and 19% from post treatment sewage were ESBL-producing, although this increase rate was not statistically significant ($p = 0.367$). The antibiotic resistance for pre and post sewage treatment isolates were determined (Table 2, 3). All 60 *E. coli* isolates were resistant to penicillin. The 100% of ESBL-producing isolates before and after the sewage treatment process were resistant to penicillin, ciprofloxacin and ceftriaxone. However, these isolates were mostly susceptible to Aminoglycosides. In general, in comparison to the pre treatment sewage isolates, in the post treatment isolates the resistance to gentamicin, clindamycin, imipenem and pipracillin was lower, but resistance to Co-trimoxazole and amikacin was higher ($p > 0.05$). In non-ESBL isolates, the rate of antibiotic resistance was higher for most antibiotics in post treatment isolates, but it was not statistically significant.

Isolates	Antibiotics	Pre treatment isolates (No. 20)			Post treatment isolates(No. 20)			p value
		Resistant No (%)	Intermediate No (%)	Susceptible No (%)	Resistant No (%)	Intermediate No (%)	Susceptible No (%)	
	Ciprofloxacin	20(100)	0(0)	0(0)	20(100)	0(0)	0(0)	1
	Clindamycin	17 (85)	3 (15)	0(0)	16(80)	4(20)	0(0)	0.677
	Co-trimoxazole	18 (90)	0(0)	2(10)	19 (95)	0(0)	1(5)	0.548
	Amikacin	7(35)	2 (10)	11 (55)	5(25)	5(25)	10 (50)	0.435
	Gentamicin	8(40)	3 (15)	9(45)	7(35)	2(10)	11(55)	0.792
	Ceftriaxone	20 (100)	0(0)	0(0)	20(100)	0(0)	0(0)	1
	Imipenem	12 (60)	2 (10)	6(30)	9(45)	4(20)	7(35)	0.557
	Penicillin	20 (100)	0(0)	0(0)	20(100)	0(0)	0(0)	1
	Piperacillin	17 (85)	3 (15)	0(0)	16 (80)	4(20)	0(0)	0.677

Table 2
Antibiotics susceptibility of ESBL-producing *E. coli* isolates from pre and post sewages treatment phases.

Isolates	Antibiotics	Pre treatment isolates (No. 10)			Post treatment isolates(No. 10)			p value
		Resistant No (%)	Intermediate No (%)	Susceptible No (%)	Resistant No (%)	Intermediate No (%)	Susceptible No (%)	
	Ciprofloxacin	1 (10)	3(30)	6 (60)	3(30)	2 (20)	5(50)	0.524
	Clindamycin	8(80)	2 (20)	0	8(80)	2 (20)	0	1
	Co-trimoxazole	8(80)	1 (10)	1 (10)	9(90)	0	1 (10)	0.589
	Amikacin	2 (20)	2 (20)	6 (60)	1 (10)	5(50)	4(40)	0.364
	Gentamicin	1 (10)	5(50)	4(40)	1 (10)	6 (60)	3(30)	0.89
	Ceftriaxone	4(40)	2 (20)	4(40)	7(70)	1 (10)	2 (20)	0.403
	Imipenem	1 (10)	2 (20)	7(70)	1 (10)	4(40)	5(50)	0.607
	Penicillin	10(100)	0	0	10(100)	0	0	1
	Piperacillin	8(80)	2 (20)	0	8(80)	1 (10)	1 (10)	0.513

Table 3
Antibiotics susceptibility of non ESBL-producing *E. coli* isolates from pre and post sewages treatment phases.
Multidrug resistant strains (MDR) were determined by resistance to at least three antimicrobial classes. All ESBL-producing isolates (20 pre plus 20 post treatment) showed resistance to three antibiotic classes and were therefore regarded as MDR (Fig. 1). However, among non-ESBL isolates, 70 and 90 percent were MRD for pre and post treatment sewage, respectively.

Frequency of antibiotic resistance genes of isolates

The results of antibiotic resistance genes and their frequency for ESBL producing isolates were determined (Table 4). For post treatment sewage isolates, the frequency of the *bla* CTX-M gene was 25% less than pre treatment isolates. In contrast,

the frequency of *bla* CTX-M-1 and *bla* CTX-M-2 was higher by 15% for post treatment sewage isolates. In case of *qnr* and *bla* SHV genes, there were no remarkable changes in their frequency rates for pre and post treatment sewage isolates.

Table 4
The rate of antibiotic resistance genes in ESBL-producing isolates from pre and post sewage treatment phases.

Antibiotic resistance genes	Pre treatment isolates (No. 20)		Post treatment isolates (No. 20)		p value
	Contain genes No (%)	Lack of genes No (%)	Contain genes No (%)	Lack of genes No (%)	
*qnr*A	0 (0)	20 (100)	0 (0)	20 (100)	1
*qnr*B	2 (10)	18 (90)	0 (0)	20 (100)	0.147
*qnr*S	0 (0)	20 (100)	1 (5)	19 (95)	0.311
*bla*TEM	18 (90)	2 (10)	19 (95.0)	1 (5.0)	0.345
*bla*CTX-M	18 (90)	2 (10)	13 (65)	7 (35)	0.058
*bla*CTX-M-1	14 (70)	6 (30)	17 (85)	3 (15)	0.256
*bla*CTX-M-2	12 (60)	8 (40)	15 (75)	5 (25)	0.311
*bla*SHV-5	0 (0)	20 (100)	0 (0)	20 (100)	1
*bla*SHV-12	1 (5)	19 (95)	0 (0)	20 (100)	0.311

Discussion

E.coli is one of the bacteria isolated from hospital sewage and can be a source for dissemination of antibiotic resistance genes (17). Since antibiotic resistance genes are often located on plasmids or transposons that capable to transfer to other microorganisms, the presence of these genes in hospital sewage can be a serious threat to public health (5). Therefore hospital wastewater should be properly collected, treated and returned to the natural environment (1).

In our study, all pre and post sewage treatment isolates were totally resistant to penicillin and highly resistant to other classes of antibiotics indicating the presence of high resistant strains of *E. coli* in hospital sewage. The both pre and post sewage treatment isolates was mostly susceptible to aminoglycosides (gentamicin and amikacin). The resistance rate of ESBL-producing isolates to most antibiotics tested was slightly lower in post sewage treatment isolates; however, the resistance rate in non-ESBL isolates for most antibiotics was higher in post treated isolates. It has been shown that in many cases, sewage treatment process cannot significantly eliminate resistant strains or resistance genes (7). Furthermore, there are some reports that showed the increase in both antibiotic resistance rates and antibiotic resistance genes for post treatment sewage isolates (18, 19). For instance, research has shown that sewage treatment process caused an increase in antibiotic resistance of *E. coli* to nalidixic acid, sulfamethoxazole, cephalexin and ceftriaxone (20). These results suggest that the effect of sewage treatment process on various classes of antibiotics may be different.

The rate of ESBL-producing *E.coli* strains have been reported higher than our results which may reflect difference in the regional frequencies of resistance genes (7, 21). In the present study, the rate of ESBL-producing *E. coli* strains was slightly higher in the post treatment sewage isolates, which may reflect the transferring of EBSL among bacteria within sewage. Since the ESBL genes mostly located on plasmid in *E.coli* the horizontal transferring of ESBL genes between bacteria is reasonable. The *bla* SHV, including *bla* SHV-5 and *bla* SHV-12 have been found in hospital sewage isolates (8). A high incidence of SHV strains in untreated wastewaters in Australia has been reported, possibly due to the transmission of these genes in liquid environments (6). In our study, the frequency of *bla* SHV-5 and *bla* SHV-12 genes showed no significant changes in isolates of pre and post treatment sewage. The rate of CTX-M ESBL subgroups are raising among enterobacteriaceae isolates (22).

The low effect of sewage treatment processes on *bla* CTX-Ms can be attributed to the presence of this group in a wider range of gram-negative and even gram-positive microorganisms present in sewage. The majority of the microorganisms entering the sewage are gram negative with intestinal origin (23). The ESBLs genes in the presence of antibiotics can give bacteria a selective and competitive survival advantage inside hospital sewage (24). A research on the sewage treatment effect on resistance to third-generation cephalosporins in the enterobacteriaceae showed that the frequency of the *bla* CTX-M gene in the post treatment sewage isolates increased because of easily transmission of these genes among gram-negative bacteria (25). It has been shown that the prevalence of *bla* CTX-M-1 and *bla* CTX-M-2 genes increased by 15% among isolates of post treatment sewage process (23). These findings are consistent with our results for the increased rate of *bla* CTX-M-1 and *bla* CTX-M-2 genes after swages treatment. Studies on hospital sewage isolates have also indicated a low frequency of *qnr* genes. The *qnr* genes can be easily transferred through plasmids to other bacteria and, together with chromosomal mutations, can result in the high levels of resistance to fluoroquinolones (6, 15, 26). In a large study in China (2002–2005), the frequency of *qnr*A, *qnr*B and *qnr*S genes in 514 *E. coli* isolated from hospital sewage was 0.4, 1.2 and 2.7%, respectively (6) which support our results for the low frequency of *qnr* genes among isolates.

Conclusions

In conclusion, the dissemination of antibiotic resistance strains and resistance genes of *E. coli* through hospital sewage is a serious concern for the efficacy of antibiotics globally. As it has been previously reported, despite the widespread use of active sludge system to treat hospital sewage, they are not able to adequately reduce the antibiotic resistance strains of bacteria (27). Therefore, the efficacy of hospital sewage plants requires regular surveillance to find out their impact on the elimination of resistant strains of bacteria.

Abbreviations

E. coli:*Escherichia coli*; ESBL: extended-spectrum beta-lactamases; PCR: Polymerase chain reaction; EMB: Eosin methylene blue; MDR: Multidrug resistant strains.

Declarations

Ethics approval and consent to participate

The protocol was approved by the Ethics Committee of Kermanshah University of Medical Sciences (97297).

Consent to publish

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article (Table 2, Table 3, Table 4, Fig. 1)

Competing interests

The authors declare that they have no competing interests to disclose.

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Authors' Contributions

AK participated in study design, sample collection, processing, bacterial culture, data analysis, acquisition of fund and preparing the manuscript. JSZ participated in sample collection, processing and bacterial identification. AB participated in data analysis and interpretation and assisted in manuscript preparation. FNZ participated in bacterial culture, identification. RC participated in bacterial identification and PCR. All authors read and approved the final manuscript.

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Figures

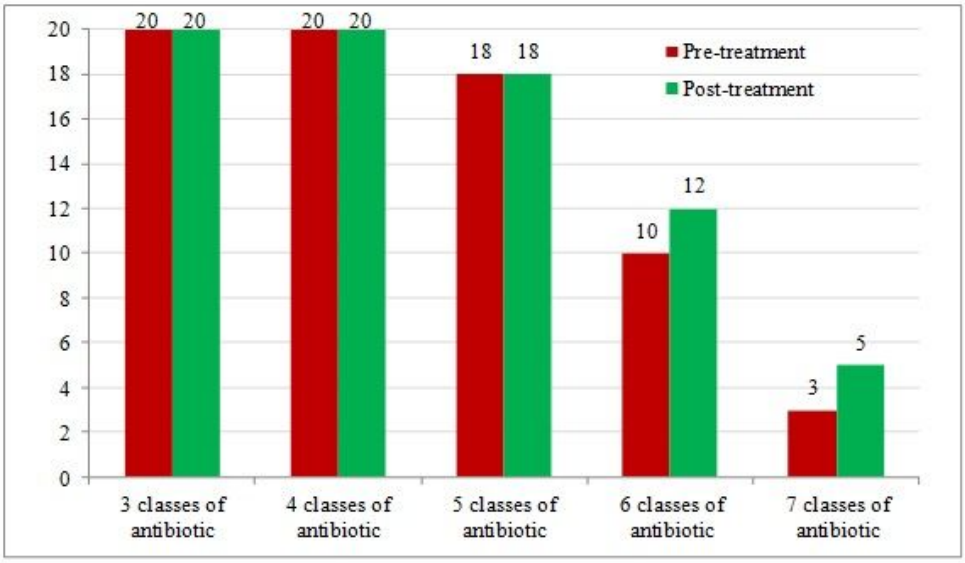

Figure 1

Frequency of multidrug-resistant of EBSL-producing isolates for pre (n=20) and post treatment sewage (n=20).