Original Article

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Evaluation of Antibiotic Resistance in Extended-spectrum Beta-lactamase (ESBL) Genes in the E. coli Isolates of Urinary Infections

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ABSTRACT

BACKGROUND AND OBJECTIVE: Extended-spectrum beta-lactamase (ESBL) enzymes hydrolyze cephalosporins and penicillins. This study aimed to determine the frequency of Escherichia coli strains producing SHV, TEM and CTX-M β-lactamase genes and their association by inducing antibiotic resistance.

METHODS: In this cross-sectional study, 55 E. coli strains were isolated from urinary samples and cultured on eosin methylene blue (EMB) agar and CHROMagar. After biochemical examinations, antibiotic susceptibility test was performed using the disk-diffusion method according to the guidelines of the Clinical & Laboratory Standards Institute (CLSI). In addition, the presence of blaCTX-M, blaTEM and blaSHV genes was evaluated using specific multiplex polymerase chain reaction (PCR) primers.

FINDINGS: In this study, the highest antibiotic resistance was observed against penicillin and erythromycin (96% and 94.5%, respectively), while the highest susceptibility was reported for ciprofloxacin and imipenem (67.2%). Out of 55 samples, 26(47.27%) had the TEM gene, and CTX-M gene was detected in 41 (74.54%) samples. Moreover, TEM and CTX-M genes were simultaneously detected in 32.72% of the samples, while in six samples (10.9%), neither of these genes were present. The SHV gene was not detected in any of the samples.

CONCLUSION: According to the results of this study, the production of ESBL was identified in 70% of the investigated E. coli isolates. Therefore, accurate and timely medical care, as well as the use of appropriate antibiotics, is required to prevent the outbreak of ESBL-producing E. coli strains.

KEY WORDS: Escherichia coli, Extended-Spectrum Beta-Lactamase (ESBL), Disk-Diffusion, Multiplex PCR..

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Introduction

Escherichia coli is the most common cause of urinary infections (1). E. coli strains are usually divided into four phylogenetic groups of A, B1, B2 and D (1, 2). Uropathogenic E. coli falls under the B2 category and is associated with gender (1); female individuals within the age range of 10-40 years tend to be more commonly affected by community-acquired infections (3, 4). Beta-lactam antibiotics are a broad class of antibiotics, which are all categorized into one group because of their similar core structure (5). β-lactamase antibiotics act through obstructing the completion of peptide

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and glycan synthesis by inhibiting the production of cross-bridges. This leads to the interruption of cell wall biosynthesis and cell function, eventually causing deformation and turgidity (6). Microorganisms produce enzymes, which results in the destruction of active drugs; such examples are the β-lactamases produced by gram-negative bacteria (6). β-lactamases belong to the family of hydrolytic enzymes, which are able to hydrolyze and convert β-lactamase antibiotics into derivatives without antibacterial properties (7). Varieties of genes are responsible for the expression of β-lactamases and they could be located on the chromosome or plasmid surface initially. The resistance of gram-negative bacteria is commonly due to the presence of different groups of β-lactamase enzymes, which may be chromosomal or plasmid. In gram-negative bacteria, the production of β-lactamase enzymes has been mainly associated with the presence of bacteria such as Enterobacteriaceae, Haemophilus influenzae, Moraxella catarrhalis, Neisseria gonorrhoeae, Vibrio cholerae and Pseudomonas aeruginosa (8). Furthermore, plasmid enzymes with high-level expression, such as SHV and TEM, are considered to be among these factors (7). In order to be effective, β-lactamase inhibitors need to have a β-lactam ring for establishing an acyl-enzyme intermediate in case of β-lactamase enzyme attack, and become hydrolyzed slowly (8). Among different β-lactamase enzymes, CTX-M is known to affect a broader range of β-lactamase antibiotics. The first endemic case of CTX-M was reported in Latin America and Eastern Europe; there were also reports of the extensive spread of this gene in Western Europe in countries such as Greece, France, England, Spain and Mongolia after the year 2000 (9). One of the strains of E. coli, which is resistant against cefotaxime, is referred to as CTX-M-1; this strain lacks any TEM or SHV characteristics. In addition, CTX-M-1 performs hydrolytic function toward cefotaxime (10). The TEM enzyme is the first-known ESBL widely developed in the family of Enterobacteriaceae and is considered as the most frequent type of β-lactamases. Moreover, this enzyme has been transferred to other bacteria including Haemophilus influenzae and Neisseria gonorrhoeae (4). In several of these strains, the gene coding for SHV β-lactamase is located on the chromosome; however, this gene gradually enters the plasmid and spreads in different bacterial strains (11).In recent years, the development of resistance against the antibiotics used for the bacteria of the Enterobacteriaceae family has raised concerns among medical experts since it results in unsuccessful treatments. This study aimed to determine the frequency of E. coli strains producing ESBL genes, such as SHV, TEM and CTX-M, using the multiplex PCR method. Furthermore, the association between these genes was evaluated through the induction of antibiotic resistance in the E. coli strains isolated from urinary infections

Methods

In this cross-sectional study, based on the previous studies and a confidence interval of 95%, 150 urine samples were collected from the clinical laboratories of Tehran, Iran and transferred to our laboratory. The samples were cultured on blood agar, MacConkey agar, eosin methylene blue (EMB) agar and CHROMagar and were incubated at 37°C for 24 hours. After confirming the presence of E. coli, biochemical tests, including IMViC and Triple Sugar Iron Sugar (TSI), were performed, and 55 bacterial E. coli strains were identified. Following that, the disk-diffusion method was used for antibiograms (e.g., Kirby-Bauer) according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) (12). Several bacterial colonies were taken by Anas and resolved in sterile saline until becoming equal to half the turbidity of the McFarland standards. Afterwards, the colonies were cultured on Mueller-Hinton agar. In addition, antibiotic discs were placed on the culture media at

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specific distances and were incubated at 37°C. The results were recorded after 24 hours (12, 13).For this study, antibiotic discs including cefotaxime 30 µg (CTX), erythromycin 15 µg (E), gentamicin 10 µg, tetracycline 30 µg (TE), co-trimoxazole (trimethoprim/ sulfamethoxazole [TMP/SMX]) 25 µg, ampicillin 25 µg (AM), imipenem 10 µg (IPM), amikacin 30 µg (AN), ciprofloxacin 30 µg (CP) and penicillin 10 µg were purchased from HiMEDIA Laboratories Pvt. Limited, India. Moreover, the standard E. coli strain of ATCC 35218 was used as the positive control to evaluate the quality control of the examinations. In this study, DNA extraction was performed using the kit produced by the Iranian Biological Resource Center (MBK0041). In this test, the 15-second initial denaturation step was performed at 95°C, followed by a 30-second denaturation step at 94°C. Afterwards, the connection step was performed for 40 seconds at 61°C, the extension step lasted 2 minutes at 72°C (cycles: 35) and the final extension step was carried out for 10 minutes at 72°C (14). The primers used for the multiplex PCR in this study are shown in table 1. The combinations used for this test are as follows: 1) 12.35 µl distilled water; 2) 2 µl 1X PCR buffer; 3) 0.7 µl MgCl2; 4) 0.5 µl DNTP mix (5 mM); 5) primers 0.6 µl each; 6) 0.25 µl Taq polymerase enzyme and 7) 3 µl of DNA sample in a total volume of 20 µl (14).

In this study, multiplex-PCR was performed using the BioRad device. To evaluate the outcomes, the samples were placed on 2% agar gel and were stained using the Bio-Rad Duac gel imaging system. Statistical analysis of the data was performed using descriptive-statistics in SPSS V.19.

Results

In this study, the highest level of antibiotic resistance was reported against penicillin and erythromycin (96% and 94.5%, respectively), and the greatest susceptibility was observed toward ciprofloxacin and imipenem (67.2%) (table 2).

Table 1. Primers used in Multiplex-PCR Test

Multiplex-PCR Test: Primer Sequence (5' to 3')	Product Length (bp)	Target Gene
ATG CGT TAT ATT CGC CTG TG	747	bla-SHV.SE
TGC TTT GTT ATT CGG GCC AA		bla-SHV.AS
TCG CCG CAT ACA CTA TTC TCA GAA TGA	445	TEM-164.SE
ACG CTC ACC GGC TCC AGA TTT AT		TEM-165.AS
ATG TGC AGC ACC AGT AAA GTG ATG GC	593	CTX-M-U1
TGG GTA AAG TAA GTG ACC AGA ATC AGC GG		CTX-M-U2

Table 2. Susceptibility of the Isolates to Different Antibiotics in the Disk-diffusion Method

Antibiotic	Susceptibility N(%)	Average Susceptibility N(%)	Resistance N(%)
Cefotaxime	7(12.8)	6(10.9)	42(76.3)
Erythromycin	-	3(5.4)	52(94.6)
Amikacin	4(7.3)	47(85.4)	4(7.3)
Tetracycline	13(23.6)	4(7.3)	38(69.3)
Co-trimoxazole	9(16.3)	7(12.7)	39(71)
Ampicillin	-	7(12.8)	48(87.2)
Ciprofloxacin	37(67.2)	2(3.7)	16(29.1)
Imipenem	37(67.2)	4(7.3)	14(25.5)
Penicillin	-	2(3.7)	53(96.3)
Gentamicin	0	25(45.4)	30(54.6)

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Out of 55 investigated samples, 26 had the TEM gene, and CTX-M gene was detected in 41 samples. In addition, both TEM and CTX-M were present in 18 samples, while neither of them was found in six samples (fig 1). Also, none of the studied samples contained the SHV gene (fig 2). According to the results of the correlation coefficient, there was a significant association between CTX-M and imipenem at 5%, and CTX-M and cefotaxime at 1%. The presence of this gene caused the bacterial resistance to increase toward imipenem antibiotic, and there was a more significant increase in the bacterial resistance against cefotaxime.

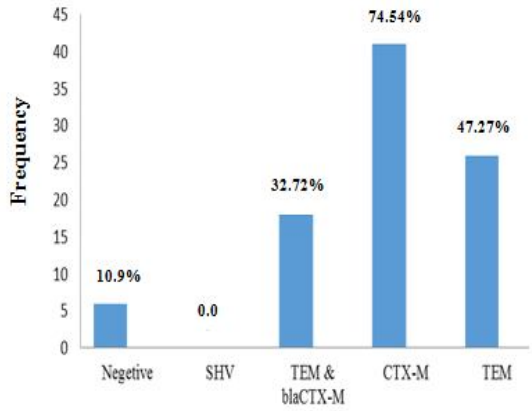


Figure 1. Distribution of the Frequency of the Studied Genes in terms of Number and Percentage

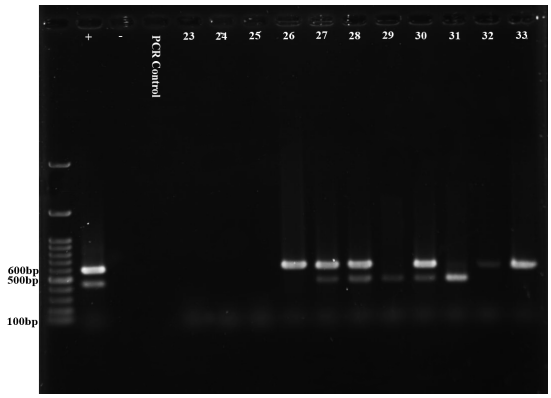


Figure 2. Results of Multiplex-PCR from left to right: 50 bp Marker, Positive Control, Samples (No. 27-31) with TEM (445 Bp) Gene, Samples (No. 26, 27, 28, 30, 33) with CTX-M (593 Bp) Gene

Discussion

According to the results of this study, antibiotic resistance against penicillin, cefotaxime, gentamicin, erythromycin, tetracycline, co-trimoxazole and ampicillin was observed to be over 50%. To date, E. coli has been known as the predominant microorganism involved in 80-90% of all urinary infections across the world (3). In one study, Rafati et al. reported E. coli to be the main cause of 20% of neonatal infections (15). In another study performed on 188 urinary samples in Brazil, Dias Neto et al. isolated E. coli strains from 26% of the samples and reported the highest resistance of the bacteria against ampicillin (27%) (16). Similarly, the results obtained by Tamberkar et al. indicated the highest antibiotic resistance toward ampicillin (87%) and co-trimoxazole (91%), respectively, while the lowest resistance was found to be against nitrofurantoin (29%) (17). In another study conducted by Tankhiwale et al. on E. coli strains, the greatest antibiotic resistance was reported in co-trimoxazole (82%) and ampicillin (79.9%), respectively, while the lowest resistance was reported to be against nitrofurantoin (38%) and ceftizoxime (41.3%), respectively (18). Furthermore, the findings of Zamanzad et al. were indicative of a high resistance of E. coli strains towards ampicillin and co-trimoxazole (19). In several studies performed in Europe and North America during the 1990s, antibiotic resistance to ampicillin accounted for more than 30% (20). According to the study by Tadesse et al., resistance to antibiotics such as ampicillin, sulfonamides, tetracycline and gentamicin has been on a rising trend within the past decades. In addition, multidrug-resistant E. coli has been a major health concern since its prevalence rose from 7.2% to 63.6% during the 1950s-2000s, and the most common type of cooperative resistance phenotype has been observed in tetracycline and streptomycin (29.7%), as well as tetracycline and sulfonamides (29%) (21). In another study in this regard, Bouzari et al. reported the highest resistance against

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ampicillin, and the greatest susceptibility among different bacterial strains was toward nalidixic acid, gentamicin and ciprofloxacin (22). Similarly, Mirsalehian et al. observed the highest level of resistance against ampicillin (98.05%), while the lowest resistance was reported toward imipenem (2.91%) (23). In the present study, multi-drug resistance was estimated at 60% to five drugs or more. In another study conducted in Taiwan in 2005, the antibiotic resistance of E. coli strains, which were isolated from hospital infections using the minimal inhibitory concentration (MIC) method, was mostly against ciprofloxacin (37.3%); in Turkey, the level of this resistance has been reported to be about 33% (24, 25). According to the results obtained by Kiffer et al., the frequency of resistant E. coli strains isolated from different hospital sections was reported to be 14.6% against cefotaxime (26); in China, the level of this antibiotic resistance was estimated at 2.7% (27). In the current study, approximately 7.3% of the E. coli strains were resistant to amikacin. Moreover, imipenem resistance was calculated to be 25.5%, while there are no reports on this antibiotic in the studies conducted in other countries. In Turkey, 8% of the isolated E. coli strains from the intensive care unit (ICU) of different hospitals were observed to be resistant to imipenem (25). he inconsistencies between the findings of the aforementioned researches and the present study could be due to the patterns of antibiotic use, geographical diversities, differences in resistance patterns in different areas and indiscriminate use of antibiotics in our country. In the present study, 89% of the E. coli strains were identified as the producers of ESBL. In a study by Ling et al. performed in China, the frequency of ESBL production in the E. coli strains was reported to be 16% (27). In another study by Duttaroy et al. conducted in India, 187 E. coli strains and Klebsiella were investigated, and 53 isolates (29.1%) were identified as the producers of ESBL (28). In another study conducted in France on 3062 isolates of Enterobacteriaceae, 16.2% of the E. coli strains were found to be producers of ESBL (29). In an extensive study performed during 1998-2000 in different countries, the resistance level of β-lactamase-producing E. coli strains was calculated for seven antibiotics. According to the results, there was a significant level of antibiotic resistance in these bacterial strains (30). These bacteria tend to spread in a similar manner to other hospital infections (e.g., contaminated hands of hospital personnel or contaminated medical devices such as urinary, vascular, and arterial catheters). Enzymes such as CTX-M, SHV and TEM vary in prevalence among different members of the Enterobacteriaceae family. In the present study, the prevalence of ESBL enzymes in the E. coli strains was reported to be 74.5% for CTX-M, 0% for SHV and 47.2% for TEM. In the study by Mirsalehian et al., 60% of the E. coli samples isolated from the patients admitted in the ICU were producers of the ESBL enzymes (23). In the study by Melzer et al., 60.8% of the bacteria that caused death were functioning due to the presence of ESBL-producing E. coli strains (31). According to the findings of Tasil et al. in Turkey, the production of ESBL enzymes by E. coli strains was estimated at 17%, while the results obtained by Villagas in Columbia reported this rate between 3.3-4.7% (32, 33). On the other hand, the study of Zhou conducted in Shanghai indicated that 47.4% of the isolated E. coli from the studied patients were producers of the ESBL enzymes (34). In the comparison between the results of the current research and the aforementioned studies, it was concluded that the proportion of the ESBL produced by the isolated strains of bacteria varied in each country, as well as different hospitals, depending on the infection control system and treatment methods applied in each health care center.In the present study, 49 samples of positive CTX-M were identified among the investigated isolates indicating a total prevalence of 74.5% for this gene. This finding was consistent with the prevalence reported in similar resistant isolates in other studies conducted in different countries; for

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instance, the prevalence of CTX-M was estimated at 44.1% in the resistant isolates investigated in South Korea (35). According to the study by Eisner et al. (2006) in Austria, the prevalence of CTX-M was determined at 58% in the E. coli strains that could produce this gene (36). Furthermore, Monstein reported that SHV, CTX-M and TEM genes were identified in 3, 2 and one samples, respectively, while all these genes were found to be frequent in one sample, and CTX-M and TEM were simultaneously detected in 13 samples (14). In another study by Soltan Dallal et al., 79% of the E. coli isolates could produce ESBL among 161 samples, which is similar to the results of the current study (89%) (37).Moreover, Soltan Dallal et al. investigated the prevalence of the TEM gene, and the resistance rate to this gene was reported to be 57.8%, which is consistent with the findings of the present study; according to our results, the resistance to the TEM gene was 47.2% (37). This consistency between the findings of these studies could be due to the similar sources of the investigated bacteria since the samples used in both studies were collected from different health care centers of Tehran, Iran. In another study conducted by Pak-Leung Ho in Hong Kong, six samples out of 46 investigated E. coli isolates were observed to be producers of ESBL. Moreover, seven samples among the resistant bacteria were observed to contain CTX-M plasmid (38). In the current study, the SHV gene was not identified in any of the isolated E. coli strains. According to the study by Shahcheraghi et al. conducted to identify the SHV gene in the isolates of E. coli and Pseudomonas aeruginosa, the prevalence of this gene accounted for 6% and 28% of the studied bacteria, respectively; this finding is consistent with our results (11). On the other hand, 47.2% of the investigated samples in the present study contained the TEM gene, while the prevalence of TEM was estimated at 52.7% in a study in Turkey performed on the intestinal bacteria samples provided from a hospital. According to the alarming statistics in Taiwan, the prevalence of this gene was estimated at 81% among different isolates of E. coli, Klebsiella pneumoniae and Enterobacter (24). In the study by Shahcheraghi et al. performed on E. coli strains, the prevalence of the TEM gene was reported to be 24%, while it was estimated at 48.7% in the study by Zamanzad and 84.6% in the study by Mirsalehian et al. (1, 11, 19, 23). In conclusion, the results of the present study indicated that in order to achieve success in patient treatment and prevent antibiotic resistance development, different patterns of antibiotic resistance need to be accurately determined according to CLSI guidelines

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