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Isolation of Beta-Lactamase Producing Genes (SHV, CTX-M1, CTX-M2, CTX-M3) in *Escherichia Coli* Isolated from Pregnant Woman Patients

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ABSTRACT

Background: In recent decades, extended spectrum beta-lactamase (ESBL) generating bacteria have increased universally. Among the most important causative agents of nosocomial infections throughout the world, *Escherichia coli* as main ESBL-producing bacteria are so highly regarded. Trends in the treatment of infections by such bacteria have led to a global concern.

Methods: All strains were cultured and identified by the Clinical Microbiology Laboratory and were recovered from blood and urine cultures. *In-vitro* presence of ESBL was confirmed with Clinical and Laboratory Standard Institute double disc and PCR for CTX-M1, CTM-M2, and CTX-M3 method.

Results: The results of this study showed that Escherichia coli samples were resistant to AN (42.85%), GM (28.57%), AM (35.71%), AMC (35.71%), CZ (35.71%), and AZM (50%) antibiotics. While the most susceptible to antibiotic was ampicillin (64.28%), the least resistance to antibiotics was gentamicin.

Conclusion: The current situation of multiple bacterial antibiotic resistances has become a worrisome issue in UTI. Multi-drug-resistant *E. coli* can be readily encountered in hospital settings during daily clinical practice, and urologist should act timely. The management of such infections is extremely important for the future, with particular reference to prevention of new antibiotic resistance patterns.

Introduction

Urinary tract infection (UTI) refers to the presence of microbial pathogens within the urinary tract and is one of the most common infections in outpatient and hospitalized patients.[1] Urinary tract infections are more common in women than in men. So that almost half of the women have experienced at least one urinary tract infection during their lifetime. Studies in different societies indicate that gram negative bacilli are the most common etiologic factor in UTI and among them, *E. coli* accounts for more than 80% of cases of acute infections in the urinary tract. [2]

Typically, *E. coli* strains are divided into four phylogenetic groups A-B1-B2-D.[3] Uropathogen *E. coli* is more commonly classified as Group B2 and is related to gender[4]. Most infections in the community occur in women less than 10 years of age or in women aged 20 to 40 years [5]. Beta-lactams are a bunch of antibiotics that fall into a bundle due to their common central building.[6]

Beta-lactam antibiotics act to inhibit the formation of transverse bridges due to the prevention and suppression of the peptide and glycans that this action disrupts the cellular biosynthesis and cellular actions, resulting in cell deformation and lysis.[7]

Microorganisms produce enzymes that destroy active drugs, such as beta-lactams produced by gram-negative bacteria.[7] Beta-lactams are a family of hydrolytic enzymes that, by hydrolysis of beta-lactam antibiotics, convert them into antibacterial-free derivatives.[8]

The responsible genes for beta-lactamase are diverse and can either be located on the chromosome or at the plasmid level. Most of the resistance to gram-negative bacteria is due to beta-lactamase enzymes which have a variety of enzymes that are chromosomes and plasmids. In gram-negative bacteria, the production of beta-lactamase enzymes has been reported extensively by Enterobacteriaceae, Haemophilus influenza, Moraxella, Neisseria gonorrhea, Vibrio cholera and Pseudomonas aeruginosa[9]. The TEM and SHV enzymes, which are plasmid and widely distributed, are included in this group.[8] CTX-M enzymes are effective on a wider range of β-lactams compared to other members. The purpose of this study was to isolate beta-lactamase producing genes (SHV, CTX-M1, CTX-M2, CTX-M3) in *Escherichia coli* isolated from pregnant woman patients in Zabol.

Materials and Methods

Isolation of Bacterial: This descriptive cross-sectional study was performed by Zabol University of Medical Sciences in Imam Khomeini and Ali ibn Abi Talib Hospital, from urine samples of patients referred. A total of 14 positive samples were obtained. All positive culture specimens were tested on EMB and Blood Agar medium. A small amount of liquid was transferred to the above environments under sterile conditions and incubated for 24 to 48 hours at 37°C. After colony growth, they were identified by coloring morphology and diagnostic tests which had *E. coli* isolates. Samples were measured through disc diffusion method in terms of resistance to antibiotics which included AZM-AN-CZ-AMC-AM.

MIC: MIC testing was performed on the Muller Hinton Broth medium in microplate and from Segma's antistatic agents. Based on the definition of MIC, it is a concentration of antigens that stop bacterial growth. A suspension of bacteria that was prepared as small as McFarland, there are as many as 15 million bacteria per millimeter in the suspension.

MIC for antibiotics cefazolin, ceftriaxone, ceftazidime, ampicillin, ampicillin and cefotaxime were performed by microplate method in different antibiotic dilutions. Microplate method was used for dilution of 2512-625-625-6-6-32- 16-8-4-2-1 μg / ml. After obtaining Stoke volume (5120), dilution of antibiotic was made in the Hull Broth Mueller environment and bacterial suspensions were added to various dilutions. After mixing the suspensions in anti-odorant dilutions, they were incubated in the microplate wells for 18 hours

and placed at 37°C in the incubator. After this time, the turbidity formed in the wells caused by bacterial growth was observed and MIC dilutions were read. By observing the turbidity in each well, the previous well was considered as the minimum inhibitory concentration.

PCR reaction: In order to detect OXA gene β-lactamase, genomic DNA of isolates was performed by using phenol-chloroform method. To identify the beta-lactamase gene, SHV, CTX-M, CTX-M2, CTX-M3 was performed using primers of Table 1. Finally, the PCR reaction was carried out in a volume of 25 µl containing 12 µl Master mix, 1 µL of each primer, 3 µl of DNA and 8 µl of sterilized deionized water, according to the primer temperatures in Table 2. The PCR product was electrophoresed on the presence of the gene on 2% agarose gel and finally, stained with ethidium bromide, and photographed and captured with ultraviolet UV.

Table 1. Primers for each gene

Gene name	Primer
SHV F	AAGATCCACTATCGCCAGCAG
SHV R	ATTCAGTTCCGTTTCCCAGCGG
CTX-m1 F	GACGATGTCACTGGCTGAGC
CTX-m1 R	AGCCGCCGACGCTAATACA
CTX-M2f	F-GCGACCAGGTTAACTACAATCC
CTX-M2R	R-CGGTAGTATTGCCCTTAAGCC
CTX-M3F	F-CGCTTTGCCATGTGCAGCACC
CTX-M3R	R-GCTCAGTACGATCGAGCC

Statistical Analysis: Data were analyzed by SPSS software version 20 using descriptive statistics.

Results

The results of this study showed that *Escherichia coli* samples were resistant to AN (42.85%), GM (28.57%), AM (35.71%), AMC (35.71%), CZ (35.71%), and AZM (50%) antibiotics. While the most susceptible to antibiotic was ampicillin (64.28%), the least resistance to antibiotics was gentamicin (Table 1).

The minimum inhibitory concentration of different antibiotics against Escherichia coli was investigated, so that the lowest inhibitor concentration for antibiotic Ceftazidime was 8 µg/ml and the highest inhibitory concentration was 32 µg/ml. The lowest inhibitory concentration for Imipenem antibiotic was 256 µg/ml, two strains were inhibited at this concentration and the lowest was 4 µg/ml, the highest inhibitory concentration for ampicillin antibiotic was 256, with 4 strains being inhibited at this concentration, and the lowest was 8 µg/ml, with 2 strains inhibited. The highest inhibitory concentration of cefazolin antibiotics was 256 that one strain has grown at all concentrations of this antibiotic. The highest and lowest antibiotic concentrations of ceftriaxone were 256 and 8 µg/ml respectively, while 5 strains of E. coli did not grow in any concentration of cefotaxime antibiotics and the highest inhibitory concentration was 128 µg/ml and the lowest was 8 µg / ml (Table 2).

The results of this study showed that of the 14 samples tested, 3(21.42%) for the SHV gene, 6(42.85%) for the CTX-M1 gene, 4(28.57%) for the CTX-M2 and 1(7.14%) for the CTX-M3 gene were positive. These results indicate the low incidence of SHV, CTX-M1, CTX-M2, CTX-M3 gene in *E.coli*. Figure 1 shows positive examples of ESBL genes.

Table 2. The temperatures of the PCR reaction for each gene

	PCR reaction steps				
Primer	Denaturation	Annealing	extension	Number of cycles	Reaction product
SHV					
CTX-M1	94 180 s	94 60s	55 30 s	35	499
CTX-M2	94 180s	94 60s	55 30 s	35	351
CTX-M3	94 180 s	94 60s	55 30 s	35	307

Table 3. Percentage of antibiotic resistance of *E. coli* isolated from patients

	AN	GM	AM	AMC	CZ	AZM
R	42.85	28.57	35.71	35.71	35.71	50
S	57.14	50	64.28	57.14	42.85	35.71
I	0	21.42	0	7.14	21.42	14.28

Discussion

In a study by Mohajeri et al., investigated the production of broad-spectrum beta-lactamases in *E. coli* isolated from urinary tract infections and antibiotic resistance patterns in Kermanshah, the results showed that the highest sensitivity was observed for imipenem (100%), amikacin (97%), nitrofurantoin (95.5%), gentamicin (85%), cefepime (75%), ceftazidime (74%), ofloxacin (73.5% %), Ciprofloxacin, ceftriaxone and aztreonam (71%) and cefotaxime (70%), on the other hand, the highest resistance rates were observed for ampicillin (77%), carbenicillin (76%), piperacillin (74%) and cotrimoxazole (62.5%), respectively.

Resistance to third-generation cephalosporins was 63-75%. Fifty-four cases (27%) produced ESBL isolates, and most of them, 47 (87%), were able to produce all four ESBL enzymes.[10]

In a study by Mousavi et al., who examined an outbreak of CTX-M-2 beta-lactamase resistance gene in *E. coli* isolated from urinary tract infection in Sanandaj province, the results of phenotypic tests showed that out of 100 strains of *E. coli*, 27 (27%) strains produced ESBL. Through PCR Method, it was found that among these, 2 (7.40%) strains produced CTX-M-2.[11]

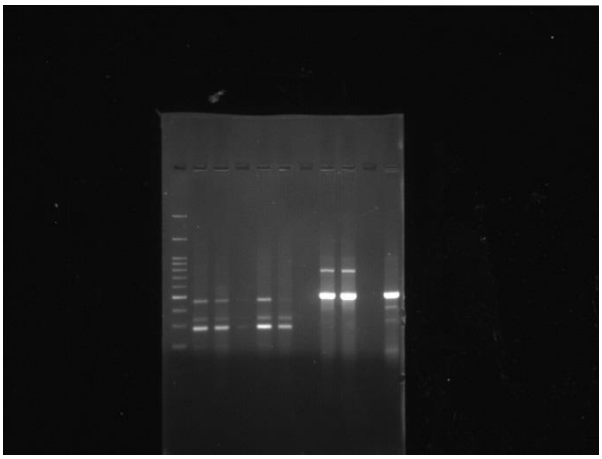

Figure 1. The result of agarose gel electrophoresis of PCR product for genes (SHV, CTX-M1, CTX-M2, CTX-M3).

In the study of Mira Elmi et al. who examined the prevalence of TEM-SHV-CTX-M genes isolated from *E. coli* strains of urinary tract infections and their antibiotic resistance, the results showed that the highest resistance to penicillin and erythromycin antibiotics were reported with 96% and 94.5% frequency respectively, and the highest sensitivity to ciprofloxacin and ampicillin antibiotics with a frequency of 67.2% was reported.

From the 55 specimens tested, 26 samples (47.27%) had the TEM gene and in 41 samples, 74.54% of the CTX-M gene was identified.

Table 4. Minimum Inhibitory Concentration (MIC) of various antibiotics on *E. coli*

Bacterial code	Ceftazidime	Imipenem	Ampicillin	Cefazolin	Ceftriaxone	Cefotaxime
1	16	64	32	32	64	did not grow
2	16	64	32	64	64	did not grow
3	16	64	256	64	256	did not grow
4	16	64	256	64	64	did not grow
5	16	64	128	256	128	did not grow
6	32	64	8	64	128	128
7	32	64	8	64	128	128
8	8	64	16	64	32	64
9	8	32	64	128	32	64
10	8	16	256	64	32	32
11	8	256	256	128	32	32
12	8	256	64	grow	16	32
13	32	8	128	16	8	16
14	32	4	32	16	32	8

Also, in 32.72% of the samples, both TEM and CTX-M were detected simultaneously. In addition, the SHV gene was not detected in any of the specimens.[12]

In the study of Mohammadi et al., the results showed that isolated *E. coli* was resistant to ampicillin (43.87%), ciprofloxacin (31.31%), tetracycline (41.08%), nalidixic acid (41.69%), nitrofurantoin (3.62%), amikacin (9.75%), trimethoprim sulfamethoxazole (40.46%), tobramycin (7.65%), ceftriaxone (34.19%), gentamicin (18.51%) and ampicemin (21.46%). [13]

Sadeghi isolated 221 strains of Klebsiella and 255 strains of *E. coli* from Tehran and Tabriz cities and tested the ESBL production with TDT and E-test tests. The prevalence rate of *E.coli* species in admitted and outpatient patients in Tehran was 6.1% and 1.7%, respectively. The numbers for Tabriz were 4.67% and 1.1% for Escherichia coli.[14]

In Shahcheraghi study on 200 strains of *E. coli* isolated from different clinical specimens showed that 52.5% had an ESBL gene, of which 24% had TEM gene and 6% of the strains had SHV gene. [15]

Mirzaee examined 160 isolates of *E. coli* in terms of CTX β-lactamase production through PCR method which was 37.8% positive. The CTX-M group was 35.78% and the CTX-M-III group accounted for 2.1% of the positive cases.[16]

In the Soltan Dallah study on 200 isolates of *E. coli*, 64% (128 strains) of isolates produced eSBL. TEM and SHV genes were 74 (57.8%) and 7 (5.5%) cases, respectively.[17]

In the study of Shayan and Bekayan who examined the prevalence of ESBL and AMPc in E. *coli* samples in Zahedan city, the results showed that out of 90 samples, 37 samples (62.7%) and 3 samples (5%) were identified as ESBL and AMPc and PCR results showed that 29 samples (49.1%) and 3 samples (5%) carrying TEM and CMY-2 genes.[18]

In the study of Sultan Dallal et al., The results of the phenotypic tests showed that of 188 strains of *E. coli*, 82 strains (43.6%) were producing ESBL. The PCR method showed that 69 strains (84.1%) were strains producing CTX-M-1.[19]

In a study by Riyahi Zaniani et al. who examined the prevalence of TEM and SHV genes in Escherichia coli producing ESBL, the results showed that 43.9% of *E. coli* produced ESBL and the prevalence of SHV and TEM genes was 14.4% and 20.6% in *E. coli* samples.[20]

In a study by Ghorbani-Dalini et al. who examined the prevalence of ESBL producing genes in *E. coli* samples, the results showed that the highest resistance of the samples was to the cefixime, trimester primers-sulfamethoxazole, and ampicillin and penicillin antibiotics. While the prevalence of TEM 45, SHV 17, and CTX-M 11 genes was 83.33%, 31.48% and 20.37%, respectively.[21]

The results of Khaledi et al. Showed that 108 (28.4%) of the 380 *E. coli* producing broad-spectrum beta-lactamases. *E. coli* producing beta-lactamase was resistant to Amikacin (3%), ampicillin (99.5%), trimethoprim-sulfamethoxazole (90.7%), gentamicin (21%), ceftriaxone (98%), cefixime (100%), ceftazidime (100%) antibiotics.[22]

In a study by Zamani et al., Which examined the prevalence of CTX-M gene in *E. coli* producing broad-spectrum beta-lactamases in Shiraz, the results indicated that from 202 ESBL-producing *E.coli*, 185 (91.5%) possessed CTX-M genes. CTX-M-1 subtypes were found in 98% of the isolates. The CTX-M gene was identical to CTX-M-15.[23]

In the study of Farrokhnazar et al., isolated *E. coli* was resistant to ceftazidime of 89 samples (44.5%) and cefotaxime of 98 samples (49%).[24]

Pakzad examined the prevalence of *E. coli* resistance to quinolones and frequency of qnrA, qnrB and qnrS in non ESBLs (extended spectrum beta-lactamases) and ESBLs producing *E. coli* with blaSHV and blaTEM. The result showed that out of 150 isolates, forty-two (28%) ESBLs producing and one hundred and eight (72%) non-ESBLs producing *E. coli* were identified. 64.2% (n = 24) of *E. coli* producing ESBLs and

4.62% (n = 5) of non-ESBLs *E. coli* were resistance to ciprofloxacin. 95.2% (n = 40) and 26.1% (n = 11) of the isolates harbored blaTEM and blaSHV, respectively. 23.8% (n = 10) had both genes. 37.5% (n = 9) and 20.8% (n = 4) of ESBLs producing *E. coli* were positive for qnrA and qnrB respectively. qnrS was not identified in any isolates.[25]

The study of Rezai, genotype ESBL-producing *E. coli* isolates from pediatric patients for ESBL genes and determine their association with antimicrobial resistance. About 30.5% of isolated E. coli was ESBL-producing strain. The TEM gene was the most prevalent (49%) followed by SHV (44%), CTX (28%), VEB (8%), and GES (0%) genes. The ESBL-producing E. coli isolates were susceptible to carbapenems (66%) and amikacin (58%) and showed high resistance to cefixime (99%), colistin (82%), and ciprofloxacin (76%).[26]

Out of 200 UPEC, 22% (n = 44) were ESBL-producing; 0% showed the least resistance to imipenem, and 97.7% showed high resistance to carbenicillin and ampicillin. The ESBLs CTX-M, SHV, TEM, OXAI, and OXAII were detected in 93.3%, 68.2%, 43.2%, 31.8%, and 22.7% of isolates respectively.[27]

Nakhaei Moghaddam investigated the TEM, CTX and SHV ESBLs among urinary *E. coli* isolates in Mashhad, a city in northeast of Iran. ESBL producers of E. *coli* isolates were 33.3%. Among 37 ESBL-producing isolates, 35 (94.6%), 21 (56.8%) and 5 (13.5%) were shown to have bla_{CTX}, bla_{TEM} and bla_{SHV}, genes respectively. Co-resistance to non-beta lactam antibiotics was observed more with ESBL producers ($P < 0.05$).[28]

Mohammadi Tabar determined the pattern of antimicrobial resistance and investigates the prevalent ESBL phenotype and the *ctx-M* gene in *E. coli* isolated from patients with urinary tract infections (UTI) in Semnan. One hundred ninety samples (4.16%) were identified as *E. coli*. Twenty-one (26.6%) of *E. coli* were ESBL positive and 73.4% were ESBL negative. There was 100% susceptibility to imipenem. Twenty (68.97%) out of 29 isolates were positive for the *ctx-M* gene, as detected by PCR.[29]

The purpose of the study of Nakhaei Moghaddam was to determine the antibiotic resistance and CTX-M extended-spectrum β-lactamases (ESBL) carrying *E. coli* isolated in hospitals from two different areas in Iran. Resistant of isolated bacteria from urinary samples of patients in Mashhad was higher than of urinary isolates in Qom to the β-lactam antibiotics of ceftazidime, cephalothin, cefotaxime, cefepime, cefpodoxime and cefixime. The difference was significant for cephalothin (70.5% versus 49%). Significant difference was not observed between susceptibility of isolates from two areas to the non-β-lactam antibiotics ($P > 0.05$). Based on the DDS test, 24 isolates were positive and 6 isolates were suspicious from 49 bacteria isolated in Qom. Among isolates from Mashhad, 21 were ESBL+ and 5 were suspicious. By ESBL confirmatory test, 48 of 93 isolated bacteria (51.6%) were ESBL-producing that 25 and 23 isolates of these bacteria were from Qom and Mashhad isolates, respectively. Thus, there was no significant difference between the β-lactamase-producing bacteria from the two studied cities (51% versus 53.5%). The ESBL phenotype was detected in 48 (51.6%) isolates, 42 of which were carrying bla_{CTX-M} gene.[30]

Another study was carried out to evaluate the molecular properties of these genes in clinical isolates of Enterobacteriaceae by polymerase chain reaction, restriction digestion and sequencing. The finding suggests that CTX-M Type B- lactamases are widespread in the studied community (96.3%).[31]

Goudarzi in his study surveyed the frequency of bla CTX-M genotype in ESBL producing *E. coli* isolated from hospitalized patients with urinary tract infection and determined their antibiotic resistance pattern. PCR and sequence analysis showed that 75 (55.5%) isolates produced bla CTX-M genes. In vitro susceptibility of ESBL producing E. coli showed that all of them were resistant to amoxicillin and penicillin. The rates of resistance to the majority of

tested antibiotics varied among 61% to 100% with the exception of amikacin (14.7%) and imipenem (2.7%). The results showed that the frequency of bla CTX-M was strikingly high (93.3%) in patients with UTI.[32]

The study of Altinkum, was to compare the epidemiological feature of CTX-M, TEM and SHV producing pathogenic *E. coli* and *K. pneumoniae* strains in outpatients and hospitalized patients. The distribution rate of *bla* genes in ESBLs producing *E. coli* isolates was found as 94.8% *bla*CTX-M, 25% *bla*TEM and 15.6% *bla*SHV.[33]

In a study by Memariani, who investigate the prevalence of bla CTX-M, bla SHV and bla TEM β-lactamase genes among enteropathogenic *E. coli* (EPEC) isolates in Tehran, Iran, the result indicated that of 42 EPEC, eight isolates carried the bla CTX-M1. None of the isolates carried bla CTX-M2 and bla CTX-M9. The bla CTX-M15 variant was identified in all of bla CTX-M1 -positive isolates. Furthermore, bla SHV and bla TEM genes were detected in 40.5% (n = 17) and 19% (n = 8) of all EPEC isolates, respectively. No significant association was observed between the existence of bfp gene and presence of those β-lactamase genes (P > 0.05). MLVA analysis revealed high genetic diversity among bla CTX-M15 -positive isolates.[34]

The aim of the study of Najar Peerayeh was to assess the prevalence of multidrug resistance of ESBL-producing urine isolates of *E. coli* and *K. pneumoniae* collected in Tehran hospitals, as well as the molecular characterizations of some ESBL genes, with an emphasis on occurrence rates by sex. The result showed that the ESBL phenotype was detected in 55.5% of *E. coli* and 46.4% of *K. pneumoniae* isolates. Presence of $bla_{\text{CTX-M-1}}$ was dominant in both organisms. The prevalence of $bla_{\text{CTX-M-1}}$ carrying isolates among ESBL-producing *K. pneumoniae* and *E. coli* isolates were 49.1% and 85.7%, respectively. Among ESBL-producing isolates, 68.5% of *E. coli* and 59.3% of *K. pneumoniae* isolates carried the bla_{TEM} genes, and simultaneous carrying of $bla_{\text{CTX-M}}$ and bla_{TEM} genes was observed in 68.5% of *E. coli* and 33.3% of *K. pneumoniae* isolates. The resistant rate to ceftazidime, cefotaxime, and cefepime was significantly higher in *K. pneumoniae* and *E. coli* isolates from male patients' urine samples. A significant higher rate of $bla_{\text{CTX-M-1}}$, bla_{TEM}, and co-$bla_{\text{CTX-M}}$-1-bla_{TEM} genes were seen for *E. coli* and *K. pneumoniae* isolates in male patients' urine.[35]

In a study by Nojoomi**,** who determined the production of ESBL and prevalence of $bla_{\text{CTX-M1}}$, bla_{SHV}, and bla_{TEM} among *E. coli* blood isolates in Tehran, the results clearly defined that in the broth dilution test, 19 (82%) isolates showed MIC ≥ 1, and 18 (78.3%) isolates were ceftazidime resistant. In the combined disk test, 19 (82%) isolates were ESBL producers. The results of the MIC and ceftazidime resistance were the same for ESBL selection. The results of MIC, in fact confirmed the disk diffusion in determining the phenotypic ESBL production. The frequency of $bla_{\text{CTX-M1}}$, bla_{SHV}, and bla_{TEM} genes among blood ESBL producing isolates was 26% (n = 6), 8.6% (n = 2), and 0%, respectively. Isolates that showed higher MIC were positive for these genes.[36]

Conclusion

Based on the results of this study and other studies, the widespread use of broad-spectrum beta-lactam antibiotics has increased the spread of ESBL enzymes in Iran and throughout the world and the use of these antibiotics is becoming more and more limited. The widespread use of beta-lactam antibiotics, especially cephalosporins, has increased the prevalence of ESBL enzymes in *E. coli* (the most common cause of urinary tract infections). Therefore, the complete identification of ESBLs by experiments, the restriction of the use of beta-lactam antibiotics and the use of inhibitory antibiotics on beta-lactamase function can maintain the efficacy of beta-lactam antibiotics as much as possible.

Conflict of Interests

Authors have no conflict of interests.

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Author contributions

All authors of this article have the same contribution to perform this project.

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