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Journal of **A**pplied **B**iotechnology **R**eports

Original Article

Assessment of Third Generation Cephalosporin (Ceftazidime and Ceftriaxone) Resistant *Escherichia Coli* Strains Isolated from Zahedan Hospitals by Tracing the *TEM* Gene

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Abstract

Escherichia coli is a Gram-negative, facultative anaerobic, rod-shaped bacterium and member of the Enterobacteriaceae family. *E. coli* is common in various infections, including hospital-acquired urinary tract infections. Ceftriaxone and ceftazidime are most commonly-used antibiotics to treat infections caused by Enterobacteriaceae. The purpose of this study was to determine the antimicrobial resistance pattern of *E. coli* strains isolated from patients referred to the selected hospitals in Zahedan by tracing the *blaTEM* beta-lactamase gene. Over a 12 month period, 200 clinical samples were examined. Antibiotic susceptibility was determined by disk diffusion test and microdilution method and the presence of *bla TEM* gene was evaluated by PCR. 130 isolates were potentially extended-spectrum beta-lactamase-producing and 72 isolates contained the *TEM* gene. The results of the present study indicate a high rate of antibiotic resistance among *E. coli* isolates to ceftriaxone and ceftazidime. Therefore, it is recommended to perform antibiogram tests before prescribing antibiotic therapy.

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Keywords: *Ecsherichia coli*, Disk Diffusion Test, Extended Spectrum Beta-lactamases (ESBLs)

Submission Date: 3/25/2017
Accepted Date: 6/2/2017

Introduction

Increased use of antibiotics and the tendency of individuals to arbitrarily consuming antibiotics have caused pathogenic organisms use of defense mechanisms to be adapted to antibiotics and in this way the bacterial resistance to antibiotic has emerged. Bacterial resistance against antibiotics is gradually increasing, so that it is now regarded as one of the challenges and concerns of microbiologists [1]. Mechanisms of bacterial resistance to antibiotics are various, complex and different for each antibiotic. In some cases the resistance is created by chromosome and in other cases this is related to the extra-chromosomal DNA, such as plasmids. One of these mechanisms is the ability to express extended-spectrum beta-lactamase (ESBL) producing enzymes in bacteria [2-4]. Extended Spectrum Beta-lactamase group of enzymes have the ability to hydrolyze oxyimino-beta-lactam and has been found in most of the members of the Enterobacteriaceae, including *E. coli* [3]. ESBLs give the property to the bacteria to be resistant not only to penicillin, aztreonam, and cephalosporins, but to other antibiotics, such as aminoglycosides, trimethoprim, sulfamethoxazole, and quinolones [5]. Beta-lactamase enzymes are divided into four classes: A, B, C, and D. ESBLs are in group A that are able to hydrolyze penicillin, aztreonam, and cephalosporins [6]. Unique beta-lactamase is growing significantly. Transferable plasmids acquire genes encoding lactamase enzymes and are transferred among bacteria, such as *Escherichia coli* and *Klebsiella* that do not these enzymes as a chromosomal gene [7]. The prevalence of ESBLs-producing strains among clinical isolates has increased steadily in recent years, which has led some restrictions in antibiotic therapy options. Organisms that cause urinary tract infection (UTI-causing microorganisms), especially *E. coli* strains, are capable of producing large-scale of ESBLs [8]. Two main dericatives of TEM-type beta-lactamases are TEM-1 and TEM-2. TEM-1 was first isolated in 1965 from an *E. coli* strain isolated from the patient named Temoneria, so it was named TEM. TEM transfer to other bacteria is dependent on plasmids and transposons. TEM-1 is able to hydrolyze ampicillin at a higher rate than carbenicillin, oxacillin, or cephalothin and is inhibited by clavulanic acid. However, the number of TEM beta-lactamase mutants have been found which are capable of hydrolyzing third generation of cephalosporins and yet are resistant to beta-lactamase inhibitors and are identified as Inhibitor Resistant TEM

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(IRT) [9]. Worldwide, *Escherichia coli* is considered as an important Gram-negative bacilli in urinary tract infections [10]. Widespread of *Escherichia coli* resistant strains in hospitals across the world, being awareness of their prevalence in each district and hospital is necessary for planning of experimental treatment policy. Knowing the patterns of antibiotic resistance in bacterial populations helps to guide the rational use of antibiotics and lead to prevent the further spread of resistant strains [11]. The routine examinations, which are performed in clinical laboratories for antibiotic susceptibility testing, cannot identify beta-lactamase-producing strains. Sometimes resistant isolates are wrongly reported susceptible to broad-spectrum antibiotics, such as cefotaxime, ceftazidime and ceftriaxone [10]. Since the interpretation criteria of Clinical and Laboratory Standards Institute (CLSI) for resistance threshold of Enterobacteriaceae against third-generation cephalosporins is changing, it is recommended to replace results of the resistance to cephalosporins that are reported on the basis of minimum inhibitory concentrations (MIC) instead of the negative or positive resistance by antibiotic discs, regardless of whether ESBLs-producing isolates [12].

The purpose of this study is to determine the antibiotic susceptibility pattern of ESBL-producing *E. coli* by tracing the *blaTEM* gene in clinical samples.

Materials and Methods

In this empirical study, from 2015 to 2016, 200 strains of *Escherichia coli* from various clinical specimens, such as wound, blood, secretions, urine, etc. were collected from three hospitals in Zahedan (Imam Ali, Bu Ali, and Nabie Akram), and confirmed by biochemical tests. Antibiotic susceptibility was determined by disk diffusion method (Kirby-Bauer) according to the recommendations of CLSI, for seven antibiotic: ceftriaxone (CRO), ceftazidime (CAZ), cefotaxime (CTX), gentamicin (GM), cefepime (CPM), ciprofloxacin (CIP), piperacillin (PIP), and imipenem (IMP). All antibiotic discs and powders were purchased from MAST Group Ltd (mastdiscsTM. AST, UK).

Determination of antibiotic resistance patterns of isolated samples by disk diffusion method (Kirby-Bauer)

According to the CLSI protocol suspension of bacterial colonies dissolve in the 2 ml sterile saline solution and then the 0.5 McFarland turbidity standard was provided. The suspension compared with control tube (0.5 McFarland) and turbidity was the same for both tubes. Then, near the flame, bacterial suspensions ($1\chi10^6$ bacteria) were cultured densely on Mueller-Hinton (MHA) agar medium so that the entire surface of plate was smeared (a lawn culture of the isolated bacteria on Mueller-Hinton agar was made). The antibiotic discs were placed on the medium by sterile forceps 2 cm from the plate wall and with 25 mm apart [13]. Isolates that have reduced susceptibility to ceftazidime and ceftriaxone were studied using confirmatory test for ESBL production by the combination disk method. Through the combination of the discs, ceftazidime (30 µg) and ceftazidime (30 µg) in combination with clavulanic acid (10 µg), ceftriaxone (30 µg), and ceftriaxone (30 µg) in combination with clavulanic acid (10 µg) was used. The results were interpreted according to CLSI recommendations. Therefore, if inhibition zone diameter of the combination discs be equal with inhibition zone diameter of the disc alone ($A \geq 5$ mm) is considered ESBL positive. *E. coli* ATCC 25922 strain was used as positive control and *Klebsiella pneumoniae* ATCC 700603 was used as negative control [14].

After incubation at 37°C for 16-18 hours, beta-lactamase-producing strains by an increase of ≥5 mm in zone of inhibition for ceftazidime + clavulanic acid compared to ceftazidime alone and ceftriaxone + clavulanic acid compared to ceftriaxone alone was confirmed as ESBL producing strains, according to the recommendations of CLSI (2011).

Broth dilution method to determine the minimum inhibitory concentrations (MIC)

Broth dilution method was used to determine the minimum inhibitory concentrations (MIC) of ceftazidime and ceftriaxone. Pure antibiotic powders were prepared. Proper solvents for each antibiotic powder were selected, sodium bicarbonate solution for ceftazidime and distilled water for ceftriaxone.

To prepare serial dilutions of antibiotics, at first 128 µl of antibiotic stock with the concentration of 10 mg/ml added to a tube and 872 µl of sterile Mueller-Hinton broth medium was added to this tube to make the concentration of 1280 µg/ml of the relevant antibiotic. Then, 125 µl of tube A was poured into tube B and 875 µl of sterile Mueller - Hinton broth medium was added to this tube to make 160 µg/ml dilution. In the next step 125 µl solution of tube B was poured into tube C and 875 µl of sterile Mueller-Hinton broth medium was added to this tube to obtain 20 µg/ml concentration of the antibiotics. Then, antibiotic dilutions in tubes A, B, and C are used to prepare serial dilutions for MIC test. To perform the test, concentration of 0.25 to 512 µg per ml of each antibiotic was prepared.

Genetic studies

After performing the phenotypic tests (disk diffusion and MIC) and identification of resistant and ESBLs-producing bacteria, resistant isolates were selected for DNA extraction. Boiling method was used for DNA extraction (Petit et al., 2004). To confirm the presence of isolated DNA, samples were electrophoresed on a 1% agarose gel and for determination of DNA concentration, UV spectrophotometer was used, n which the used wavelength was 260 nm and OD value was read up to 1-1.5. PCR assay was performed to investigate the pesence of lactamase gene (*blaTEM*) with 848 bp .The PCR reaction conditions were as Table 1. The sequence of the exploited primers was as follows:

Forward primer: 5'GAGTATTCAACATTTCCGTGTC3'
Reverse primer: 5'TAATCAGTGAGGCACCTATCTC3'

100 bp Ladder (Thermo scientific, USA) was used to confirm the molecular weight of the PCR products. Gel electrophoresis of PCR products was performed on a 1.5 % agarose gel. After the electrophoresis, power supply shut down and gel removed from the tank and placed carefully inside the gel documentation unit and after adjusting and sharpening, the image of the gel was photographed.

Table 1. PCR conditions.

Steps of PCR	Temperature (°C)	Time
Initial denaturation	94	5 min
Denaturation	94	30 s
Annealing	60	30 s
Extension	72	50 s
Final extension	72	5 min
Cycle number	30	

Results

Of the 200 *E. coli* strains isolated from clinical samples168 samples were isolated from urine, 17 samples from wounds, 12 samples from blood, 2 samples from abscess, and 1 sample from ascitic fluid. Antibiotic resistance pattern of isolated clinical strains was as follows: cefotaxime 66%, ceftazidime 65.5%, ciprofloxacin 62.5%, ceftriaxone 60.5%, gentamicin 36.5%, and cefepime 36%, while none of the isolates showed resistance to imipenem. Among the 131 samples which were resistant to ceftazidime, 114 samples were isolated from urine, 9 samples from wound, 7 samples from blood, and one sample from ascitic fluid. Of the 121 samples that were resistant to ceftriaxone, 103 samples isolated from urine, 11 samples from wounds, 6 samples from blood, and one sample from the abscess (Table 2).

Table 2. Antibiotic resistance pattern of clinical strains of *E. coli* by disk diffusion method.

	Antibiotic	The percentage of (%)		
		Resistant	Intermediate	Susceptible
1	Ceftazidime	65.5	9	25.5
2	Ceftriaxone	60.5	7	32
3	Cefotaxime	66	9.5	23.5
4	Cefepime	36	10	54
5	Gentamicin	36.5	12.5	51
6	Ciprofloxacin	62.5	5	32.5
7	Imipenem	0	0	100

In the combination disk method, an increase of ≥ 5 mm in the zone of inhibition for ceftazidime + clavulanic acid and ceftriaxone + clavulanic acid indicates ESBL-producing strains. In this test 97 strains (47%) were ESBL-producing strains. The results of the minimum inhibitory concentrations (MIC) tests are shown in Table 3.

Discussion

Urinary tract infections that are caused by *Escherichia coli* have a widespread distribution worldwide. It is noticeable that *E. coli* strains are of highly resistance to various antibiotics and apparently seem that this resistance level is increasing rapidly.

Table 3. Results of the minimum inhibitory concentration of resistant strains to ceftazidime and ceftriaxone.

MIC	No. of strains resistant to ceftazidime (CAZ)	No. of strains resistant to ceftriaxone (CRO)
2 µg/ml	15	5
4 µg/ml	13	23
8 µg/ml	10	33
16 µg/ml	8	16
32 µg/ml	13	17
64 µg/ml	17	10
128 µg/ml	29	10
256 µg/ml	21	5
512 µg/ml	4	2

It also indicated that the 72 isolates (38.55 %) were positive for the *blaTEM* gene. Image of TEM amplification is shown in Fig. 1.

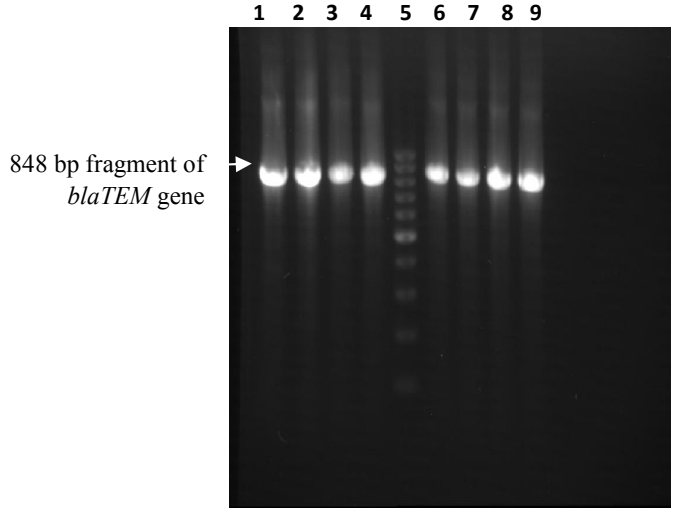

Figure 1. PCR results of *E. coli* strains; line 1: The positive control; Lines 2-4 and 6-9: Clinical samples; Line 5: 100 bp Ladder .

The important factor in the development of bacterial resistance is the production of beta-lactamase enzymes by bacteria. Increase in nosocomial infections and in severe cases mortality of them is outcome of such resistance [8]. Most of the samples obtained from hospital infections are resistant to numerous antibiotics. In the present study, nearly 70% of clinical isolates were resistant to third generation cephalosporin antibiotics such as ceftazidime and ceftriaxone. Most resistant clinical samples are originated from hospitals. Broad-spectrum cephalosporins resistance in *Escherichia coli* and *Klebsiella pneumoniae* is due to production of ESBLs that can hydrolyze monobactams and cephalosporins [15].

Phenotypic methods are useful screening method for distinguishing between ESBL-producing strains and non–ESBL-producing strains that use other mechanisms for resistance to beta-lactam antibiotics [16]. An enzymatic mechanism of resistance by ESBL-producing bacteria in combination with defects in outer membrane permeability and efflux complicates the interpretation of antibiotic resistance of bacteria. Since it is possible that treatment with third-generation cephalosporins or aztreonam may lead to failure report of strains resistant to these antibiotics is essential. Furthermore, the detection of ESBLs-producing strains helps to prevent cross-transmission of such strains among patients [16].

The increasing resistance in Gram-negative bacilli has caused problems in the empirical antibiotic therapy in the world. Asia has a long history of ESBL-producing bacteria. Also It is well established that there are many differences in the prevalence and genetic of ESBLs in hospitals and especially in different countries [17]. This was confirmed in our study. So when we compared our phenotypic tests with other Colleagues who had done similar tests, we found that the prevalence of ESBLs-producing organisms dramatically increases day by day [18].

In a study conducted in India, the incidence of infection with ESBL-producing *E. coli* was reported 79%. In the present study, the incidence of infection with ESBL-producing *E. coli* was reported 56%. Meanwhile, it has been reported that the production of ESBLs in Japan, Thailand, Singapore, and Philippines is more than 20% and even in some centers its incidence has increased to more than 60%. The same data were previously seen in South American [19]. ESBLs production rate in Asia is varied in different countries. In a phenotyping study in India, the prevalence of ESBL-producing *E. coli* isolates, was reported 46.51% [20]. In Taiwan, the prevalence of ESBL-producing *E. coli* isolates was reported 1.5-16.7% [21]. In a study in Pakistan, the prevalence of ESBL-producing *E. coli* isolates, was reported 41% [22].

In another study in Pakistan, the prevalence of ESBL-producing *E. coli* isolates, was reported 56.9 % [23]. Report of ESBLs produced by *Escherichia coli* in various parts of Iran is different (8.9-67%) [24]. In a study conducted by Fazly and colleagues in Mashhad, the prevalence of ESBL-producing *E. coli* isolates was reported 57.5% [24].

In a study by Hoban *et al*., it was confirmed that *E. coli* strains have remained as important gram negative bacilli in urinary tract infections worldwide [10]. Previous studies have proven that the prevalence of resistant pathogenic *E. coli* in both inpatient and outpatient [25].

Studies in which antibiotic resistance phenotype and MIC values were close to values in the current study, are as follows: Nasa and colleagues in a study in 2011 in Delhi showed that the rate of ESBL-producing *E. coli* was 71.8 % [25]. Bertrand and colleagues reported the rate of ceftriaxone resistance in the Middle East is less than values in our study (42.5%). However, the value of MIC was similar (128 µg/ml) [26]. In a study conducted in 2009 in Tehran, 59.2% of the *E. coli* isolates were ESBLs-producing. 29.8% of ESBLs-producing isolates had the MIC equal to 128 µg/ml that was the most common MIC [27]. In our study also the most common MIC was 128 µg/ml.

In a study conducted by Mirzaei and colleagues the rate of third-generation cephalosporin resistance (ceftazidime, cefotaxime, cefepime) was 56.69% and the most common MIC for ceftazidime was obtained 256 µg/ml [28]. In other studies, significant differences founded in the rates of resistant bacteria than the present study that can be caused by indiscriminate use of ceftriaxone and ceftazidime in the region. For example, the ceftazidime resistance rate in the study conducted by Hoban *et al*., was 14.3% vs. 65%, which are very different. This value is for listed worldwide isolates. In this study, the presence of ESBL for the Middle East has been reported 16.2 %. Although the production of ESBL indicates resistant to third-generation cephalosporins, other factors, such as changes in membrane proteins (including PBP2A) are effective on the cephalosporins resistance rate. A study was conducted by Okesola in Nigeria, the rate of resistance to ceftriaxone and ceftazidime has been reported, 37.5% and 43.4%, respectively (by disc diffusion method) [29], whereas in our study the rate of resistance to ceftriaxone and ceftazidime were 65.5% and 61%, respectively

In another study was conducted by Thean in Singapore, until 2009 the value of ceftriaxone resistance rate that was obtained by disc diffusion method, increased to 21.7% [30], whereas in the present study, the rate of resistance to ceftriaxone were reported 61%. The data of the present study in comparison to the results of these studies indicates 1.5-3 fold increase.

Lactamase gene that is specifically responsible for antibiotic resistance in our study is the *TEM*. However, beta-lactamase enzyme types and changes in membrane proteins, such as PBP2A involved in antibiotic resistance isolates. Our molecular studies revealed that 60%of ESBLs-producing *E. coli* contained *TEM* gene, whereas in a study conducted in 2007, 85.6% of ESBLs-producing strains contained *TEM* gene. Its prevalence is higher than in our study. In another study in Turkey, 32.6% of ESBLs-producing *strains* contained *TEM* gene [31] that prevalence of TEM beta-lactamase enzyme in this study was lower than in our study.

In a study conducted in 2011 in Tehran, 128 isolates (64%) were ESBLs-producing isolates, which 74 of them (57.8%) contained *TEM* gene [32]. Thus, the prevalence of *TEM* gene in this study was close to our study.

In another study conducted in Tehran, of 59.2% of *E. coli* isolates that were ESBLS-producing, 46.4% contained *TEM* gene [27]. Thus the prevalence of *TEM* gene in this study was also close to our study.

Conclusion

ESBLs production should be considered as a major threat for consumption of broad-spectrum cephalosporins. Today, we are faced with increasing prevalence of ESBL-producing strains. The results of the present study indicate high rates of antimicrobial resistance among *Escherichia coli* strains isolated from this region to ceftriaxone and ceftazidime that there is a significant difference between

results of this study and results of studies in other parts of the world and even some studies of the Middle East and Iran. These results were predictable due to the high rate of prescribing these two antibiotics. Many clinical laboratories are not aware of the importance and the methods of diagnosis of ESBLs and they have not new strategy to prevent the spread of this resistance mechanism. These reasons are responsible for the lack of an appropriate response to stop the global spread of beta-lactamase-producing bacteria [29]. Therefore, it is recommended for the treatment of bacterial infections, at first, beta-lactam susceptibility pattern be determined accurately by laboratory professionals, then a beta-lactam in combination with beta-lactamase inhibitor be used. Accordingly the spread of ESBLs among different strains of bacteria is reduced [30].

Distribution, abundance and diversity of minimum inhibitory concentrations were obtained from the study of this population implicitly suggest that *E. coli* isolates produce various types of resistance factor against common cephalosporins.

Acknowledgements

The authors are deeply grateful and thank the Infectious and Tropical Diseases Research Center in Zahedan and Falavarjan Islamic Azad University for providing the necessary facilities to conduct this study.

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