Original Article

Antibiotic Resistance Pattern and Genotype of Beta-Lactamase Producing *Escherichia coli* Isolates from Urinary Tract Infections in Zabol-Souteast of Iran

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Introduction: Extended spectrum beta-lactamase (ESBL) producing *Escherichia coli* generate a major problem for clinical therapeutics and epidemiological study. The incidence of ESBL producing strains among clinical isolates has been steadily increasing during the past few years, and remains an important cause of failure of therapy with cephalosporins. The aim of this study was to determine the antimicrobial susceptibility pattern and prevalence of ESBLs in *E. coli* isolates taken from different clinical specimens by phenotypic and genotypic techniques. **Methods:** In this descriptive study, a total of 100 *E. coli* isolates collected from different clinical specimens were used. The antibiotic resistance pattern to twelve antimicrobial agents was determined by disk diffusion method. The ESBLs producing strains were confirmed by double-disk-diffusion test, and the *CTX-M, TEM, SHV*, and *OXA* were detected by PCR. **Results:** The prevalence of ESBL producing *E. coli* was 56%. The results show that 95% of ESBL producing *E. coli* isolates tested were resistant to ceftriaxone and cefotaxime, 93% to ceftazidime, 86% to azithromycin, 79% to cefazolin and 43% to imipenem. Among the ESBL producing *E. coli*, 48%, 30% and 11% were positive for *CTX-M, TEM* and *SHV* genes, respectively. *OXA* was not found in all isolates. **Conclusion:** ESBL producing isolates of *E. coli* have been increasingly recognized and there is a need to carefully formulate therapeutic strategies to control infections in teaching Hospitals. The high percentage of drug resistance in ESBL producing *E. coli* suggests that routine detection of ESBL is required by reliable laboratory methods. *J Med Microbiol Infec Dis, 2014, 2 (4): 153-158.*

Keywords: *Escherichia coli,* Multi-drug resistance, Extended spectrum beta-lactamase, PCR.

INTRODUCTION

Escherichia coli is an important opportunistic pathogen that frequently causes urinary tract infections and pneumonia in immunocompromised individuals. It is the most common cause of Gram-negative septicemia and nosocomial infections [1]. The worldwide development of multi-drug resistant (MDR) strains of *E. coli* is a growing major public health issue and a strong concern for the medical community [2]. Production of β-lactamases is a major means by which Gram-negative bacteria exhibit resistance to β-lactam antibiotics [3, 4]. Extended spectrum β-lactamases (ESBLs) are a group of enzymes that can hydrolyze a variety of β-lactams including cephalosporins like ceftazidime, cefotaxime, ceftriaxone and monobactams like aztreonam in addition to penicillins but, do not hydrolyze cephamycins like cefoxitin. Most of the ESBLs also have the ability to hydrolyze fourth generation cephalosporins like cefepime [5, 6]. Until recently, most infections caused by ESBL-producing *E. coli* (ESBLEC) had mostly been described as nosocomially acquired, nursing home related or health care associated pathogens, *i.e.* affecting patients who had typically been in hospitals or other health care facilities like nursing homes [7, 8]. Infections with ESBL-producing strains have been associated with higher mortality rates and lower rates of favorable clinical responses to antibiotic regimens. In the last decades however, these infections have increasingly been recognized in patients who had no prior

contact with the health care system [9, 10]. Detection of ESBLs is primarily based on phenotypic testing, such as evidencing a synergy image using the double-disc synergy test performed with clavulanate and any extended-spectrum cephalosporin discs [11, 12]. In recent years, alternative strategies aimed at replacing or complementing traditional phenotypic methods have been proposed. Standard PCR and DNA sequencing are still the most widely used technique [13, 14]. Assessment of phenotypic and genotypic characteristics of these isolates would be necessary for understanding the resistance mechanisms and their potential spread. We designed this study to find the prevalence of ESBL producing *E. coli* isolates collected from different clinical specimens, to detect their drug resistance pattern and to look for the *blaTEM*, *blaSHV*, *blaCTX* and *blaOXA* genes in such organisms.

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MATERIAL AND METHODS

Bacterial identification. In a retrospective cross-sectional study, a total of 100 non-duplicate isolates of *E. coli* were collected from clinical specimens of patients admitted in Amir al Momenin Hospital of Zabol City, Iran, from July 2011 to September 2012. These isolates were obtained from culture of specimens from wounds, pus, urine, sputum, blood culture of the patients. Bacteria were identified as *E. coli* based on their colony morphology, staining characters, motility and other relevant biochemical tests as per standard methods of identification.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed for all collected *E. coli* isolates by disk diffusion method on Mueller-Hinton agar (Merck, Germany). *E. coli* ATCC27853 was used as the control strain. Susceptibility was defined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [15]. Antibiogram disks containing ampicillin (AMP), amoxicillin (AMO), cefazolin (CZ), trimethoprim-sulfamethoxaxole (SXT), azithromycin (AZM), ceftazidime (CAZ 30 μg), ceftriaxone (CRO 30 μg), cefotaxime (CTX 30 μg), cefoperazone (CP), cephalothin (CF), and imipenem (IMP 10 μg) were used on Mueller Hinton agar (MHA, Hi-Media) to test antimicrobial susceptibility.

Test for extended spectrum ß-lactamas production. Screening for ESBL production was done according to criteria recommended by National Committee for Clinical Laboratory Standards (NCCLS) [15]. Two discs, ceftazidime (30 mg) and cefotaxime (30 mg) were used for *in vitro* sensitivity testing by Kirby-Bauer disk diffusion method. Zone diameters were read using NCCLS criteria. An inhibition zone of ≤22 mm for ceftazidime and ≤25 mm for ceftriaxone indicated a probable ESBL producing strain requiring phenotypic confirmatory testing.

Phenotypic confirmatory methods. Disk diffusion method was used to confirm ESBL production by *E. coli* strains. Ceftriaxone (30 μg) *vs.* ceftriaxone/clavulanic acid (30/10 μg) and cefotaxime (30 μg) *vs.* cefotaxime/clavulanic acid (30/10 μg) were placed onto Mueller Hinton agar plate lawned with the test organisms and incubated overnight at 35°C. Regardless of zone diameters, a ≥5 mm increase in a zone diameter of an antimicrobial agent tested in combination with clavulanic acid *vs.* its zone size when tested alone, indicated ESBL production.

Extraction of total DNA. DNA extraction was performed using an optimized boiling method. *E. coli* strains were grown in Luria-Bertani (LB) Broth (Lonza; The USA) at 37°C overnight. Bacteria were pelleted from 1.5 mL LB broth and suspended in 200 uL of sterile distilled water, then incubated at 100°C for 10 min and centrifuged. One hundred microliters of the supernatant was stored at -20°C as a template DNA stock [16].

Detection of resistance gene using PCR method. Primers used for amplification of resistance genes are shown in table 1. The reactions (25 μL) consisted of 10-pmol/L of each primer, 2 μL templates DNA, and 12.5 μL of a ready-to-use 2× PCR Master Mix Red (Ampliqon; Denmark). Amplification was carried out using the following thermal cycling conditions: initial denaturation at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 30 s and a final extension step at 72°C for 5 min. Annealing temperatures differed according to the primer pair used and were 56°C for *CTX-M-3,* 50°C for *TEM-3,* 58°C for *SHV* and 60°C for *OXA-1.* Amplified PCR products were separated on 0.8% agarose gels, stained with ethidium bromide and visualized under UV illumination. Appropriate positive and negative controls were used in all cases.

Table 1. List of PCR primer pairs used in this study

Primer name	Sequence (5'-3')	length (bp)/ T_m	Reference
blaTEM-3-F	ACCAATGCTTAATCAGTGA	857 (50ºC)	17
blaTEM-3-R	GAGTATTCAACATTTTCG		17
blaSHV-F	TCGCCTGTGTATTATCTCCC	768 (58ºC)	17
blaSHV-R	CGCAGATAAATCACCACAATG		17
blaOXA-F	GCAGCGCCAGTGCATCAAC	198 (60ºC)	17
blaOXA-R	CCGCATCAAATGCCATAAGTG		17
blaCTX-M-F	AATCACTGCGTCAGTTCAC	701 (56ºC)	17
blaCTX-M-R	TTTATCCCCCACAACCCAG		17

RESULTS

Demographic characteristics of carriers of *E. coli.* The medical records of the 300 index patients were reviewed. The mean age of the patients was 52±17.4 years, with a male to female ratio of 2.57:1. Eighty four (28%) patients were female and two hundred sixteen (72%) were male. The samples were urine (n=100, 33%), blood (n=70, 23%), pus (n =50, 17%), wound (n=30, 10%), and sputum (n =50, 17%). The most common underlying diseases were urinary infections (Table 2).

Detection rates of *E. coli* isolates according to specimen origins. Totally, 100 isolates collected from different samples of the patients were confirmed as *E. coli* by standard biochemical tests. Urinary tract infections (UTI) (46 cases (46%)), were the most common infections caused by *E. coli*, followed by pus (23 cases (23%)), and septicemia (14 cases (14%)), Wound swab (10 cases (10%)) and Sputum (7 cases (7%)) (Table 2). The most common underlying diseases were urinary infections.

Antimicrobial susceptibility of clinical isolates. The antibiotic resistances of the isolates are shown in figure 1. The analysis of drug resistance patterns showed that, of the 100 isolates of *E. coli*, the maximum resistance (100%) was to ampicillin and the least resistance (22%) was to colistin.

A moderately high resistance of 96%, 78%, 76%, 70%, 67% and 66% were shown to amoxicillin, ceftriaxone, cefazolin, co-trimoxazole, cefotaxime, and ceftazidime respectively. Moderate resistance of 59%, 41%, and 33% were shown toward the azithromycin, cefoperazone, and imipenem respectively (Figure 1).

ESBL pattern. Out of 100 strains of *E. coli* tested for ESBL, 56 (56%) were found as ESBL-positive with the highest frequency (19 isolates 33.92%) from urine, followed by, pus (17 isolates 30.35%), wound swab (6 isolates 10.71%), and sputum (3 isolates 5.35%), septicemia (19 isolates 19.64%) (Table 2). Among the 56 ESBL producers, all were resistant to ampicillin followed by 99% to amoxicillin, 95% to ceftriaxone and cefazolin, 93% to ceftazidim, 86% to azithromycin, 79% to cefazolin, 75% to co-trimoxazole, 64% to cefoperazone, 52% to cephalothin and 43% to imipenem (Figure 2).

bla genotyping in *E. coli* isolates. All the ESBL producing isolates were studied for the presence of ß-lactamase determinants. Genotypically, ESBL genotypes were detected in 54/56 (95%) of isolates. Three of four ESBL

genotypes were found among these isolates (Figure 3) with predominance of *CTX-M*-type (48/54: 89%) followed by *TEM*-type (30/54: 56%) and *SHV*-type (11/54: 20%). Of these isolates, 25/54 (46%) had two types of ESBL genes. Twenty five (25/54: 46%) of *CTX-M*-type positive isolates were *TEM*-type positive. Ten (10/54: 19%) of *CTX-M*-type positive isolates were *SHV*-type positive (Figure 3).

DISCUSSION

ESBL-producing organisms pose unique challenges to clinical microbiologists, clinicians, infection control professionals and scientists engaged in finding new antibacterial molecules. ESBL-producing *E. coli* are usually found in those hospitals where antibiotic use is frequent and the patients are in critical condition. This study was carried out in major university teaching hospitals in southeast Iran where there is no record of investigation of molecular epidemiology of ESBL *E. coli*. Nosocomial bacterial infections constitute a substantial cause of morbidity and mortality in developing countries such as Iran.

Table 2. Frequency and percentage of samples yielding ESBL producing isolates

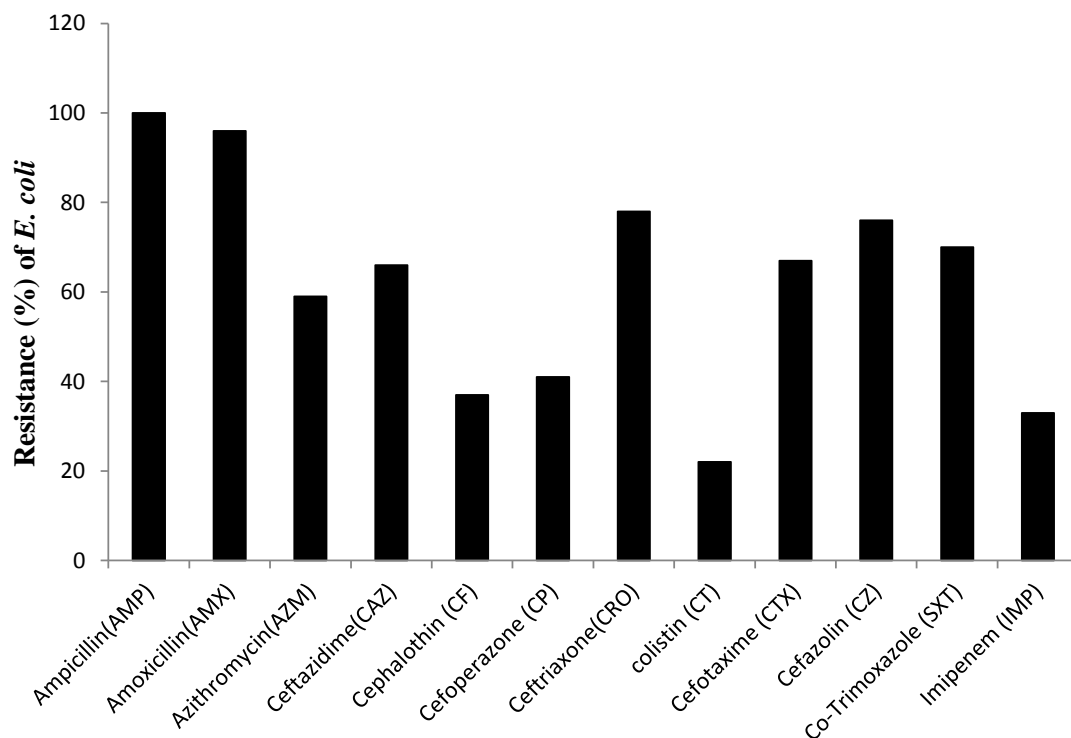| Source (N of Sample)	Frequency and percentage of E.coli isolates		Frequency and percentage of E.coli yielding ESBL production	
	Frequency of *E.coli* isolates	Percentage (%)	Frequency ESBL producing isolates	Percentage (%)
Urine (100)	46	46	19	33.92
Blood (70)	14	14	11	19.64
Sputum (50)	7	7	3	5.35
Pus (50)	23	23	17	30.35
Wound swab (50)	10	10	6	10.71
Total (300)	100	100	56	100

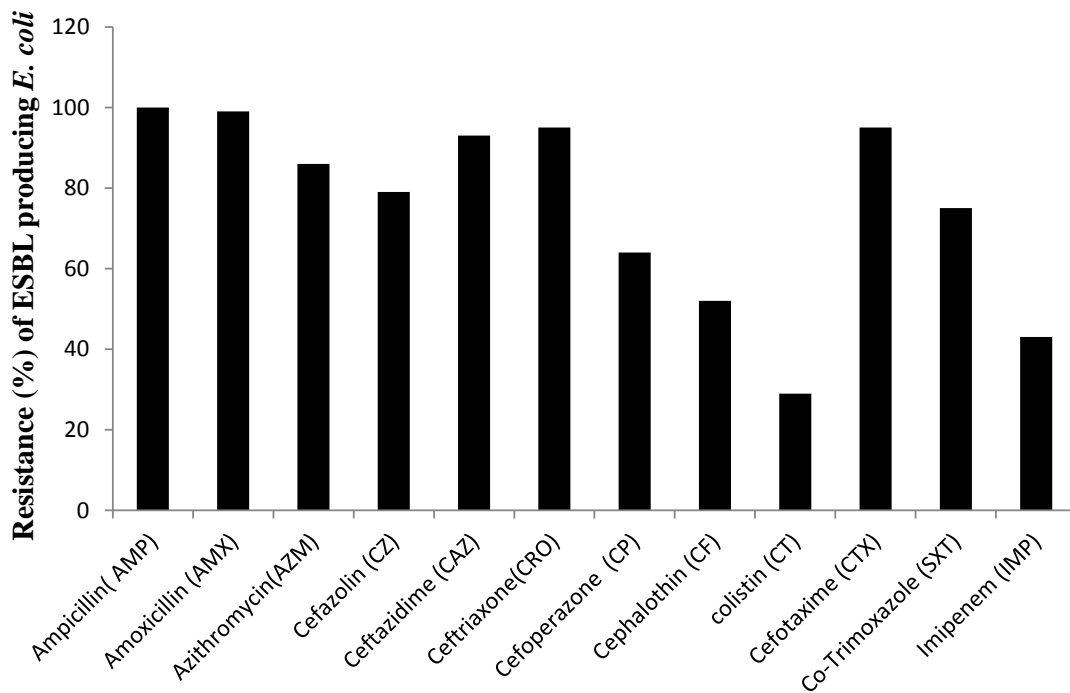**Fig. 1.** Antimicrobial susceptibility pattern of *E. coli* isolated from Hospital Samples

Fig. 2. Antimicrobial susceptibility pattern of ESBL producing *E. coli* isolated from Hospital Samples
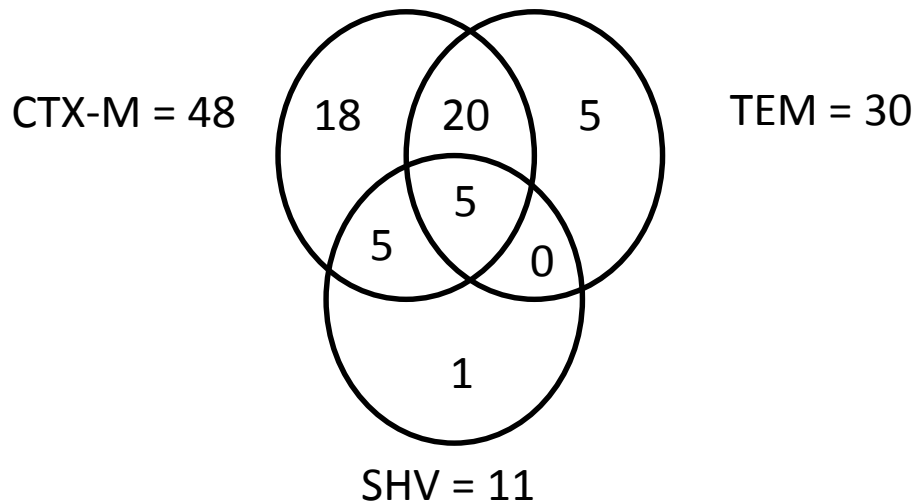
Fig. 3. Distribution of CTX-M, TEM and SHV in ESBL producing *E. coli* isolated from Hospital Samples

E. coli is known to be one of the major organisms causing nosocomial infections within the hospital and has also been implicated in community acquired ESBL [17, 18]. The observation of ESBL producing *E. coli* isolates in this study is very alarming and this could be attributed to the indiscriminate and widespread use of antibiotics, particularly beta-lactam antibiotics that are sold over the counter in pharmacy shops without doctors' prescription in southeast Iran. This misuse of antibiotics might have contributed to the emergency of ESBL producing isolates. In our study antimicrobial susceptibility testing showed that the majority

of isolates were resistant to at least one of the third-generation cephalosporines (78% to ceftriaxone, 76% to cefazolin, 67% to cefotaxime, and 66% to ceftazidime). A Study by Jeong *et al.* in 2004 in North Korea showed [19], ceftazidime and cefotaxime resistance was respectively 11% and 14%. Retrospective studies about resistance to antibiotics showed an increasing trend [20, 21]. Out of a total of 100 isolates, 56 isolates (56%) showed ESBL phenotype detected by combination disk method which is different from the reported rates of ESBLs in other countries such as India (97%), Canadian hospitals (83%), Norway (60%) and Korea

(69%) [22, 23]. The occurrences of ESBL among clinical isolates vary greatly in Iran and geographically and are rapidly changing over time. A study in the year 2010 has shown the prevalence of ESBL in northeast of Mashhad, Iran to be 44% [24] which is 37% in Tehran [25] and 42% in Isfahan [26]. Like other investigation in Iran hospitals, our results showed high ESBLs prevalence in hospitalized *E. coli* isolates (56%); however, none of the studies have provided patterns identical to those of our study. This is probably because the ESBL is located on a plasmid that can be transferred from one organism to another rather easily and can incorporate genetic material coding for resistance to other antimicrobial classes. As 52 (93%) of 66 ceftazidime resistance isolates were ESBL positive in this study (Figure 2), it appears that ESBL production has a significant role in resistance to cephalosporines rather than other mechanisms of resistance such as the loss of porins and efflux pumps in our research [27]. In this study, blood (79%) was the main source of ESBL producing isolates from all patients, followed by pus (74%), wound swab (60%), sputum (43%) and urine (41%). Most of isolates did not demonstrate any sensitivity for amoxicillin, ceftriaxone, cefazolin, co-trimoxazole and ceftazidime tests. Ampicillin demonstrated a 100% resistance towards all isolates. There are very limited treatment options available for these pathogens. So prevention remains a significant priority in controlling the development and spread of ESBL producing organisms. Based on the results of this study, a total of about 95% of ESBL producing isolated bacteria were *CTX-M*, *TEM* and/or *SHV* positive. The *CTX-M* gene has high frequency compared to *TEM* and *SHV* genes; a fact which is similar to previous studies [28, 29]. Also, in our study, the *OXA* gene was not found in ESBLs producing *E. coli* isolates. However, further studies are required for finding other genes in ESBLs producing *E. coli* isolates. During the last 2 decades, most of the ESBL found in *E. coli* and, in general, in gram-negative bacilli, has been of *TEM* or *SHV* lineage. Recently *TEM* and *SHV* types have been replaced by *CTX-M*-type ESBL[5, 30]. *CTX-M* β-lactamases have spread among Enterobacteriaceae in most parts of the world [7, 31-33]. In the middle east area, reports pointed out that CTX-M is the predominant ESBL in *E. coli* [34, 35]. Among the different ESBLs, particular attention should be paid to the worldwide increasing prevalence of the CTX-M types. This study showed high prevalence of resistance rates among clinical isolates of *E. coli* in southeast Iran compared with similar study in south Iran in 2008 [36].

In short, the prevalence of ESBLs producing organisms in southeast Iran is high. It seems necessary for clinicians and health care systems to be fully aware of ESBLs producing microorganisms. Also, the ESBLs production monitoring is recommended to avoid treatment failure and suitable infection control in Iran.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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