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Detection, susceptibility and molecular characterisation of ESBL- producing *E. coli* causing urinary tract infection

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Abstract

In this study we evaluate role of a rapid polymerase chain reaction (PCR) assay compared with traditional empiric therapy in extended spectrum b-lactamases production *E. coli* detection, using a literature-derived model. A cross-sectional study was performed. Sample were isolated from urine culture of hospitalized patients (Amir Al-Momenin Hospital, Zabol, south-eastern Iran) suffered from urinary tract infections during the years 2010- 2011. Ninety isolates of *E. coli* from urinary tract infection were collected, tested for antibiotics with disc diffusion method, minimum inhibitory concentration (MIC) for ceftazidime and resistant gene TEM were detected by PCR. The result showed forty out of ninety *E. coli* isolates were ESBLs producing organisms by disc diffusion. Antibiotic susceptibility of *E. coli* isolates was evaluated for 9 antimicrobial. However, overall, *E. coli* were resistance to 9 of the agent including ceftriaxone (100%), ceftazidime (100%) amoxicillin (100%), azithromycin (95%), cefixime (87.5%), tetracyclin (87.5%), erythromycin (100%). nalidixin acid (85%) and difloxcain (75%) respectively. The distribution of antibiotic-resistant TEM gene according to PCR was 30%. Totally 82.5% were MIC observed as ceftazidime-resistant. We conclude that the TEM gene PCR assay is a rapid, sensitive and clinically useful test, particularly for the early detection of ESBLs- producing *E. coli*.

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Introduction

The recurring theme in antimicrobial resistance has become readily apparent. Namely, the introduction of an antibacterial to the market is initially associated with exuberant use, at least partially as a result of fairly uniform susceptibility of targeted pathogens, which is followed by the emergence of resistance and rapid clonal spread. The rapidity of the development and spread of resistance is a complex process that is influenced by selective pressure, pre-existence of resistance genes and use of infection control measures. There are different mechanisms for drug resistance based on bacterial strain and type of antibiotic. Thus, defining the antibiotic resistance pattern in common pathogens is critical for conducting empirical and specific treatment against a pathogen (Fauci *et al*., 2008).Urinary tract infection (UTI) is the second most common type of infection in the body. The most common cause of UTI is Gram-negative bacteria that belong to the family Enterobacteriaceae. Members of these families include *E. coli*, *Klebsiella, Enterobacter* and *Proteus*. Also Gram- positive *Stapylococcus sp*. plays a role in the infection (Nahed *et al,* 2010). More than 80% of urinary tract infections occur in outpatients and *E. coli* accounts for more than 50% of the infections in these patients (Blomgran *et al*., 2004; Jha and B apat 2005). Multidrug-resistant organisms (MDROs), including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and certain Gram-negative bacilli (GNB) have important infection control implications that either have not been addressed or received only limited consideration in previous isolation guidelines. In addition to MRSA and VRE, certain GNB, including those producing extended spectrum beta-lactamases (ESBLs) and others that are resistant to multiple classes of antimicrobial agents, are of particular concern. In addition to *Escherichia coli* and *Klebsiella pneumoniae*, these include strains of *Acinetobacter. baumannii* resistant to all antimicrobial agents, or all except imipenem and organisms such as *Stenotrophomonas maltophilia, Burkholderia* and *Ralstonia pickettii* that are intrinsically resistant to the broadest-spectrum antimicrobial agents. In some residential settings (e.g., LTCFs), it is important to control multidrug-resistant (Jalalpour., 2011). Beta-lactam antimicrobial agents are the most commonly used treatment of bacterial infections. Resistance to beta-lactam antibiotics among clinical isolates of gram-negative bacilli is most often due to the production of b-lactamases (Kotra *et al*., 2002; Samaha-Kfoury *et al*., 2003). These enzymes are numerous and they mutate continuously in response to heavy pressure of antibiotic use and have lead to the emergence of extended spectrum beta-lactamases (ESBLs) **(**Bradford. 2001). The ESBL enzymes are capable of hydrolyzing broad- spectrum cephalosporins and monobactams but inactive against cephamycins and imipenem. In addition, ESBL producing organisms exhibit co-resistance to many other classes of antibiotics resulting in limitation of therapeutic option. For this reason, the significance of such ESBL–mediated infections has been increasingly reported worldwide (Aminzadeh *et al*., 2008). More than 200 different types of extended spectrum beta-lactamases (ESBLs) have been reported around the world so far (Bradford, 2001; Kim *et al*., 2002). Members of the family Enterobacteriaceae commonly express plasmid-encoded β-lactamases (e.g., TEM-1, TEM-2, and SHV-1), which confer resistance to penicillins but not to expanded-spectrum cephalosporins. ESBLs are beta-lactamases that hydrolyze extended-spectrum cephalosporins with an oxyimino side chain. These cephalosporins include cefotaxime, ceftriaxone, and ceftazidime, as well as the oxyimino-monobactam aztreonam. Thus ESBLs confer resistance to these antibiotics and related oxyimino-beta lactams (Johann *et al*., 2008; Moyo *et al*., 2010). ESBLs are often plasmid mediated and most of the enzymes are members of TEM and SHV families (Ramazanzadeh *et al*., 2010) that have been described in many countries (Kalematizadeh, 2008; Herna´ndez *et al*., 2005). The TEM was first reported in *E. coli* isolated from a patient named Temoniera in Greece (Mansouri *et al*., 2009). Method for detection of ESBL species and related published data are

contradictory with regard to the recommendation. The aims of the present study in the comparison of methods for detection of extended-spectrum beta-lactamases producing *Escherichia coli*.

Materials and methods

Isolation of E. coli

All 90 strains of *E. coli* were isolated from urine cultures of hospitalized patients (Amir Al-Momenin Hospital, Zabol, south-eastern Iran) suffering from urinary tract infections during the years 2010- 2011. The samples were examined microscopically by Gram's stain. Samples with Gram- negative results were inoculated on plates of nutrient agar, clede agar, macConkey's and blood agar then incubated at 37°c for 24 hour (Betty *et al*., 1998). The colony showed fermenting of lactose on macConkey agar and cled agar media were purified and identified according to their morphology as circular, rose - pink to red colonies on macConkey agar medium and yellow colonies on cled agar. The isolates were identified by biochemical reactions e.g. catalase enzyme, potassium hydroxide test, Indole and methyl red test, voges proskaur reaction, urease and citrate, H2S and oxidase test(Holt *et al*., 1994).

Detection of ESBL by Confirmatory Tests

Double Disk Synergy Test (DDST)

A disk of ceftrixon (30µg) and ceftazidime (30µg) were placed 16 to 20 mm apart from the augmentin disc (centre to centre). After incubation (37°C for 24 h) the zone of cephalosporin disc towards the clavulanic acid disc was considered as ESBL producers (Peter-Getzlaff., 2011).

Phenotypic Disc Confirmatory Test (PDCT)

The test was performed as recommended by CLSI. Disks of ceftazidime (CA) 30µg and ceftazidime-clavulanic acid CAC) 20+10µg or ceftraxon (CE) 30 µg and ceftraxon clavulanic acid (CEC) 20+10 µg were placed on MHA at a distance of 30mm between each other. Increase in zone diameter (=5mm) for CAC versus CA or CEC versus CE is confirmed as ESBL producing organisms 27. *E. coli* ATTCC 25922 was used as control strains. The tested drugs (in µg) and their potencies as follow amoxicillin (25 µg), azithromycin (15 µg), cefixime (5 µg), tetracyclin (30m.c.g), erythromycin (15 µg), nalidixic acid (30m.c.g) and difloxcain (25 µg) (Anushia *et al*., 2009).

DNA extraction and PCR

The colonies of ESBLs producing organisms were suspended in (Tris+ EDTA). The buffer and their DNA were extracted by simple boiling. The PCR method for detection of TEM gene was performed as described previously with minor modifications (Aboaba and Efuwape., 2001). Briefly, specific primers for the genes (forward primer 5´-GAGTATTCAACATTTCCGTGTC -3´; Reverse primer 5´-TAATCAGTGAGGCACCTATCTC - 3´ for TEM gene) were used for PCR amplification that produced 857 bp PCR products for TEM gene. The PCR mixture consisted of 10 pmol of each primers, 1 µl DNA sample (3 µg/µl), 1.5 mM MgCl2, 0.2 mM each dNTP, and 5 u Taq DNA polymerase (Cinagen, Iran) in a total number of 50 µl of PCR reaction. Amplification of TEM gene was performed by following program: initial denaturation at 94 °C for 2 min and 35 cycles of 1 min at 94 °C, 30 sec at 52 °C and 1 min at 72 °C. Five min at 72 °C was considered for the final extension. Then, PCR products were analyzed by agarose gels electrophoresis.

MIC of antibiotics

The MIC of antibiotics was determined using bacterial broth dilution method according to the method used by Baron and Finegold (Baron and Finegold, 1990). To study the effect of antibiotics, the Nutrient broth (Merck, Germany- PH=6.5) containing concentrations of (512, 256, 128, 64, 32, and 2 µg / ml) were prepared from each antibiotics (ceftazidime) (Mast, UK). Nutrient broth without antibiotic was used as the control media (Khaleghi *et al*., 2010). The MIC was defined as the lowest drug concentration which prevented visible growth of bacteria (Well and Stood, 1989) and *E.coli* ATTCC 25922 was used as control strains.

Statistica assessment

All the experiment and measurement were repeated at least three times. All The statistical analyses were performed using SPSS and Excel 2010 software.

Result

In the study, forty out of ninety *E. coli* isolates were ESBLs producing organisms by disc diffusion. The MIC of the ceftazidime against 40 clinical isolates of *E. coli* shown in Table 2. The MIC susceptible strains to ceftazidime were MIC≤ 8µg/ml and resistant to ceftazidime were MIC ≥ 32µg/ml. Eighty two point five percent of *E. coli* was found to be resistant to ceftazidime with an MIC ≥ 32µg/ml (33 sample) and seventeen point five percent of *E. coli* was found to be susceptible to ceftazidime with MIC≤ 8µg/ml (7 sample) (Table1).

Table 1. Antimicrobial susceptibility, MIC ceftazidim and positive TEM for *E. coli*.

Bacterial Cod	TEM Gene	Value of MIC for ceftazidim	Resistance pattern
1	+	512	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
2	-	256	$A_2, A_3, B_2, B_3, C_2, C_3$
3	+	128	$A_2, A_3, B_2, C_1, C_2, C_3$
4	-	2	$A_1, B_1, B_2, C_1, C_2, C_3$
5	-	8	$A_1, A_2, A_3, B_2, C_2, C_3$
6	-	512	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
7	+	256	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
8	-	128	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
9	+	512	$A_1, A_2, A_3, B_1, B_2, C_1, C_2, C_3$
10	+	512	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
11	-	512	$A_1, A_2, A_3, B_1, B_2, C_1, C_2, C_3$
12	+	128	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
13	-	128	$A_1, A_2, A_3, B_1, B_2, C_1, C_2, C_3$
14	-	512	$A_1, A_2, B_1, B_3, C_1, C_2, C_3$
15	+	256	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
16	-	512	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
17	-	512	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
18	-	512	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
19	+	256	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
20	-	256	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
21	-	8	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
22	-	128	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
23	-	256	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
24	+	128	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
25	-	128	A1, B1, B2, C1, C2, C3
26	-	8	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
27	-	4	$A_1, A_2, A_3, B_1, C_1, C_2, C_3$
28	-	4	$A_1, A_3, B_1, B_2, C_1, C_2, C_3$
29	+	128	$A_1, A_3, B_1, B_2, C_1, C_2, C_3$
30	+	128	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
31	-	128	$A_1, A_3, B_1, B_2, B_3, C_2, C_3$
32	-	8	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
33	-	128	$A_1, A_2, B_1, B_2, B_3, C_1, C_2, C_3$
34	-	256	$A_1, A_2, A_3, B_1, B_2, B_3, C_2, C_3$
35	-	256	$A_1, A_2, B_1, B_2, B_3, C_2, C_3$
36	-	256	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
37	-	512	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
38	+	512	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
39	+	256	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$
40	-	128	$A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3$

A_1= Ceftazidime, A_2= Nalidixin acid, A_3= Cefixime, B_1= Ceftriaxon, B_2= Azitromycin, B_3= Difloxcain, C_1= Tetracyclin, C_2= Amoxicillin, C_3= Erythromycin

Table 2. Antimicrobial susceptibility of 40 strains of *E. coli* (%).

	CAZ	NA	CFM	CRO	AZM	DIF	TE	AM	E
S	0	4(10%)	5(12.5%)	0	0	8(20%)	5(12.5%	0	0
I	0	2(5%)	0	0	2(5%)	2(5%)	0	0	0
R	40(100%)	34(85%)	35(87.5%)	40(100%)	38(95%)	30(75%)	35(87.5%)	40(100%)	40(100%)

S= Sensitive, I= Intermediate R= Resistant

CAZ= Ceftazidime, NA= Nalidixin acid, CFM= Cefixime, CRO= Ceftriaxon, AZM= Azitromycin, DIF= Difloxcain, TE= Tetracyclin, AM= Amoxicillin, E= Erythromycin.

Discussion

During the past decade, ESBL producing Gram-negative bacilli especially *Escherichia coli* and *Klebsiella pneumoniae* have emerged as serious pathogens both in hospital and community acquired infections worldwide. Recent studies revealed that patients with infection such as septicaemia with ESBL producing organisms had significantly higher fatality rate than those with non-ESBL isolates (Alipourfard and Yeasmin Nili., 2010). Among gram-negative bacteria, the emergence of resistance to extended-spectrum cephalosporins has been a major concern, initially in a limited number of bacterial species and now expanding rapidly (Al-Zahrani and Akhtar, 2005). In the study, forty out of ninety *E. coli* isolates were ESBLs producing organisms by disc diffusion. The MIC of the ceftazidime against 40 clinical isolates of *E. coli* shown in Table 2. The MIC susceptible strains to ceftazidime were MIC≤ 8µg/ml and resistant to ceftazidime were MIC ≥ 32µg/ml. Eighty two point five percent of *E. coli* was found to be resistant to ceftazidime with an MIC ≥ 32µg/ml (33 sample) and seventeen point five percent of *E. coli* was found to be susceptible to ceftazidime with MIC≤ 8µg/ml (7 sample) (Table1).

Similarly, the prevalence of the organisms in India was reported 46.51% in *E. coli* and 44.44% in *K. pneumoniae* isolates (Varaiya *et al.*, 2008). In Tehran out of 115 ESBL producing isolates, 60% were *Escherichia coli* and 40% were *K. pneumonia* (Alipourfard and Yeasmin Nili., 2010). In study Shanthi of 101 isolates, 68 (67.3%) were ESBL producers among the ESBL producers 49 were *E. coli* and 19 were *K. pneumoniae*. (Shanthi and Sekar., 2010). In Pakistan, the prevalence of the ESBLs producing *E. coli* and *K. pneumoniae* was reported 41% in *E. coli* and 36% in *K. pneumoniae* isolates (Jabeen *et al.*, 2005). In another study in Pakistan, the prevalence of the ESBLs producing *E. coli* isolates was reported 56.9% (Ullah *et al.*, 2009). Some study in Iran prevalence of ESBL in isolated *E. coli* and *K. pneumoniae* from hospitalized and non- hospitalized patients was 72.22%, 23.73% and 83.33% and 0%, respectively (Jalalpoor *et al.*, 2009; Jalalpour *et al.*, 2011) and the amplification of TEM revealed 12 isolates (30%) harbored the gene and 28 rests (70%) were negative (Table 2), these primers generated a 872bp PCR product in the PCR reaction (fig.1). The another study, the prevalence of TEM gene among *E. coli* and *Klebsiella* was 100% and 75% respectively (Dorneanu *et al.*, 2010).The frequency of TEM among the ESBLs producing isolates were and 20.6 was report (Riyahi Zaniani *et al.*, 2011). The study of Harda, ESBL-producing *E. coli* accounted for 160 of 1359 isolates (11.8%) and the number of ESBL-producing *E. coli* with TEM was 13 (8.1%)(Harda *et al.*, 2013). The study of Ahmed, ESBL-producing *E. coli* accounted for 102 of 157 isolates (11.8%) and the number of ESBL-producing *E. coli* with TEM was 27 (55.1%)(Ahmed *et al.*, 2013).The antibiotic susceptibility of *E. coli* isolates was evaluated for 9 antimicrobial. however, overall, *E. coli* were resistance to 9 of the agent including ceftriaxone (100%), ceftazidime (100%) amoxicillin (100%), azithromycin (95%), cefixime (87.5%), tetracyclin (87.5%), erythromycin (100%), nalidixic acid (85%) and difloxcain (75%)(Table2).

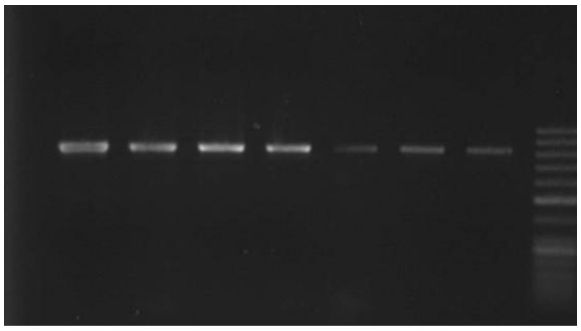

Fig. 1. PCR results for TEM gene.
Lane numbers 1-7 shows the 872 bp fragment of TEM gene and lane 8 shows the 50 bp DNA size markers.

In the study, *E. coli* were resistance to 9 of the agent including ceftriaxone (100%), ceftazidime (100%) amoxicillin (100%), azithromycin (95%), cefixime (87.5%), tetracyclin (87.5%), erythromycin (100%), nalidixic acid (85%) and difloxcain (75%). The study of Shakya, the proportions of isolates resistant to various antibiotics were, nalidixic acid (45%), tetracycline (37%), ampicillin (37%), sulfamethoxazole/trimethoprim (29%) and amoxicillin/clavulanic acid (29%) (Shakya *et al.*, 2013).The study of Iruka, the prevalence of strains resistant to tetracycline, ampicillin, chloramphenicol, and streptomycin were 9% to 35% in 1986 and 56% to 100% in 1998(Iruka *et al.*, 2000). The study of Rajabnia-Chenari *E. coli* were resistance to the agent including amikacin (15.4%), imipenem (49.65%), gentamicin (16.35%), ceftriaxone (60%), cefixime (97.24%) and nitrofurantoin (19.08%) (Rajabnia-Chenari *et al.*, 2012). The study of Mohammadi, *E. coli* were resistance to the antibiotic agent including ampicillin (98.4%), amoxicillin (83.7%), trimethoprim (56.7%) and cephalexin (48.1%) (Mohammadi *et al.,* 2010).

In conclusion, the ESBL producing isolates detected by PCR in 30% of the total isolates. PCR is a valuable tool for isolation of ESBLs in clinical and research settings. Determination of TEM gene by molecular techniques in ESBL producing bacteria may give useful data about their epidemiology and risk factors associated with these infections. Therefore, ESBL producing organisms should be promptly identified for appropriate antibiotic prescription and proper implementation of infection control measures.

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