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Phenotypic and genotypic detection of extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* isolated from urinary tract infections in Zabol, Iran

Saeide Saeidi[1], Mehdi Ghamgosha[2], Ramezan Ali Taheri[3], Yasub Shiri[4], Mahmood Solouki[4], Kazem Hassanpour[5], Gholamreza Farnoosh[6*]

[1]*Department of Agronomy and Plant Breeding, Agriculture Research Center, Zabol University, Zabol, Iran*

[2]*Neurosciences Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran*

[3]*Nanobiotechnology Research Centre, Baqiyatallah University of Medical Sciences, Tehran, Iran*

[4]*Department of Biotechnology, Faculty of Agriculture, Zabol University, Zabol, Iran*

[5]*Medical School, Sabzevar University of Medical Sciences, Sabzevar, Iran*

[6]*Applied Biotechnology Research Centre, Baqiyatallah University of Medical Sciences, Tehran, Iran*

PEER REVIEW

Peer reviewer

Jafar Amani, Ph.D. Molecular Biology, Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran. Tel/Fax: 02188600046, E-mail: jafar.amani@gmail.com

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Comments

This is a valuable research work in which authors have demonstrated TEM gene PCR assay is a rapid, sensitive and clinically useful test, particularly for the early detection of ESBLs-producing *E. coli* and the prevalence of ESBLs producing organisms in Zabol, Iran is high. It seems necessary for clinicians and health care systems to be fully aware of ESBLs producing microorganisms. Also, monitoring of ESBLs production is recommended to avoid treatment failure and for suitable infection control in Iran.

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ABSTRACT

Objective: To investigate the role of a rapid polymerase chain reaction (PCR) assay in comparison with traditional empiric therapy in detection of extended spectrum β-lactamase (ESBL) producer *Escherichia coli* (*E. coli*).

Methods: Ninety isolates of *E. coli* from urinary tract infection were collected and screening of ESBL resistance using disc diffusion method, minimum inhibitory concentration (MIC) for ceftazidime and detection of TEM resistant gene by PCR were done.

Results: The results of disc diffusion method showed that forty out of ninety *E. coli* isolates were ESBLs producing organisms. Antibiotic susceptibility of *E. coli* isolates to 9 antibacterial agents were evaluated. However, all isolated *E. coli* were resistant to all 9 antibacterial agents by these percentage: ceftriaxon (100%), ceftazidime (100%), amoxicillin (100%), erythromycin (100%), azithromycin (95%), cefixime (87.5%), tetracyclin (87.5%), nalidixic acid (85%) and difloxcain (75%). The abundance of antibiotic-resistant TEM gene according to PCR was 30%. Totally 82.5% of strains tested by MIC were observed as ceftazidime-resistant.

Conclusions: 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*.

KEYWORDS

Antibiotic resistance, Extended spectrum β-lactamase, TEM resistance gene, Polymerase chain reaction, Urinary tract infection, *E. coli*

1. Introduction

Disease-causing microbes that have become resistant to antibiotic drug therapy are an increasing public health problem. Urinary tract infections (UTIs) are just a few of the diseases that have become hard to treat with antibiotics[1].

*Corresponding author: Gholamreza Farnoosh, Applied Biotechnology Research Centre, Baqiyatallah University of Medical Sciences, Tehran, Iran.

Tel: +98 9155431709

E-mail: rzfarnoosh@yahoo.com

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Antimicrobial drug resistance is a threat for therapy failure in human medicine. The presence of enterobacteria, especially *Escherichia coli* (*E. coli*) that produces extended-spectrum β-lactamases (ESBLs), has increased during past decades in terms of the worldwide distribution of such resistance traits and of the evolution of different genes. Resistance genes of the ESBL type are mostly plasmid associated and therefore can spread among bacteria and they also harbor resistant genes to other antimicrobial classes with resulting multidrug-resistant isolates[2,3]. The rapidity of development and spreading of resistance is a complex process that is influenced by selective pressure, pre-existence of resistance genes and use of infection control measures. UTI is the second most common type of infection in body. The most common cause of UTI is Gram-negative bacteria that belongs to the Enterobacteriaceae family. Members of this family include *E. coli*, *Klebsiell*, *Enterobacter* and *Proteus*. Also, Gram-positive *Staphylococcus* sp. plays a role in infection[4]. β-Lactam antimicrobial agents are the most commonly used treatment for bacterial infections. Resistance to β-lactam antibiotics among clinical isolates of Gram-negative bacilli is most often due to the production of β-lactamases[5,6]. These enzymes are numerous and they mutate continuously in response to heavy pressure of using antibiotics and have led to development of ESBLs[7]. The ESBL enzymes are capable of hydrolyzing broad spectrum cephalosporins and monobactams but it is inactive against cephamycins and imipenem. In addition, ESBL producing organisms exhibit co-resistance to many other classes of antibiotics, resulting in limitation of therapeutic options. For this reason, the significance of such ESBL-mediated infections has been increasingly reported worldwide[8]. The methods for detection of ESBLs can be broadly divided into two groups: phenotypic methods that use non-molecular techniques, which detect the ability of the ESBL enzymes to hydrolyze different cephalosporins; and genotypic methods, which use molecular techniques to detect the gene responsible for the production of the ESBL. Clinical diagnostic laboratories use mostly phenotypic methods because these tests are easy to do, cost effective, and have been incorporated in most automated susceptibility systems, making them widely accessible. However, phenotypic methods are not able to distinguish between the specific enzymes responsible for ESBL production (SHV, TEM, and CTX-M types). Several research or reference laboratories use genotypic methods for the identification of the specific gene responsible for the production of the ESBL, which have the additional ability to detect low-level resistance (*i.e.* can be missed by phenotypic methods). Furthermore, molecular assays also have the potential to be done directly on clinical specimens without culturing the bacteria, with subsequent reduction of detection time[9].

In our study, primary antimicrobial susceptibility test of *E.coli* isolates showed the most resistance to third-generation cephalosporins such as cefixime, ceftriaxone and ceftazidime. More than 200 different types of ESBLs have been reported around the world so far[7,10]. ESBLs are often plasmid mediated and most of the enzymes are members of TEM and SHV families that have been described in many countries[11-13]. The TEM was first reported in *E. coli* isolated from a patient named Temoniera in Greece[14].

2. Materials and methods

2.1. Isolation of E. coli

All 90 strains of *E. coli* were isolated from urine culture of hospitalized patients (Zabol, southeastern city of Iran) suffered from UTIs during 2010-2011. The samples were examined microscopically by Gram staining. Samples with Gram-negative results were inoculated on plates of nutrient agar, cystine-lactose-electrolyte-deficient (CLED) agar, MacConkey′s agar and blood agar then incubated at 37 °C for 24 h[15]. Those colonies that showed fermentation of lactose on MacConkey′s agar and CLED agar were purified and identified according to their morphology as circular, rose-pink to red colonies on MacConkey′s agar and yellow colonies on CLED agar. The isolates were identified by biochemical tests such as catalase, potassium hydroxide, indole, methyl red, Voges-Proskauer, urease, citrate, H_2S and oxidase[16].

2.2. Phenotypic detection of ESBLs

Because of the clinical significance of ESBLs, specific guidelines for the detection of ESBL-expressing organisms were proposed in 1999 by the National Committee for Clinical Laboratory Standards. The presence of an ESBL is suggested if bacterial growth is observed despite a concentration of 1 μg/mL of at least one of three expanded-spectrum cephalosporins (ceftazidime, ceftriaxone or cefotaxime) or aztreonam, or growth occurs despite a concentration of 4 μg/mL of cefpodoxime. The use of more than one antibacterial agent for screening improves the sensitivity of detection of ESBLs. Phenotypic confirmatory tests include the addition of clavulanic acid to both ceftazidime and cefotaxime. A ⩾3 serial dilution concentration decrease in a MIC for either antibacterial agent tested in combination with clavulanic acid versus its MIC when tested alone constitutes a positive phenotypic test for an ESBL. If disk diffusion is used by the laboratory, a ⩾5 mm increase in zone diameter for either cefotaxime or ceftazidime tested with clavulanic acid versus its zone size when tested alone is considered a positive phenotypic ESBL test[17].

ESBLs producing strains was detected using single or

combined ceftazidime-clavulanic acid disks (MAST Co. UK). This was a combination test for phenotypic confirmatory of ESBLs[18]. Phenotypic detection of ESBLs was defined by an increase (≥5 mm) in the inhibition zone around clavulanic acid disk in comparison with zone around the disks without clavulanic acid. For assay of antibacterial activity, bacterial suspensions with concentration of 1.5×10^8 CFU/mL (0.5 McFarland standard) were prepared in nutrient broth. Oxoid combination disk method was used for detection of ESBLs producing organisms. In this method the bacteria were cultured on a Muller-Hinton agar plate, then amoxicillin (25 μg), azithromycin (15 μg), cefixime (5 μg), tetracyclin (30 μg), erythromycin (15 IU), ceftazidime (30 μg), ceftriaxone (10 μg), nalidixin acid (30 μg) and difloxcain (25 μg) disks (Mast, UK) were placed on media with 20-30 mm distance to other disks. The plates were incubated for 18-24 h at 37 °C[19,20].

2.3. DNA extraction and PCR

The colonies of ESBLs producing organisms were suspended in Tris-EDTA buffer and their DNA were extracted by simple boiling[21]. The PCR method for detection of TEM gene was performed as described previously with minor modifications[22]. Briefly, specific primers for the TEM gene (forward primer 5′GAG TAT TCA ACA TTT CCG TGT C3′; reverse primer 5′TAA TCA GTG AGG CAC CTA TCT C3′) were used for PCR amplification that produced 872 bp PCR products. The PCR mixture consisted of 10 pmol of each primers, 1 μL DNA sample (3 μg/μL), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each dNTP, and 5 IU *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 seconds 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 gel electrophoresis.

2.4. MIC of antibiotics

The MIC of antibiotics was determined using bacterial broth dilution method according to the method used by Baron and Finegold[23]. To study the effect of antibiotics, the nutrient broth (Merck, Germany, pH=6.5) containing concentrations of (512, 256, 128, 64, 32, 16, 8, 4 and 2 μg/mL) were prepared from each antibiotics (ceftazidime) (Mast, UK). Nutrient broth without antibiotic was used as the control media[24]. The MIC was defined as the lowest drug concentration which prevented visible growth of bacteria[25].

2.5. Statistic assessment

All the experiments and measurements were repeated at least three times. All the statistical analysis was performed using SPSS and Microsoft Excel 2010 software.

3. Results

3.1. Antibiotic susceptibility

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 agents. however, *E. coli* were resistant to 9 of the agents including ceftriaxon (100%), ceftazidime (100%), amoxicillin (100%), azithromycin (95%), cefixime (87.5%), tetracyclin (87.5%), erythromycin (100%), nalidixin acid (85%) and difloxcain (75%) (Table 1).

Table 1

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

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

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

3.2. Assessment of MIC

The MIC of ceftazidime against 40 clinical isolates of *E. coli* was shown in Table 2. The MIC of susceptible strains to ceftazidime were ≤8 μg/mL and MIC of resistant strains to ceftazidime were ≥32 μg/mL. 82.5% of *E. coli* strains was found to be resistant to ceftazidime with MIC ≥32 μg/mL (33 samples) and 17.5% of *E. coli* strains was found to be susceptible to ceftazidime with MIC ≤8 μg/mL (7 samples).

3.3. Amplification of antibiotic-resistant genes

The amplification of TEM gene revealed that 13 isolates (32.5%) harbored the gene and another 27 strains (67.5%) were negative (Table 2), the primers generated a 872 bp PCR product in PCR reaction (Figure 1).

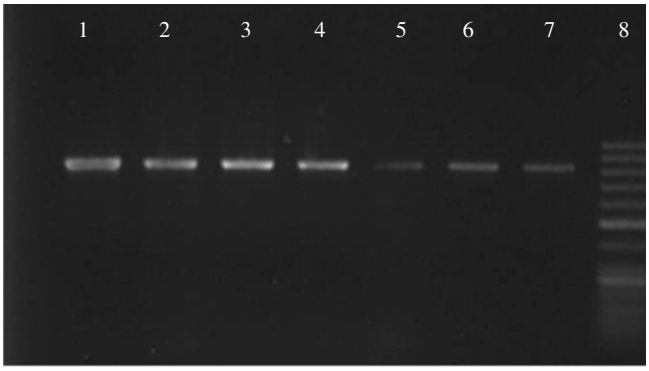

Figure 1. PCR results for TEM gene.
Lane numbers 1-7 shows the 872 bp fragment of TEM gene and Lane 8 shows the 50 bp DNA size marker.

Table 2
Antimicrobial susceptibility, MIC of ceftazidim and positive TEM strains of *E. coli*.

Bacterial code	TEM gene	MIC for ceftazidim	Resistance pattern
1	+	512	A1, A2, A3, B1, B2, B3, C1, C2, C3
2	-	256	A2, A3, B2, B3, C2, C3
3	+	128	A2, A3, B2, C1, C2, C3
4	-	2	A1, B1, B2, C1, C2, C3
5	-	8	A1, A2, A3, B2, C2, C3
6	-	512	A1, A2, A3, B1, B2, B3, C1, C2, C3
7	+	256	A1, A2, A3, B1, B2, B3, C1, C2, C3
8	-	128	A1, A2, A3, B1, B2, B3, C1, C2, C3
9	+	512	A1, A2, A3, B1, B2, C1, C2, C3
10	+	512	A1, A2, A3, B1, B2, B3, C1, C2, C3
11	-	512	A1, A2, A3, B1, B2, C1, C2, C3
12	+	128	A1, A2, A3, B1, B2, B3, C1, C2, C3
13	-	128	A1, A2, A3, B1, B2, C1, C2, C3
14	-	512	A1, A2, B1, B3, C1, C2, C3
15	+	256	A1, A2, A3, B1, B2, B3, C1, C2, C3
16	-	512	A1, A2, A3, B1, B2, B3, C1, C2, C3
17	-	512	A1, A2, A3, B1, B2, B3, C1, C2, C3
18	-	512	A1, A2, A3, B1, B2, B3, C1, C2, C3
19	+	256	A1, A2, A3, B1, B2, B3, C1, C2, C3
20	-	256	A1, A2, A3, B1, B2, B3, C1, C2, C3
21	-	8	A1, A2, A3, B1, B2, B3, C1, C2, C3
22	-	128	A1, A2, A3, B1, B2, B3, C1, C2, C3
23	-	256	A1, A2, A3, B1, B2, B3, C1, C2, C3
24	+	128	A1, A2, A3, B1, B2, B3, C1, C2, C3
25	-	128	A1, B1, B2, C1, C2, C3
26	-	8	A1, A2, A3, B1, B2, B3, C1, C2, C3
27	-	4	A1, A2, A3, B1, C1, C2, C3
28	-	4	A1, A3, B1, B2, C1, C2, C3
29	+	128	A1, A3, B1, B2, C1, C2, C3
30	+	128	A1, A2, A3, B1, B2, B3, C1, C2, C3
31	-	128	A1, A3, B1, B2, B3, C2, C3
32	-	8	A1, A2, A3, B1, B2, B3, C1, C2, C3
33	-	128	A1, A2, B1, B2, B3, C1, C2, C3
34	-	256	A1, A2, A3, B1, B2, B3, C2, C3
35	-	256	A1, A2, B1, B2, B3, C2, C3
36	-	256	A1, A2, A3, B1, B2, B3, C1, C2, C3
37	-	512	A1, A2, A3, B1, B2, B3, C1, C2, C3
38	+	512	A1, A2, A3, B1, B2, B3, C1, C2, C3
39	+	256	A1, A2, A3, B1, B2, B3, C1, C2, C3
40	-	128	A1, A2, A3, B1, B2, B3, C1, C2, C3

A1: Ceftazidime; A2: Nalidixin acid; A3: Cefixime; B1: Ceftriaxon; B2: Azitromycin; B3: Difloxcain; C1: Tetracyclin; C2: Amoxicillin; C3: Erythromycin.

4. Discussion

During the past decade, ESBL producing Gram-negative bacilli especially *E. coli* and *Klebsiella pneumoniae* (*K. pneumoniae*) have emerged as serious pathogens both in hospital and community acquired infections worldwide. β-Lactam antibiotics such as long spectrum cephalosporins and carbapenems are the preferred treatment of enterobacterial infections[26]. Recent studies revealed that patients with infection such as septicemia with ESBL producing organisms had significantly higher fatality rate than those with non-ESBL isolates[27]. Among Gram-negative bacteria, the emergence of resistance to extended-spectrum cephalosporins has been a major concern, initially there were a limited number of bacterial species and now they are expanding rapidly[28]. Our investigations were carried out on testing the distribution of antibiotic resistance to ceftazidim and presence of TEM gene in isolated *E. coli* from urine culture of hospitalized patients. The isolated *E. coli* population showed a considerable resistance to antibiotics that are using frequently. Based on the results of this study, the prevalence of ESBL producing *E. coli* was high (44.4%) by disc diffusion tests and in PCR method the distribution of TEM gene in isolated ESBLs producing organisms were (32.5%). Therefore, we concluded that prevalence of ESBLs producing organisms is high in Zabol, Iran. Similarly, the prevalence of the organisms in India was reported 46.51% in *E. coli* and 44.44% in *K. pneumoniae* isolates[21]. In Tehran out of 115 ESBL producing isolates, 60% were *E. coli* and 40% were *K. pneumoniae*[27]. In one study, among 101 isolates, 68 (67.3%) were ESBL producers that 49 of them were *E. coli* and 19 were *K. pneumoniae*[29]. 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[30]. In another study in Pakistan, the prevalence of the ESBLs producing *E. coli* isolates was reported 56.9%[31]. The frequency of TEM gene among the ESBLs producing isolates was reported 20.6%[32]. Although in a study the authors concluded that expression of β-lactamase genes depend upon the environmental conditions such as the presence of antibiotics and gene presence shown by PCR does not necessarily indicate its expression[33]. In summary, the prevalence of ESBLs producing organisms in Zabol, Iran is high. It seems necessary for clinicians and health care systems to be fully aware of ESBLs producing microorganisms. Also, monitoring of ESBLs production is recommended to avoid treatment failure and suitable infection control in Iran.

This study confirms the large dissemination of the gene TEM among *E. coli* in southeast of Iran, and the results of

antibiotic susceptibility revealed a high rates of resistance against the third-generation cephalosporins which are widely used in treatment of the urinary tract infections. These results should draw the attention of the Iranian medical authorities and developing countries to serious consequences of increasing antimicrobial resistance. Therefore, it is important to increase efforts to monitor and control spreading of antimicrobial resistant strains in hospitals and community. We concluded that the TEM gene PCR assay is a rapid, sensitive and clinically useful test, particularly for the early detection of ESBLs-producing *E. coli*.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Disease-causing microbes that have become resistant to antibiotic drug therapy are an increasing public health problem. UTIs are just a few of the diseases that have become hard to treat with antibiotic. Antimicrobial drug resistance is a threat for therapy failure in human medicine. Using of rapid PCR assay in comparison with traditional empiric therapy in detection of ESBL producer *E. coli* is necessary.

Research frontiers

In this study TEM gene PCR assay was used for early detection of ESBLs-producing *E. coli*.

Related reports

ESBL producing Gram-negative bacilli especially *E. coli* and *K. pneumoniae* have emerged as serious pathogens both in hospital and community acquired infections worldwide. β-Lactam antibiotics such as long spectrum cephalosporins and carbapenems are the preferred treatment of enterobacterial infections.

Innovations and breakthroughs

This study confirms the large dissemination of the gene TEM among *E. coli* in southeast of Iran, and the results of antibiotic susceptibility revealed a high rate of resistance against the third-generation cephalosporins which are widely used in treatment of the urinary tract infections.

Applications

The results of the sutdy should draw the attention of the Iranian medical authorities and developing countries to serious consequences of increasing antimicrobial resistance.

Peer review

This is a valuable research work in which authors have demonstrated that TEM gene PCR assay is a rapid, sensitive and clinically useful test, particularly for the early detection of ESBLs-producing *E. coli* and the prevalence of ESBLs producing organisms in Zabol, Iran is high. It seems necessary for clinicians and health care systems to be fully aware of ESBLs producing microorganisms. Also, monitoring of ESBLs production is recommended to avoid treatment failure and suitable infection control in Iran.

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