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Genotypic versus Phenotypic methods to detect Extended-Spectrum Beta-Lactamases (*ESBLs*) in Uropathogenic *Escherichia coli*

Yazdi M[1], Nazemi A[1*], Mirinargasi M[1], Jafarpour M[1], Sharifi SH[2]

[1]*Department of Biology, Tonekabon Branch, Islamic Azad University, Tonekabon, Iran*
[2]*Department of Medical Biology, Ankara University, Turkey*

ABSTRACT

The rapid spread of resistance to broad-spectrum beta-lactams in pathogenic strains of bacteria has recently become a major health problem in the world. It causes antibiotics ineffectiveness, increased severity of illness and cost of treatment. The aim of this study was to compare phenotypic and genotypic methods to determine the susceptibility pattern of E. coli isolates to beta-lactam antibiotics. A total of 246 E. coli samples were isolated from different clinical laboratories located in the city of Tehran and confirmed by biochemical tests. The antibiotic susceptibility of E. coli isolates were determined by disc-diffusion method. Antimicrobial agents tested included Cefoxatime, Ceftazidime, Imipenem, Nalidixic acid, and Ciprofloxacin. The combined disc test was used to confirm the results. The results were compared with Clinical and Laboratory Standards Institute (CLSI). All samples were thereafter investigated for the presence of CTX-M, TEM, and SHV genes by PCR. Out of 246 E. coli isolates tested, 116 were resistant to Cefoxatime and Ceftazidime, of which 109 (44.3%) were ESBL positive by combined disc test. However, the number of isolates determined positive for ESBL by genotypic method was 143 (58.1%). Of 109 isolates determined positive by phenotypic method, 41 (37.6%) included all three genes. A number of 34 (13.8%) isolates showed to be ESBL positive by PCR but negative by combined disc test. The results of this study showed that some antibiotic sensitive isolates were carrying resistance genes. Such strains have the potential to turn into resistance. Therefore, the genotypic method due to detection of resistance genes has a higher specificity and sensitivity in compare to the phenotypic methods, and is suggested to be used as the method of choice for detection of ESBL producing strains of E. coli.

Keywords: ESBL; *Escherichia coli*; CTX-M; TEM; SHV genes.

INTRODUCTION

One of the most important resistant mechanisms in Gram-negative bacteria against beta-lactam antibiotics is induced by production of beta-lactamase enzymes [1]. The new broad-spectrum antibiotics such as Cephalosporins used in treatment of bacterial infections has led to the production of a new class of broad-spectrum enzymes called beta-lactamase [2]. Indeed, occurrence of point mutations in the sequence of the primary beta lactamase gene results in production of different enzymes [3]. Beta-lactamase enzymes are classified into four main groups including A, B, C, and D based on their inhibitory mechanism, type of substrate, and physical characterization such as molecular weight and isoelectric point. According to this classification, broad-spectrum beta lactamases are categorized among group A [4,5]. The gram-negative bacteria have rapidly expanded resistance to broad-spectrum beta-lactam antibiotics during the past two decades [6]. More than 200 types of ESBLs have been found worldwide, most

belonging to the Enterobacteriaceae family [7]. *Escherichia coli* is of the bacteria with the ability to produce ESBL enzymes. As a member of Enterobacteriaceae family, *E. coli* causes some hospital infections such as sepsis, enteritis gastroenteritis, neonatal meningitis, and urinary tract infections [8]. The detection of *E. coli* is of importance for infection control, reduction in use of antibiotic, and epidemiological surveillance. The ESBL producing *E. coli* can be detected by either phenotypic or genotypic methods. Since different results have be achieved by different phenotypic methods used [9], the genotypic methods seem to be necessary for accurate identification of such resistant strains. The aim of this study was to detect ESBL producing *E. coli* isolated from people with urinary tract infection, and compare the frequencies obtained by the phenotypic and genotypic methods.

MATERIALS AND METHODS

Sample Preparation

A total of 246 Uropathogenic *E. coli* samples were obtained from cultured urines on EMB and Blood agar medium, collected from patients with urinary tract infection. The sample collection was done through some private clinical laboratories located in Tehran, Iran, and all participated patients were suffering from the highest level of infection with a colony count> 100,000 CFU/ml. The *E. coli* strains were confirmed using IMVIC biochemical tests.

Antibiotic susceptibility test

Antimicrobial susceptibility testing was carried out by the Kirby-Bauer disc diffusion method according to CLSI recommendations. Antibiotic discs including Cefotaxime (30 µg) and Ceftazidime (30 µg) (Mast, USA) was applied for susceptibility test. Samples showing an inhibition zone size of ≤ 22 mm with Ceftazidime and ≤ 27 mm with Cefotaxime were considered as potential ESBL producer and were further investigated for confirmation of ESBL production by combination disc diffusion test.

Screening for ESBL producing isolates

A Ceftazidime and a Ceftazidime + Clavulanic acid (30 µg/10 µg) discs (Mast, USA) were placed at a distance of 25 mm on a Mueller-Hinton Agar (Difco, USA) plate inoculated with a bacterial suspension of 0.5 McFarland turbidity standards (Difco, USA) and incubated overnight at 37 °C. A ≥ 5 mm increase in the diameter of inhibition zone for the combination disc versus Ceftazidime disc confirmed ESBL production. The resistance of all samples to other antibiotic including Imipenem (10 µg), Nalidixic acid (30 µg), and Ciprofloxacin (5 µg) (Mast, USA) was also determined.

Genotypic Assay

The Boiling method was used to extract DNA from bacterial samples [10]. SHV, TEM, and CTX-M beta-lactamase genes were detected by PCR. PCRs were carried out using thermal cycler (BioRad, USA) in a total volume of 25 µl containing 10 pmol of each three pair of primers (Sigma, USA), 25 µmol of dNTPs, 5 µl of template DNA, 2.5 µl of 10X *Taq* buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3)], 2 mM MgCl2 and 2.5 U of *Taq* polymerase (Fermentas, USA). The Primer sequences and cycling conditions used for three different PCRs are shown in Table 1. PCR products were separated by gel electrophoresis on 1% agarose gel. In order to confirm the accuracy of genes amplified in this study, a PCR product of each gene was sent for sequencing to the Macrogen Company (South Korea) and the result was confirmed by NCBI Blast Tool.

Table 1. Primers and cycling conditions used for amplification of SHV, CTX-M and TEM genes

Resistance gene	Sequence (5' to 3')	Size (bp)	Cycling conditions	Reference
SHV	GATGAACGCTTTCCCATGATG CGCTGTTATCGCTCATGGTAA	214	95°C for 5 min; 35 cycles of 95°C for 60s, 61°C for 60s, 72°C for 60s; 72°C for 5 min	11
CTX-M	TTTGCGATGCATACCAGTAA CGATATCGTTGGTGCCATA	590	95°C for 5 min; 35 cycles of 95°C for 60s, 60°C for 30s, 72°C for 60s; 72°C for 5 min	12
TEM	ATGAGTATTCAACATTTCCG GTCACAGTTACCAATGCTTA	847	95°C for 5 min; 35 cycles of 95°C for 60s, 58°C for 60s, 72°C for 60s; 72°C for 5 min	11

Figure 1. Phenotypic confirmation test for detection of ESBL *E. coli*

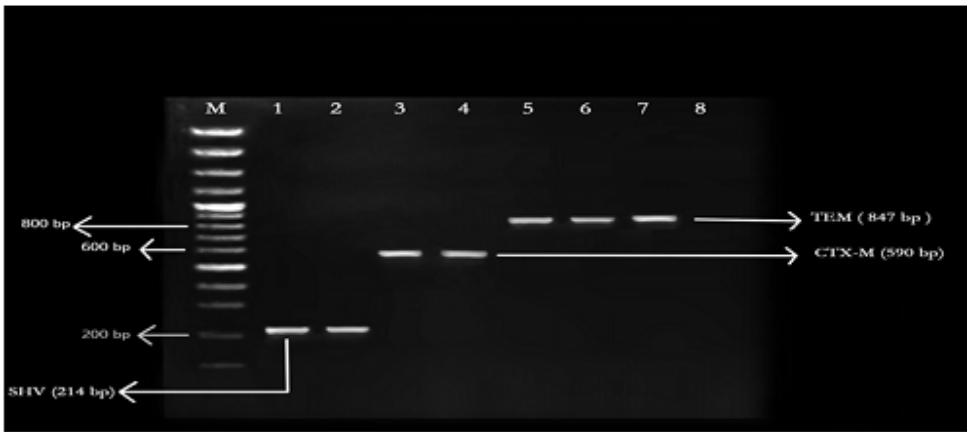

Figure 2. PCR products of SHV, TEM and CTX-M genes run on 2% agarose gel. Lanes 1–7: *E. coli* isolated from patients; lane 8: negative control; lane M: 100bp size marker.

RESULTS AND DISCUSSION

Of 246 *E. coli* isolates collected from urine samples, 116 (47.1%) showed resistance to the third generation Cephalosporins (Cefotaxime, Ceftazidime), 123 (50%) to Nalidixic acid, 82 (33.3%) to Ciprofloxacin, and 20 (8.2%) to Imipenem. The results also showed that 62 out of 246 (25.2%) isolates were sensitive to all antibiotics tested, while 143 (58.1%) isolates showed multi-drug resistance. The combined disk test done on 116 isolates resistant to third generation Cephalosporins also showed that 109 isolates were ESBL producing strains (Figure 1).

The genotyping results of ESBL producing isolates obtained by PCR amplification of SHV, CTX-M and TEM genes are shown in Table 2. Of 109 ESBL positive isolates, 77 (70.6%) were carrying SHV, 75 (68.8%) CTX-M, and 95 (87.1%) TEM genes, while 40 (36.6%) isolates included all three genes together. In addition, 68 (62.3%) isolates included SHV and TEM, 61 (55.9%) TEM and CTX-M, and 54 (49.5%) SHV and CTX-M genes together. Out of 137 isolates shown susceptible to the third generation Cephalosporins by combined disc test, 34 (13.8%) were also ESBL positive by genotypic method as they were carrying SHV and TEM genes (Table 2). 103 out of 246 isolates (41.9%) had none of these three genes. All 143 ESBL positive isolates included either SHV or TEM genes.

Table 2. Presence/absence of SHV, CTX-M and TEM genes in samples resistant/susceptible to the third generation Cephalosporins by PCR

Total samples (N= 246)	SHV	CTX_M	TEM	SHV & CTX_M	SHV & TEM	CTX_M & TEM	CTX_M & TEM & SHV
ESBL positive (N=109)	0	0	7	14	27	20	41
ESBL negative (N=137)	7	0	20	0	7	0	0

CONCLUSION

Beta-lactamase genes are of the factors that increase resistance to beta-lactam antibiotics such as broad-spectrum Cephalosporins in bacteria. The enhanced pathogenicity of the bacteria carrying these genes increases the mortality risk of infected individuals and faces the community to serious health problems [13,14]. The results obtained by this study revealed that out of 246 samples tested, 143 (58.1%) were ESBL positive by genotyping method, while the number of ESBL positive samples identified by phenotypic method was 109 (44.3%). The difference observed in detection of ESBL positive isolates by two different methods (13.8%) may be justified by the lower sensitivity of phenotypic method and the influence of environmental factors on the incidence of resistance. Garrec and his colleagues showed that various phenotypic methods could lead in different results regarding the detection of ESBL positive isolates [9]. They achieved a sensitivity of 96% when testing at least Cefotaxime, Cefepime, and a third compound (Ceftazidime, Cefpodoxime, or Aztreonam). Therefore, in order to increase the sensitivity to 100%, they proposed a two-step strategy using phenotypic methods. The lack of constant sensitivity of different phenotypic methods has also been emphasized by some other studies [15]. In contrast, the genotypic method using specific PCR amplification of resistance genes seems to have 100% specificity and sensitivity. The cost of molecular method is particularly reduced for the bacteria belonging to the enterobacteriaceae family as their DNA is easily extractable by boiling method, a quick and cost effective DNA extraction method. Our study also showed that all ESBL positive samples comprised either SHV or TEM genes. Therefore, it seems that these two genes are the appropriate candidates for the molecular screening of ESBL positive samples. Incorrect identification of antibiotic resistance may lead to inappropriate antibiotic prescription, which in turn may direct bacteria to produce new resistance genes by selective pressure. Therefore, due to detection of resistance genes, the genotypic method is suggested to be used as the method of choice for detection of ESBL producing strains of *E. coli*.

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