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Full Length Research Paper

Molecular detection of TEM broad spectrum β-lactamase in clinical isolates of *Escherichia coli*

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Resistance to β-lactam antibiotics, along with clinical isolates, frequently results to production of β-lactamase enzymes. In recent years, the production of extended spectrum β-lactamases (ESBLs) among clinical isolates, especially *Escherichia coli* has greatly increased. On the other hand, β lactamase genes have several subfamilies, and designing universal primers could be valuable to detect all of them. The beta lactamase enzyme producing *E. coli*, resistant to β-lactam antibiotics, created many problems for the patients. The TEM gene is responsible for β-lactamase resistance. The purpose of this study was to find out the percentage of *E. coli* strains that carry TEM in genes. In total, 500 clinical samples were collected from different Hospitals in Tehran. All the samples were isolated on EMB and MacConkey agar and incubated at 37°C for 24 h. The identification was carried out by conventional biochemical tests. Out of the 500 samples, 200 were identified as *E. coli*. The TEM gene was determined by PCR method on the isolates, which were already identified as Phenotypic by disk diffusion agar and combined disk. Out of the 200 isolated *E. coli* strains, 128 (64%) were producing ESBls. The PCR results show that 74 isolates of *E. coli* (57.8%) had the TEM gene. Our findings show that the majority of the ESBL positive clinical isolates of *E. coli* carried the TEM gene.

Key words: *Escherichia coli*, β-lactamase enzymes, TEM-type extended spectrum beta-lactamases.

INTRODUCTION

β-Lactamase enzymes are the most important factor among Gram-negative bacteria that contribute to the resistance to β-lactam antibiotics (Chaïbi et al., 1999; Chouchani et al., 2007). The recent discovery of new classes of antibiotics, such as expanded cephalosporins, as well as their frequent use has led to the emergence of bacterial pathogens expressing extended spectrum β-lactamases (ESBLs), which have been derived from principal β-lactamases (TEM-1, TEM-2, and SHV-1) through the substitution of one or more amino acids in their catalytic sites (Vakulenko and Golemi 2002; Tenover et al., 2003).

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Abbreviation: ESBLs, Extended spectrum β-lactamases.

Table 1. Primers used for amplification.

Target (s)	Primer	Sequence (5`to 3 ` as synthesized)	Expected amplicon size (bp)
TEM	TEMF	AGATCAGTTGGGTGCACGAG	618
	TEMR	CAGTGCTGCAATGATACCG	

A major mechanism of β-lactam resistance in bacteria is the expression of TEM-type β-lactamases (Mammeri et al., 2001). Recently, 167 TEM β-lactamases that are commonly found in the Enterobacteriaceae family were identified, several of which were from Escherichia coli (Bradford, 2001; Gupta, 2007). The increased incidence of organisms expressing β-lactamase enzymes has led to difficulties in treatment using antibiotics that are currently available. Therefore, definitive identification of resistant strains is paramount for the control of Nosocomial infections (Paterson and Bonomo, 2005). According to the recommendations of the Clinical and Laboratory Standards Institute (CLSI), isolates that show reduced susceptibility to β-lactam antibiotics, especially third generation antibiotics, such as cephalosporins, are potential ESBL-expressing strains and can be confirmed in phenotypic tests using clavulanate (Clinical and Laboratory Standards Institute [CLSI], 2005). However, the use of such phenotypic methods is not sufficient for the unequivocal detection of strains expressing these enzymes, and additional molecular techniques are required in addition to phenotypic assays (Netzel et al., 2007). Therefore, we sought to determine the prevalence of ESBLs in clinical isolates of *E. coli* and to detect TEM using molecular methods. Additionally, as β-lactamase genes have several subfamilies, universal primers that are able to detect all β-lactamase variants would be an invaluable diagnostic tool. Therefore, we designed a set of universal primers that were capable of amplifying all genes within the TEM cluster and subsequently evaluated their efficacy using PCR.

MATERIALS AND METHODS

More than 500 bacterial isolates were recovered from clinical samples including urine, diarrheal stool, blood and wounds from hospitals in Tehran during a six months period (December 2008 to May 2009). From these samples, 200 E. coli isolates were detected by IMVIC standard biochemical tests. All these E. coli isolates were subsequently stored in skim milk at -70°C until they were required for further tests.

Screening and phenotypic identification of ESBLs

Disk diffusion on Muller-Hinton agar was used for the initial screening of *E. coli* isolates expressing ESBLs. In this method, microbial suspensions with concentrations of 0.5 McFarland were evenly spread on the surface of Muller-Hinton agar plates, after which various antibiotic disks were then placed on the plates at a distance of 2.5 cm from each other. The antibiotics tested included cefotaxime (30 µg), ceftazidime (30 µg), gentamicin (10 µg), amoxicillin (30 µg), imipenem (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), cotrimoxazole (1.25 µg), ciprofloxacin (5 µg) and chloramphenicol (30 µg) (Mast Diagnostics Ltd., UK). The plates were incubated for 24 h at 37°C. The zone of inhibition (ZOI) around each dick was then measured using a ruler and was compared with the standard set by CLSI. The strains were then classified as resistant, intermediate, or sensitive to the antibiotics (CLSI, 2005; Netzel et al., 2007). Isolates that were deemed resistant to cephalosporin were selected for confirmatory tests using the Combined Disk method. In this test, the strains were tested for resistance to ceftazidime (30 µg), ceftazidime (30 µg) / clavulanate (10 µg), cefotaxime (30 µg) and cefotaxime (30 µg) / clavulanate (10 µg) (Mast Diagnostics Ltd., UK). After the plates were incubated for 24 h at 37°C, ESBL production was measured. Strains were identified as producing ESBL if there was an increase in the ZOI by ≥5 mm in the presence of clavulanic acid compared with the ZOI without clavulanic acid (Deshpande et al., 2006; Song et al., 2007). Resistant isolates were further evaluated using PCR.

PCR and sequencing of the β-lactamase genes

Genomic DNA was extracted from strains expressing ESBLs using the extraction kit (Bioneer, Seoul, Korea) according to the manufacturer's instructions. PCR was performed to amplify the bla TEM gene using universal primers (Table 1). Each reaction contained 2.5 ¬µl 10X buffer¬, 2¬ µl 50 mM MgCl2, 1 ¬µl 10 mM dNTP, 1.5 µl 50 pmol each for the forward and reverse primer, 1¬ µl 5 U Taq polymerase, 2¬ µl 50 pmol template DNA, and 14.5 µl H_2O in a final volume of 25 µl. The PCR was carried out under the following conditions: initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and elongation at 72°C for 1 min. The final elongation step was conducted at 72°C for 10 min. The PCR amplicons were subsequently resolved by electrophoresis on a 0.8% agarose gel. The amplicon with the expected size (based on the predicted size of the TEM cluster) was excised from the gel using a kit (Fermentas, Germany) and sent to Macrogen Research, Seoul, Korea for sequencing analysis. The sequences were then aligned with the known TEM sequences in the NCBI database. The DNA isolated from K. pneumoniae ATCC 7881 was used as a positive control for bla TEM expression. However, a reaction that did not contain template DNA was used as a negative control.

Design of primers

One hundred and twenty (120) submitted sequences that are related to the *E. coli* TEM gene are present in GenBank. All sequences were aligned using a MEGA 4 program that identified sequences with over 90% homology. The conserved sequences

Table 2. Pattern of resistance of 200 *E. coli* isolates to 10 antimicrobial agents.

Antibiotic	Resistance	Intermediate	Sensitive
Imipenem	1 (0/5%)	0	199 (99/5%)
Ciprofloxacin	109 (54.5%)	16 (8%)	75 (37.5%)
Gentamicin	78 (39%)	4 (2%)	118 (59%)
Chloramphenicol	61 (30.5%)	36 (18%)	103 (51/5%)
Streptomycin	143 (71.5%)	29 (14.5%)	28 (14%)
Cefotaxime	128 (64%)	7 (3/5%)	65 (32/5%)
Cepatozidin	111 (55.5%)	11 (5/5%)	78 (39%)
Cotrimoxazole	161 (80.5%)	3 (1/5%)	36 (18%)
Nalidixic acid	148 (74%)	13 (6/5%)	39 (19.5%)
Amoxicillin	189 (94.5%)	3 (1/5%)	8 (4%)

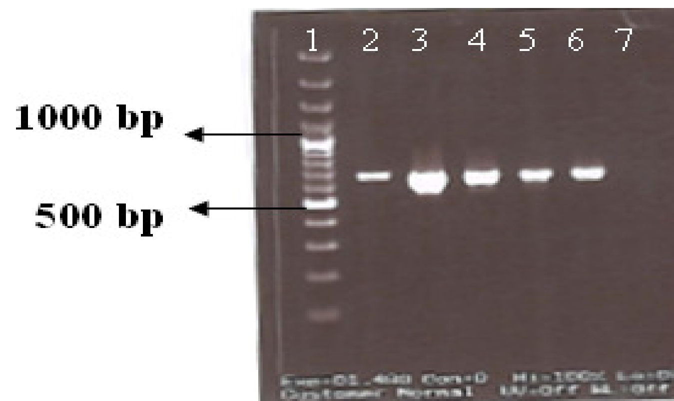

Figure 1. PCR amplification of *TEM* gene. Lane 1: 100 bp DNA marker; lane 2: *bla* TEM gene from the positive control; lanes 3, 4, 5 and 6: *bla* TEM gene from clinical isolates; and lane 7: negative control.

that had a high degree of homology were selected and used to design universal primers via the Gene runner software. The designed primers were subsequently tested in silico against submitted sequences in a BLAST analysis. PCR was also performed on the isolated DNA samples.

RESULTS

Two hundred (200) clinical isolates of *E. coli* were used for this study. Of these, 125 (62.5%) were from urine and urinary catheters, 48 (24%) were from diarrheal stool, 18 (9%) were from blood, 5 (2.5%) were from wounds and 4 (2%) were from other clinical samples. The pattern of resistance to 10 antimicrobial agents among these 200 *E. coli* isolates is shown in Table 2. Majority of the isolates showed a high degree of resistance to oxyiminocephalosporins while remaining in the susceptible range of imipenem. Approximately, 70% of the isolates tested exhibited multi drug resistance (MDR) phenotype.

In this sudy, using the disk diffusion method, 128 (64%) of the *E. coli* isolates were noted to be resistant to ceftazidime and cefotaxime. These were putatively dubbed positive for ESBL expression and were selected for further analysis using the Combined Disk assay. In this study, 89.8% (115/128) were confirmed to be ESBL producers. β-Lactamase-expressing *E. coli* isolates were more common in urinary samples (80%). PCR was performed on genomic DNA isolated from all 128 resistant strains using universal primers. These data showed that among these isolates, 57.8% (74/128) were positive for the blaTEM gene (Figure 1). However, TEM sequence analysis also showed approximately 90% sequence similarity to the submitted sequences that are related to this gene in GenBank.

DISCUSSION

There are several phenotypic tests that can be used to

detect ESBLs. However, with the emergence of novel β-lactamase enzymes, the tests currently recommended by the CLSI are no longer adequate for the definitive diagnosis of antibiotic resistant pathogens (Pérez-Pérez and Hanson, 2002; Goossens and Grabein, 2005). In this study, we identified 89.8% of the isolates tested as ESBL producing strains according to the recommendations set forth by the CLSI. We performed PCR to assess the prevalence of β-lactamase enzymes (TEM) on all the 128 (64%) isolates. Due to the inherent variation in the sequence between members of the TEM gene subfamily, a set of universal primers that could amplify all the genes from the TEM family was designed and these results were confirmed by alignment of sequences using Basic Local Alignment Search Tool (BLAST). Among the 128 *E. coli* Basic Local Alignment Search Tool (BLAST) isolates that were deemed as ESBL-expressing strains, 57.8% of them tested positive for the bla TEM gene. Two isolates that were designated as negative for ESBL expression in phenotypic assays were found to express the TEM gene, possibly due to expression of novel β-lactamase enzymes, such as AmpC. Therefore, the use of molecular methods coupled with phenotypic tests is essential for the definitive identification of these types of β-lactamase enzymes (Coque et al., 2008).

Recently, co-production of ESBLs and AmpC β-lactamases in some pathogens, particularly *E. coli*, is increasing and therefore provides these strains with a mechanism for a broader spectrum of resistance. In light of these findings, diagnostic laboratories should adopt conclusive methods to detect the bacteria expressing these enzymes, since they play a significant role in the control of antibiotic resistant pathogens (Coudron et al., 2000; Rupp and Fey, 2003). The number of β-lactamase enzymes is rapidly increasing worldwide due to the continuous mutation of their coding sequences. The majority of the genes found today were originally derived from the TEM β-lactamase family (Gupta, 2007; Harada et al., 2008). In this study, the bla TEM gene was detected in 57.8% of the isolates; however, other ESBL genes, such as SHV, OXA and CTX-M, may play a role in the antibiotic resistance seen in the other isolates (Zhang et al., 2009). In comparison, the results of the incidence of bla-TEM gene detection observed in this study was either similar (Fang et al., 2008, Hosoglu et al., 2007), higher (Valvered et al., 2007), or lower than some others (Tasli and Bahar, 2005; Chaïbi et al., 1999). *E. coli* strains play an important role in hospital infections, since TEM β-lactamase *E. coli* are resistance to penicillin antibiotic. Therefore, detection of these genes in bacterial isolates recovered from clinical samples is essential for the prescription of drugs to treat such infections and the bacterial pathogens that are resistant to particular antibiotics. Many clinical laboratories do not take into account the expression of novel β-lactamases and their inability to be detected in phenotypic tests. Moreover, previous reports have not attempted to study the expression of novel ESBLs in bacteria (Soltan Dallal et al., 2010). Therefore, our data strongly suggest that clinical diagnostic laboratories should work towards improving their diagnostic methods in order to conclusively detect ESBL-expressing organisms.

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