PREVALENCE OF TEM β-LACTAMASE RESISTANCE GENE IN ESCHERICHIA COLI ISOLATED FROM TEHRAN MILAD HOSPITAL

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ABSTRACT: Resistance to β-lactam antibiotics in Escherichia coli (E. coli) is known as a major problem in hospital setting and often caused by β-lactamase enzymes, especially extended spectrum β-lactamases (ESBLs). Due to infections caused by ESBL-producing E. coli, the aim of this study was to identify TEM in E. coli isolated from Milad Hospital by polymerase chain reaction (PCR). A total number of 100 bacterial isolates were collected from urine, blood, mucous and wound samples by biochemical test. Antibiotic resistance test was performed by disk diffusion method. ESBL screening was performed by combination disk test. PCR was optimized for identification of TEM gene using specific primers for all the samples. PCR product was cloned by using the TA Cloning. PCR limit of detection (LOD) and specificity were performed. Among the 100 samples, 17 samples were phenotypically detected as ESBL-producing E. coli. Cloning occurred because of the production of positive control. The LOD of the test was calculated as 10 copy numbers of DNA, and the specificity of this test is 100%. PCR analysis showed that 56% of samples were carrying TEM gene. The growth of antibiotic resistant bacteria especially ESBLs is become the serious public health issue. It is recommended to utilize the molecular assays like PCR in order to analysis the prevalence of ESBL genes, which it causes well-timed treatment, as well as prevention of spread of these resistances and reduction of time for being hospitalization finally the diminishing of death is the result.

Key words: ESBL, Escherichiae coli, PCR, TEM

INTRODUCTION

Escherichia coli (E. coli) are a common bacterium that causes nosocomial infections such as bacteremia, cholecystitis, cholangitis, neonatal meningitis, pneumonia and is responsible for over 85% of urinary tract infections (Struelens et al. 2004). β-lactam antibiotics are one of the treatment options for these infections. The effectiveness of these antibiotics has changed dramatically due to the resistance in Gram-negative bacteria especially E. coli. The main resistance mechanism includes production of β-lactamase enzymes which decrease the ability of hydrolyzing β-lactam antibiotics (Bakhtiar et al. 2011).

Extended spectrum β-lactamases (ESBLs) are β-lactams capable of creating resistance to penicillins, first, second and third generation of cephalosporins, aztreonam (not cefamycin or carbapenem) by hydrolysis of the β-lactam ring. These enzymes can usually be inhibited by clavulanic acid (Giske et al., 2009).

There are several types of ESBL, including TEM, SHV, CTX-M and OXA that are common among ESBL while PER, VEB, TLA, GES and BES are uncommon and derived from TEM-1, TEM-2 and SHV-1 enzymes by point mutations in their encoding genes. That the latter enzymes were first identified in 1980 in Germany (Paterson and Boram, 2003).

These genes are located on bacterial plasmids and can move easily between bacterial species, result in bacterial resistance to multiple antibiotics. The high prevalence rate of ESBL-producing organisms is a high risk for health care systems and inhibits the available treatment options (Ahmed et al. 2013).

Currently the identification of ESBLs is viable through either phenotypic detection including double disk synergy test (DDST) and combination disk test or genotypic detection including polymerase chain reaction (PCR). Phenotypic detection is based on techniques performed in clinical laboratories on a regular basis. On the other hand, phenotypic methods are cheap and easy to access; however, they are not suitable for classifying different types of ESBL. Genetic methods rely on molecular techniques which can identify gene encoding enzymes. Nowadays these methods, which can clarify different types of ESBLs, are used instead of phenotypic techniques (Thirapranthith, 2012).

Objectives

E. coli is one of the major species responsible in production of ESBL enzymes which play a significant
role in causing nosocomial infections particularly urinary tract infection. Furthermore, due to the increasing prevalence rate of these strains in hospital infections, the objective of the present study revolves around detection and investigation of TEM gene among ESBLs producing E. coli strains isolated from Tehran Milad Hospital using PCR.

MATERIALS AND METHODS

Bacterial isolates

A total number of 100 bacterial isolates were collected from urine, blood, mucous and wound samples of the patients who had been referred to Milad hospital between February 2013 until April 2014. The isolates were identified using biochemical tests.

Determination of antibiotic resistance

Antibiotic resistance was determined using standard disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Cockerill et al., 2011). For this purpose, the microbial suspensions were prepared from overnight cultures and they were compared with the 0.5 McFarland turbidity standard. The bacterial isolates were cultured on Mueller-Hinton agar (MHA) (Oxoid, UK) and the antibiotic disks were placed on the medium with standard distance. The noted plates were incubated overnight and the results were determined according to the growth inhibition zone. The used disk included cephalosporins such as ceftazidime (CAZ) (30 µg), ceftriaxone (CRO) (30 µg), β-lactams: ampicillin (AM) (10 µg), fluoroquinolones such as ciprofloxacin (CIP) (5 µg), nalidixic acid (NA) (30 µg), oxolinic acid (OX) (5 µg), aminoglycosides: gentamicin (CN) (10 µg), tetracyclines: tetracycline (TE) (30 µg) sulfonamides: cotrimoxazole (25 µg) (SXT) carbapenems such as imipenem (IPM) (10 µg) and other factors such as nitrofurantoin (F) (300 µg) which were purchased from the Mast company (Mast, UK).

Phenotypic screening of extended-spectrum β-lactamase

The ceftazidime resistant strains were tested for ESBL production according to CLSI guidelines (Cockerill et al., 2011), using combination disk test with ceftazidime 30 µg (Mast, UK) and ceftazidime-clavulanic acid 30/10 µg (Mast, UK). After an overnight incubation at 37°C, ESBL-producing E. coli were identified by increasing the size of inhibition zone diameter about 5mm or more around ceftazidime-clavulanic acid disk in comparison to ceftazidime disk.

DNA extraction

For the purpose of DNA extraction the DNG-Plus kit (Sinaclon, cat no: DN811730, Iran) was used.

Specific primers of TEM gene

The PCR primers that were used in this study are listed in Table 1 (Sharma et al., 2010).

Optimization of PCR test

PCR was carried out in a 25 µl volume containing: 2.5 µl of 10X PCR reaction buffer, with MgCl, 50 mM (0.75 µl), 0.5 µl deoxy-nucleoside triphosphate mix (dNTPs, 10 mM), 0.5 µl of each primer (10 µM), and 5 µl DNA template with 0.5 µl Taq DNA polymerase (5 U/µl).

PCR reaction was carried out by Thermocycler (Major Science, USA) under following condition for investigation of TEM gene: Initial denaturation for 3 minutes at 94°C, denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C and extension for 2 minutes at 72°C, which the last three steps in 35 cycles and final extension for 5 minutes at 72°C.

T/A Cloning

Amplion was cloned by T/A Cloning kit (Thermo Scientific, cat: K1213, USA) for sequencing and construction of positive control that this method includes ligation, transformation and clone selection.

Limit of Detection (LOD) of PCR test

PCR test to determine the LOD was performed using a spectrophotometer, in order that E. coli DNA concentration was measured at OD: 260nm. Genomic DNA content was calculated according to Genome Copy Number (GCN) formula. In the next step serial dilutions from the template DNA were prepared (from 10^5 fold to 10^3) and finally the minimum concentration of genomic DNA which represents the number of bacteria was evaluated using PCR test.

Specificity of PCR test

Specificity of the PCR reaction was examined by studying the obtained DNA from negative TEM E. coli, Salmonella spp. Mycobacterium tuberculosis, Mycoplasma pneumoniae, Candida albicans, Brucella spp and negative TEM Klebsiella spp.

PCR on sample

The PCR test was done on the DNA of samples by using specific primers and optimized temperature profile to recognize of TEM gene. The PCR products were analyzed by electrophoresis using 1.5% agarose gel and stained with SYBR Safe (Sinaclon, cat no PR891603, Iran).
RESULTS

Isolation of *E. coli* and antimicrobial resistance

A total number of 100 isolates identified as *E. coli* by biochemical tests and antibiotic resistance rate are shown in Table 2.

Phenotypic detection of ESBL

Result of combination disk method showed that 17 isolates were phenotypically ESBL positive.

Optimization of PCR test

The result of optimized PCR test was done for studying of existence of TEM gene (Fig. 1).

Cloning of PCR product

Amplicon was cloned by T/A Thermo Scientific Kit. In Fig. 2 one positive clone confirmed by PCR test on White colony (Fig. 2).

LOD test

LOD of PCR showed that TEM gene amplification was done using only 10 copies of the DNA. Electrophoresis results revealed that there is no band on electrophoresis gel for the dilutions less than 10 copies of the bacterial DNA (Fig. 3).

Specificity test

Specificity of TEM gene primers were evaluated on different DNA organisms. The results of these evaluations are shown in Fig. 4. As it is shown the PCR test was correctly able to identify TEM gene while the DNA of other microorganisms cannot be proliferate by these primers. As a result, this reflected the high specificity of the primers that they can be surely used to detect TEM gene. In fact, the specificity of the primers for proliferation of TEM gene was 100 %.

Results of PCR test on samples

The PCR results determined that 26% of the isolates were carrying TEM gene which can be visualized on agarose gel at 1080 bp (Fig. 5).

DISCUSSION

The β-lactamase genes especially ESBL which are mostly plasmid are considered as an important factor increasing β-lactam antibiotic resistant such as extended spectrum cephalosporins. Globally, there is an increasing trend of organisms carrying such genes (Giske et al, 2009).

The infections related to ESBL ranges from central venous line-related bacteremia, cholangitis, intra-abdominal abscesses, peritonitis and urinary tract infections which are considered major concerns for the medical community as they inhibit the treatment options, increase treatment costs, increase hospital length of stay and increase morbidity and mortality amongst patients (Freeman et al, 2012).

The ESBL gene isolation and obtaining information regarding the frequency rate of ESBL from clinical samples in the hospitals of Tehran can suggest useful information regarding the epidemiology and prevention of the factors involved in spreading ESBL infections which ultimately stops the development of such bacterial resistances. Furthermore, the noted also aids the physicians to prescribe more suitable and efficient antibiotics which can aid and abet the process of treatment in the context of noted infections. Consequently, in order to reduce the risk of treatment failure, it is recommended that relevant enzymes identification tests are implemented complementary to antibiogram on a routine basis.

This study examined the phenotypic rate of ESBL-producing *E. coli* as well as the prevalence rate of TEM carrying gene amongst patients diagnosed with urinary tract infections is investigated in Milad hospital. In the present study the antibiotics resistances are reported according to the following:

100% resistance to ampicillin, 82% resistance to cefalexin, 62% resistance to tetracycline, 60% were resistant to co-trimoxazole, 56% resistance to nalidixic acid, 45% resistance to gentamicin, 43% resistance to ceftriaxone, 41% resistance to ciprofloxacin, 38% resistance to ofloxacin, 7% resistance to imipenem and 3% resistance to nitrofurantoin.

Carbapenems are widely used to treat infectious diseases in Iran; however, bacterial resistance especially to imipenem limits the use of this drug in the course of treatment (Gholipour et al, 2014). Unfortunately, according to the present study, there is a 7% imipenem resistance amongst the isolates contrary to the findings presented by Gholipour et al (2014) and Alsulaiman et al (2013) which did not report any imipenem resistance. On the other hand, resistance to ampicillin and cefalexin in the present study respectively 100% and 82% are similar to the findings presented by Adeyankinnu et al (2014). Furthermore, the resistance to a wide range of cephalosporins from one side and the prevalence of ESBL genes amongst large plasmids which are resistant to other antimicrobial factors such as aminoglycosides, fluoroquinolones, tetracycline and sulfonamides further inhibits the treatment of infections caused by ESBL-producing genes (Motayo et al, 2013). In the present study, the resistance to gentamicin, co-trimoxazole, ciprofloxacin and ofloxacin are 45%, 60%, 41% and 38%
respectively against Igwe et al. (2014) study confirming resistance rates of 80%, 99%, 60% and 70% respectively which are relatively higher rates of resistance.

The frequency rate of *E. coli* in ESBL-producing genes is determined 17% using combination disk test which is in line with the findings presented by Najar-Peerynd et al. (2013) reporting 18.3% in 2013. However, according to the literature, there is a significant prevalence rate of *E. coli* in the region which is reported 45% in India (Sood et al., 2012) and 64.4% in Pakistan (Hussain et al., 2011) accordingly. The increasing prevalence of ESBL-producing genes amongst developing countries is higher compared to developed countries which can be due to factors such as lower income, higher bed occupancy rate in health facilities, higher incidences of self-medication such as over use of prescription only
Prevalence of TEM β-lactamase resistance gene in *E. coli*

![Image](image_url)

**Fig. 5:** The results of PCR test on samples. Lane 1: Marker 1Kb DNA ladder (Thermo Scientific, USA), Lane 2: Positive control, Lane 3 and 7: Negative samples, Lane 4, 5, 6, 8 and 9: Positive samples containing TEM gene, 1080 bp, Lane 10: Negative control.

**Table 1:** Primer used for amplification of TEM gene.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide Sequences, 5'-3'</th>
<th>Amplificon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-F</td>
<td>AAAAAATCTTGAAGAGC</td>
<td>1080</td>
</tr>
<tr>
<td>TEM-R</td>
<td>TTACCAATGCTAATCA</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2:** Total percentages of isolates resistant to selected antibiotics.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>E. coli NO.</th>
</tr>
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<tbody>
<tr>
<td>Cefazolin</td>
<td>82%</td>
</tr>
<tr>
<td>Cephalosporin</td>
<td>41%</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>38%</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>45%</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>62%</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>3%</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>60%</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>43%</td>
</tr>
<tr>
<td>Imipenem</td>
<td>7%</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>100%</td>
</tr>
</tbody>
</table>

Antibiotics, inadequate attention to personal hygiene especially in the hospital setting and less effective infection control (Ahmed et al., 2013).

Phenotypic methods can identify the presence of ESBL; however, the determination of the resistance is deemed complicated as some strains may be classified as susceptible strains in vitro despite being resistant. Consequently, diagnosis of ESBL requires extensive molecular techniques such as PCR which ultimately increases the efficiency of the treatment and hampers further development of resistant strains. Molecular analysis shows that 56% of the isolates have TEM carrying gene. In the study conducted by Gholipour et al. (2014) on 107 ESBL-producing isolates, 13 (12.14%) isolates were identified as TEM carrying.

Saedi et al. (2014) confirms 32.5% frequency rate of TEM gene among the isolates which is lower than the present study’s findings. According to the studies conducted by Abreu et al. (2011) and Varkey et al. (2014) the frequency rates of this gene are reported 70% and 75% respectively which are higher than the present study’s findings. According to the study conducted in Sudan by Ahmed et al. (2013) 65% of ESBL-producing *E. coli* strains were identified using phenotypic techniques. The study then conducted PCR testing on the ESBL-producing strains confirming 55.1% TEM carrying gene which is similar to the present study’s finding. However, similar to Ahmed study, the majority of conducted PCR techniques in other studies have also been conducted solely on the isolates identified as ESBL-producing strains using phenotypic techniques whilst the current study conducted PCR on all the isolates which showed higher frequency of ESBL-producing strains. Accordingly, results suggest that the prevalence rate of TEM carrying gene in *E. coli* strains varies from one country to another which can be due to implemented infection control and treatment programs. The high prevalence rate of TEM carrying gene in the present study which is a growing trend is due to misused used of antibiotics in Iran. Furthermore, resistance to cephalosporin, penicillin and imipenem must be monitored. This study contained the highest reported resistance rate to penicillin and cephalosporin which are related to TEM carrying gene. The ESBL-producing organisms showed resistance to other classes of antibiotics especially carbapenems, aminoglycosides, sulfonamides and fluoroquinolones which further inhibit the treatment options.

**CONCLUSION**

The increase in antibiotic resistant bacteria especially ESBL has become a key concern within public health. The best way to reduce the relevant infection risk is the logical usage of antibiotics and the fitting ESBL diagnosis prior to selection of the antibiotics accordingly. Consequently, it is totally necessary to equipment laboratory in quick and accurate molecular techniques such as PCR to evaluate the resistant strain and it can ultimately be used to determine the treatment options and in controlling the spread of resistant strains.

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**REFERENCES**


