Original Article

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Early Detection of blaTEM in Klebsiella Isolates by the Molecular Polymerase Chain Reaction Method

S. Bostandoost Nikoo (MSc)[1], M.H. Shahhosseini (PhD)[*2], M.R. Zolfaghari (PhD)[3], M. Rahbar (PhD)[4]

1.Azad Islamic University of Qom, Qom, I.R.Iran
2.Department of Microbiology, Azad Islamic University of Shar Qods, Tehran, I.R.Iran
3.Department of Microbiology, Azad Islamic University of Qom, Qom, I.R.Iran
4.Laboratory of the Ministry of Health and Medical Education, Tehran, I.R.Iran

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ABSTRACT

BACKGROUND AND OBJECTIVE: Obtaining information regarding pathogenesis and prevalence of extended spectrum beta-lactamase (ESBL) producing genes seems to be necessary, since it can promote prevention modalities and treatment of the infections caused by bacterias such as Klebsiella. The aim of this study was early identification of the blaTEM gene in Klebsiella, using polymerase chain reaction (PCR) technique.

METHODS: In this cross-sectional study, conducted form April to September 2013, 70 Klebsiella isolates were extracted from clinical samples (i.e., wound, urine, sputum and blood) using biochemical tests, including non-state fermentation and triple sugar iron, negative indole, motile and methyl red, as well as positive Voges-proskauer and urease tests. Subsequently, the frequency of ESBL producing strains was determined by means of combined disk method. DNA was extracted by boiling and was investigated for the presence of TEM gene using the PCR approach.

FINDINGS: In the 70 Klebsiella isolates, 11 cases of ESBL phenotype were observed, of which 10 cases contained TEM beta-lactamase resistance gene. In addition, 9 out of 59 samples (26%) of negative ESBL in antibiogram, were determined positive in terms of blaTEM gene using PCR method.

CONCLUSION: Given the increasing prevalence of ESBL producing strains and poor diagnosis rate of antibiotic resistance through antibiogram method, applying more accurate techniques such as PCR is highly recommended.

KEY WORDS: BlaTEM Resistance Gene, Extended Spectrum Beta-lactamases, Polymerase Chain Reaction, Antibiogram

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Introduction

Klebsiella is one of the extended-spectrum beta-lactamase (ESBL) producing bacterias and is one of the members of the Enterobacteriaceae family. This bacteria is one of the most common causes of nosocomial infections (1). Beta-lactamases are the most important beta-lactam

[*] **Corresponding Author: M.H. Shahhosseini (PhD)**
Address: , Department of Microbiology, Azad Islamic University of Shar Qods, Tehran, I.R.Iran
Tel: +98 21 44844946
Email: shahhosseiny@yahoo.com

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inhibitor enzymes in bacterias, which prevent resistance and inhibit the binding of antibiotics to the target sites, through hydrolysis of beta-lactam ring. Untill now, more than 340 beta-lactamase enzymes have been identified (2, 3). According to the molecular classification, which is based on nucleotide and amino acid sequences, four classes of enzymes have been identified (A-D). A, D and C classes function through serine mechanisms, while class B or mannan-binding lectins (MBL) is zinc dependent.

The majority of ESBLs belong to the molecular class of A in the Ambler category. TEM was the first beta-lactamase plasmid in gram-negative bacterias, which was discovered in the 1960s. This enzyme was the first beta-lactamase enzyme extracted from Escherichia coli (E. cloi), isolated from blood culture of a Greek patient named Temoniera in 1985, and it was named after him. Shortly after the extraction of this gene, TEM beta-lactamase spread all over the world, so that today it is the most common resistance mechanism to beta-lactam drugs in the gram-negative bacilli (4).In the recent years, extensive studies have been performed on ESBL, for instance, Sharma et al. conducted a study in 2009 entitled "Detection of TEM and SHV genes in Escherichia coli and Klebsiella, extracted in the intensive care unit of a hospital in India" (5). A study by Kaftandzieva et al. was carried out on the prevalence and molecular characterization of ESBL-producing E. coli and Klebsiella pneumoniae in 2011 (6).

In 2013, Ahmed and colleagues examined the the prevalence of TEM, SHV and CTX-M genes in E. coli and Klebsiella strains, isolated from urinary infections in Sudan (7). Masjedian investigated molecular resistance to broad-spectrum antibiotics in E. coli and Klebsiella pneumonia (8). In a study conducted by Yazdi et al., entitled as “The prevalence of SHV/CTX-M/TEM beta-lactamase resistance genes in E. coli extracted from urinary infection”, 246 isolates of E. coli, extracted from urine samples were investigated in Tehran, 2010 (9). Shabani and colleagues examined the frequency of TEM-1 gene in E. coli strains, which were separated from clinical specimens in Damghan, 2011 (10).

In the last two decades, the resistance of the gram-negative bacterias to beta-lactamase antibiotics have rapidly expanded, which is a serious problem in treatment of infections caused by these bacteria. Transmission and rapid spread of enzyme producing organisms increase the rate of nosocomial infections (3, 11). Given the importance of this issue, the purpose of this study was prompt detection of blaTEM genes in the Klebsiella isolates through PCR method

Methods

In this cross-sectional study, conducted from April to September 2013, 70 clinical isolates of Klebsiella bacteria were separated from clinical samples (i.e., wound, urine, sputum and blood) by means of biochemical tests, including non-fermentive condition, triple sugar iron, negative indole, motile and methyl red, as well as positive Voges-Proskauer and urease tests. The separated klebsiella isolates were studied using combined disk method, with the presence of ESBLs. Compound discs used in this method included: one ceftazidime disc (30 mg) and one hard compound disc (30 mg+10 mg ceftazidime clavulanic acid) (Span, mention the manufacturing country), the discs were placed 20 mm away from each other in the Mueller-Hinton agar (Merck, the Country). If the diameter of the inhibition zone around the clavulanic acid container disk is larger than ≥5 mm of ceftazidime disk, ESBL production is considered positive (12).

DNA extraction: In order for DNA extraction and PCR testing, DNA positive control from all the samples was applied through boiling method. First, 3 to 5 colonies of each sample were dissolved into Eppendorf vials containing 100 ml distilled water. Then, the solution was placed in a boiling-water

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bath at 100°C for 15-10 minutes. Finally, vials were centrifuged at 12,000 rounds for 10 minutes, and the supernatant solution containing the DNA was used for PCR assay. Optimisation of the PCR test for blaTEM gene detection: In order to identify the blaTEM gene, PCR test was employed using primers extracted through Sharma and colleagues' method (5). The sequences of encoding primers of blaTEM gene with the size of 1080 base pair (bp) were as follows:

TEMF: 5'-AAA ATT CTT GAA GAC G-3'
TEMR: 5'- TTA CCA ATG CTT AAT CA-3'

The thermal program used for blaTEM gene extraction was as follows: A) initial denaturation at 94°C for 3 minutes, B) PCR cycle repeated for 35 times, including denaturation step at 94°C for 30 seconds, the primer connection step performed at 50°C for 30 seconds and the amplification step at 72°C for 2 minutes C) The final amplification at 72°C for 10 minutes.

In this study, PCR reaction mixture in a test was as follows: 10xbuffer: 2.5 µL, MgC12: 0.75 µL, dNTP: 0.5 µL, forward primer: 0.5 µL, reverse primer: 0.5 µL and double-distilled water: 15 µL. In the present study, we used the positive control strain of Klebsiella pneumoniae (ATCC700603), prepared in Tehran Scientific and Industrial Research Center, and negative control strains of E. coli (ATCC35218) produced in the Pasteur Institute.

Limit of detection (LOD) in the optimized PCR test: To determine the LOD of the optimized PCR assay, DNA dilutions with certain number of bacterias were prepared, and the minimum number of bacterias needed for the reaction was determined via PCR reaction.

The specificity of the PCR test: To determine the specificity of the optimized PCR assay, online and offline methods were used. In the online method, the primer sequence was entered into the N-blast part of the NCBI website to investigate the primer homology with the other microorganisms. In the offline method, DNA of prevalent bacterias, such as negative E. coli TEM , Salmonella, Mycobacterium tuberculosis, Mycoplasma pneumoniae, Candida albicans, Brucella and Klebsiella negative TEM were extracted, and optimized PCR test results alongside with positive and negative control samples were evaluated.

Cloning: Cloning PCR product in a suitable carrier helps provide a large source of DNA, without the need for amplifying the product from a primary source in each test. In a suitable vector, the cloned PCR product can be used as a template for PCR positive control and for conducting further studies. Therefore, to obtain a reliable PCR positive control and also to determine the nucleotide sequence of the target gene, the desired amplicon was cloned into the appropriate vector. For this purpose, T/A cloning kit (Thermocintific, Mention the country) was used.

Results

Antibiogram test: From the 70 separated Klebsiella isolates, 11 samples showed ESBL phenotype and 59 samples of negative ESBL were reported. Optimization of PCR test for detecting the blaTEM resistance gene: PCR test was optimized according to thermal instructions, using DNAs extracted from the positive control samples and TEM-F and TEM-R specific primers.

The 1080 bp PCR product, the template of which was DNA of TEM positive Klebsiella bacteria, can be observed with negative control sample and alongside with the size of the marker on 5.1% agarose gel (fig 1). LOD of the test: The obtained PCR test LOD, for blaTEM gene in Klebsiella samples, equaled to 10 copies of the gene. The number of colony-forming units (CFU) and the number of DNAs were determined in each of the sample dilutionsm, which was indicated on the electrophoresis gel along with the negative and positive control samples (fig 2).

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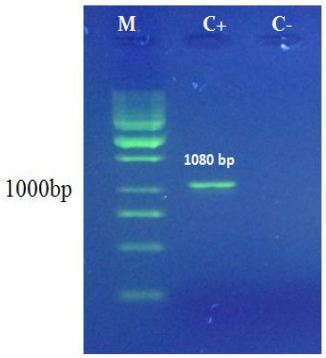


Fig1: The optimized PCR test for detects blaTEM gene in Klebsiella.
M.size marker 1kb DNA Ladder(Fermentas)
1. Positive Control
2. negative control

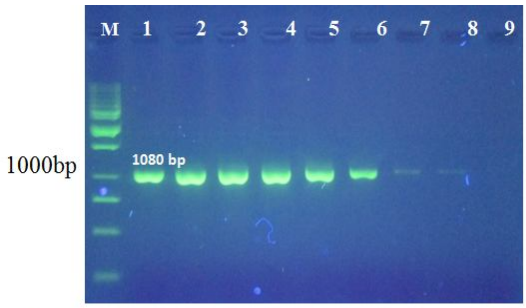


Fig:2. Limit of Detection(LOD) test of PCR detction of TEM gene.
M.size marker 1kb DNA Ladder(Fermentas)
1.Positive Control
2. Dilution 10^{-1}
3.Dilution 10^{-2}
4.Dilution 10^{-3}
5.Dilution 10^{-4}
6.Dilution 10^{-5}
7.Dilution 10^{-6}
8.Dilution 10^{-7}
9. negative control

PCR test specificity: The results of this study have shown that PCR assay was able to detect the blaTEM in Klebsiella, while the DNAs of the other microorganisms were not reproducible through primers of this test (fig 3). Cloning: The PCR product with the size of 1080 bp was cloned using pTz57R plasmid, and the pSB3946 recombinant plasmid was obtained.

Performing PCR test on 70 samples of Klebsiella: Optimized PCR assay was performed on the DNA samples extracted from Klebsiella samples (obtained from Milad Hospital), the positive control samples, the template of which were DNAs extracted from TEM-containing genes and negative samples. From the 70 samples extracted in Milad Hospital, 11 samples were reported positive through antibiogram of (Disk Diffusion) ESBL and the remaining 59 samples were negative. In the PCR assay, 10 of the 11 positive ESBL samples, and 9 out of the 59 negative samples turned positive due to the presence of TEM gene (fig 4).

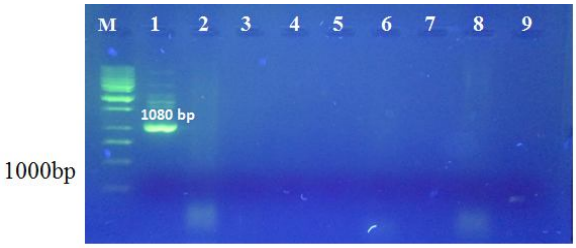


Fig3: Specificity test of PCR detction of TEM gene.
M.size marker 1kb DNA Ladder(Fermentas)
1.Positive Control
2. *Klebsiella* DNA(Without TEM Gene)
3.*Salmonella* DNA
4.*MTB* DNA
5.*M. pneumoniae* DNA
6.*C. albicans* DNA
7.*Brucella* DNA
8.*E. coli* DNA
9. negative control

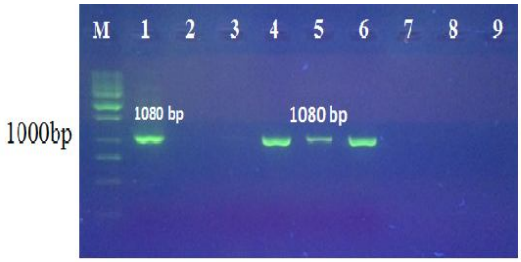


Fig4: The optimized PCR test for detects blaTEM gene in Clinical Samples
M.size marker 1kb DNA Ladder(Fermentas)
1. Positive Control
2,3,7,8. Negative Samples
4,5,6. Positive Samples

Discussion

Our results confirmed the comprehensive prevalence of ESBL and presence of blaTEM gene in the examined isolates. In this study, 15.7% of the isolate strains were ESBL producers. On the other

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hand, the phenotypic identification of ESBL Klebsiella strains was reported to be 50% by Leung Ho and colleagues in 2008 (13).

In 2009, in a study conducted by Ehlers et al., 58.4% of Klebsiella strains showed ESBL phenotype (14). Kaftandzieva and colleagues in a study performed in 2011, reported the positive beta-lactamase to be 31% (6). In addition, a study by Pornour (2010) demonstrated that 97.8% of Klebsiella strains were ESBL (15). Considering the results of this study and those of the previous ones, the obtained data indicated that the percentage of positive ESBL strains is increasing, and this upturn could be a result of resistance of bacterial strains, as well as excessive and long-term use of broad-spectrum cephalosporins in disease treatment. In general, there are various risk factors for increasing the ESBL producing bacterias as follows: the excessive and prolonged intake of broad-spectrum cephalosporin, long duration of hospitalization and using vascular and urinary catheters (16, 17). Today, the existence of ESBL in bacterias extracted from patients with multiple drug resistance is an important health problem in most countries (18, 19). Phenotypic methods can only show whether an ESBL has been produced or not; however, they cannot identifiy its type and subtype.

Therefore, the PCR technique for detection of various TEM-1 ESBLs is necessary. In our study, the frequency of genes determined by PCR was 27.14%. While, in the previous years, the frequency of TEM in Klebsiella was high in proportion to size and population of the studies. For instance, in Ehlers et al. (2009) and Bali et al. (2010) studies, the frequency of TEM gene was reported to be 24 and 74.4%, respectively (14, 20). Furthermore in a study by Akpaka and colleagues from 65 Klebsiella isolates, analyzed by Multiplex PCR method, 84.3% contained TEM gene (21). Moreover, the results of a study by Pornour et al. indicated that 74.4% of isolates were carrying TEM gene (15), which was consistent with the results of this study. It seems that this enzyme, which was highly prevalent in the Enterobacteriaceae family, has been transferred to the other bacterias by mobile genetic elements (22).

To understand the status of resistant strains and to provide guidelines and appropriate measures for preventing the spread of resistant strains, conducting similar studies is essential. Considering the fact that the result of phenotypic test for confirmation was 15.7% and the genotypic test result equaled 27.14%, it can be concluded that the phenotypic detection methods are not the best option for identification of ESBL, and genotypic methods such as PCR are more precise and sensitive in detection of these enzymes.

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