Detection of Antibiotic Resistance of Broad-Spectrum Beta-Lactams by Multiplex PCR Method

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Research

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Abstract

Background

One of the most resistant important mechanisms against beta-lactam antibiotics is Extended-spectrum beta-lactamase. These enzymes can hydrolyze penicillin, cephalosporin, Cephamycin, and monobactam. The genes of these enzymes are located on integron and can be transmitted to other strains of bacteria, including the Enterobacteriaceae.

The study of ESBL genes is critical for the report to clinicians for an appropriate antibiotic pattern. This study aimed for rapid and precise identification of ESBL genes by the Multiplex PCR method.

Methods

In this study, three pair primers were designed for common ESBL genes (TEM, AmpC, KPC) using Genscript software. The first use contains control positive genes was performed set-up of PCR. Negative control samples and the 50 isolates of the *Escherichia coli* isolated from Baqiyatallah hospital were studied with this method.

Results

The results of this research showed that primers designed for ESBL genes (TEM, AmpC, KPC) were able to simultaneously identify positive control samples. The sensitivity of the multiplex PCR technique for ESBL genes was 1 pg and specificity reported 100%.

Conclusion

This study showed that a Multiplex PCR designed with a sensitivity of 1 pg and 100% specificity can correctly detect ESBL genes. Therefore, by quickly and correctly identifying the pattern of antibiotic resistance and providing a suitable treatment pattern to physicians, the spread of antibiotic resistance genes as well as the occurrence of economic and human losses can be prevented.

Background

One of the most important achievements of medical science was the beta-lactams. The emergence of resistance to beta-lactams antibiotics is a global concern. The coding gene of this enzyme is abundant in the *E. coli* strains. This prompted the World Health Organization to emphasize the risk of developing antibiotic-resistant strains. WHO is planning to use the WHONET software and the requirement for health centers to use it to update it promptly to inform physicians about the results of local and global antibiotic resistance (1).

Phenotypic tests are the simplest methods used for the detection of antibiotic resistance all over the world. However, none of the phenotypic identification tests can detect fully ESBL antibiotic resistance. (2-

5).

The Multiplex PCP technique is one of the PCR methods in which 2 to 3 target sequences can be multiplied simultaneously. Rapid and accurate detection and reporting of results can help the physician to treat the patient. Beta-lactams are good antibiotics for the treatment of bacterial infections that are used throughout the world (2, 3, 5).

Because of the inactivation of Beta-lactams to β-lactamase enzymes, the need for the correct use of these antibiotics to prevent the distribution of antibiotic resistance genes is very important.

ESBLs are an enzyme family that, by attacking the Beta-lactam rings, inactivates the effects of Beta-lactam. (2-5).

ESBL is a group of enzymes that hydrolyze antibiotics belonging to the Penicillin, Cephalosporin, Cephamycin, and Monobactam and inactivate them. Beta-lactamase inhibitors such as Clavulanic acid, Tazobactam, and Sulbactam can inhibit bacterial β-lactamases. The most common type of ESBL is the CTX-M gene. (6- 9).

Multiplex PCR can help to solve numerous problems, including simultaneous identification of several resistance genes, lower cost, and timely reporting of results and safe treatment. In recent years, due to the widespread use of antibiotics, the advent of drug resistance is deteriorating, and as long as uncontrolled administration of antibiotics continues this problem, the prevalence of ESBLs in the strains is a matter of concern. Beta-lactam antibiotics are one of the most abundant and most prevalent prescription drugs against these microorganisms. In recent years, the emergence of MDR species of these organisms has caused many concerns for physicians to select the appropriate antibiotic in the treatment process. (10-14).

This study aimed to design and set up Multiplex PCR for the identification of ESBL Beta-lactamase genes Using the Multiplex PCR method.

Methods

Design Primer

First, a complete sequence of genes was searched on the NCBI site. Primers were designed using software Genscript. The Forward and Reverse primers were blasted and the results showed that the designed primers were suitable. Three primer pairs were evaluated by Oligo Analyzer software. Then Primers were evaluated in online silico PCR amplification software. (Table1).

Determine the sensitivity of the primers

PCR sensitivity was measured with different genome dilutions. At first, nucleic acid serial dilution was done. For all genomic dilutions were performed PCR. The lowest dilution of PCR reaction was considered

as test sensitivity.

Determine the specificity of the primer

To obtain the specificity of the primers, the PCR reaction was performed with the above conditions on the nucleic acid *Staphylococcus aureus* and *Bacillus subtilis*.

DNA extraction

DNA extraction was carried out with the Cinnaclon kit. The concentration and purity of the DNA extracted from the samples were obtained by spectrophotometry Nanodrop.

PCR reaction

The PCR reaction was performed to multiply the target genes and the reaction final volume was 20 µL. (Table 2,3).

Gel Electrophoresis

The amplified DNA amplicons with PCR were visualized on a 1% (w/v) agarose gel electrophoresis containing SYBR® Safe (Qiagen).

Results

Antibiotic sensitivity test

Antibiotic susceptibility tests were evaluated in 50 bacterial isolates isolated from Baqiyatallah hospital.

The result of the DNA concentration by Nanodrop

The concentration of DNA was measured by nanodrop was a level of 302.86 $ng/\mu L$.

Determine the sensitivity of the primer

For the determination of the sensitivity of primers ESBL, the serial dilution of concentrations of 10^{-1} to 10^{-16} was used. The least dilution that PCR reaction was positive was calculated as PCR sensitivity.

Multiplex PCR results

The results of electrophoresis of Multiplex PCR products based on the ESBLs of AmpC, TEM, KPC on 1% agarose gel were indicated in Figure1.

Frequency of antibiotic resistance genes

The confirmed primers of TEM, KPC, and AmpC genes evaluated in 50 *Escherichia coli* clinical isolates.

Discussion

Currently, the excessive use of antibiotics is one of the main reasons for the prevalence of antibiotic resistance in different regions. The increased exposure to ESBL and long-term hospitalization causes the distribution of antibiotic-resistant genes.

Beta-lactams are one of the most common antimicrobial agents for the treatment of serious infections caused by *Enterobacteriaceae* infections. The emergence of resistance to this class of antibiotics makes it difficult to decide on treatment and often leads to treatment failure. (15-20).

In this study, Multiplex PCR was used to simultaneously identify the genes TEM, KPC, and AmpC.

The sensitivity of the test for TEM, KPC, and AmpC genes was1 pg. The results showed that genotypic and phenotypic assays of clinical isolates of *E. coli* were compatible and the designed primers had a specificity of 100%.

The advantages of using Multiplex PCR contain the development of rapid diagnostic methods and reduce drug resistance. The specific primers lead to the correct diagnosis and the provision of appropriate counseling to the physician. Using this method leads to a reduction in the use of costly and wide-spread antibiotics. The use of these methods increases the readiness for rapid diagnosis of antibiotic resistance when epidemics occur. Because of identification importance MDR bacteria necessary is design appropriate methods for rapid and correct detection of antibiotic resistance. The other factor is the reduction of health costs, and it is expected that many people will be relieved of their mortality by cooperating in reducing antibiotic resistance. The advantages of primers designed in this research contain technique efficacy both in Multiplex and Real-Time PCR methods. The Tm all primers are the same therefore, all reactions take place at a certain temperature. (15- 20).

The emergence of resistance to these antibiotics is due to genetic structures such as plasmids, it is worried that these enzymes will be transmitted to other gram-negative bacteria, such as *E. coli*. (18, 19).

In this study, resistance to piperacillin and Cefotaxime was observed. However, there were no isolates of PDR with resistance to all antimicrobial agents.

MDR isolates are species that contain resistance to at least three classes of antibiotics, including all Penicillins and Cephalosporins, Fluoroquinolones, Aminoglycosides, and XDR strains, which, in addition to MDR resistance, also are resistant to Carbapenems. PDR isolates also XDR are resistant to Polymixins and Tigecycline. (19-20).

The present study showed that 100% of *E. coli* isolates had MDR resistance and 92% had PDR resistance which was very concerning.

Based on the results of this study, the highest frequency of resistance to Piperacillin and Cefotaxime antibiotics with 100% and the highest sensitivity to the Meropenem and Imipenem antibiotics was 64% and 52% respectively. In a study by Imam Qureshi et al in 2003 in Jahrom Hospital and clinics, antibiotic resistance to Ciprofloxacin, Amikacin, *Nitrofurantoin*, Gentamicin, Vancomycin was 6.7, 28.6, 34.3, 72.1, 56.2%. In this study, the resistance to these antibiotics was significantly higher and the acquisition of antibiotic resistance during time is the cause of this difference. (21)

In a study conducted by Mobashirizadeh in Esfahan isolated broad-spectrum β-lactamases from urinary tract infections in outpatients and admitted patients, the frequency of *Escherichia coli* and *Klebsiella pneumonia* producing β-lactamases was 47.97%, 41.6%, respectively. It was also found that the highest and lowest antibiotic resistance were related to Cotrimoxazole antibiotics 75% and Nitrofurantoin 16.7% respectively (22).

In a study conducted by Kaykha in Zahedan, the antibiotic resistance of *E. coli* to each of the antibiotics of Imipenem, Gentamicin, Ceftazidime, Amikacin, Nitrofurantoin was 4.5, 13.7, 44.8, 19.5, 19.3, which was less than the current study (23).

Also, in a study conducted by Heidari et al.the antibiotic resistance to antibiotics Imipenem, Gentamicin, Cefotaxime, Ciprofloxacin, Nitrofurantoin, Cotrimoxazole, Nalidixic acid was 38.2, 6 / 43.1 / 49, 7/32, 8 / 21.3 / 67.60% (23).

The increase of this antibiotic resistance was caused by mutations, such as the rate of Imipenem and Cefotaxime. The method of disc diffusion used in these studies, and the type of infection in all studies was almost the same, the difference may be due to differences in isolated geography regions and or company the disc manufacturer.

The beta-lactamase (ESBLs) genes are one of the effective factors in increasing resistance to beta-lactam antibiotics. Organisms carrying ESBL plasmid genes are more virulent and pathogenic. Therefore, timely and accurate diagnosis of this type of resistance and reporting it to a physician and appropriate medical advice can reduce patient problems and treatment. In this study, out of the 50 isolates of *Escherichia coli*, 58% isolates containing the TEM gene, 18% isolates of the KPC gene, and 84% isolates containing the AmpC gene were found. Also, in the present study, 58% of the samples contained all three KPC, TEM, and AmpC genes.

A study by Shahcheraghi and colleagues in 2006 showed that 24% of isolates contained the TEM gene. This frequency is lower than the current study, given the ability of the plasmid to distribution the gene, reveals the widespread distribution of this gene over time. (24).

In 2008, a comprehensive study of ESBL enzymes was conducted in Switzerland. This study showed that 42.9% of the isolates contained the AmpC gene.

In a study conducted by Jafari in 2018, the PCR method examined the presence of ESBL in *Escherichia coli* isolates. The study found that 52% of the samples had a TEM gene that was consistent with our

study, 10% of the samples had a SIM gene and 10% of the isolates had a GIM gene that the frequency gene GIM and SIM was lower than current study. (25, 26, 27, 28, 29, 30, Hosseini, S.A.G., et al 2020, Bahador, A., et al 2013). Current studies show that resistance to beta-lactam induced by ESBL is increasing rapidly. Possible reasons are due to improper administration of antibiotics and lack of appropriate methods for identifying antibiotic resistance, as well as lack of proper interpretation of new methods of identification.

Conclusions

Antibiotic resistance and the prevalence of broad-spectrum beta-lactamase genes and metallo-beta-lactamase in gram-negative bacteria around the world are of concern and require infection control measures including management of antibiotics and rapid identification of beta-lactamase-producing isolates. Prompt and accurate diagnosis of these strains can help physicians prescribe antibiotics and avoid high costs and long hospital stays.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree with the publication of the paper.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Davoud Esmaeili and Mohamad Ali Khosravi, participated in specimen collection and preservation as well as review of the manuscript, Mohammad Raiszadeh, participated in data collection, specimen collection, bench work, result analysis and drafting of the manuscript. Mohamad Ali Khosravi, Mohammad

Raiszadeh and Davoud Esmaeili designed the study, collected data, performed bench work, result analysis and drafted the manuscript. All authors have read and approved the manuscript.

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Tables

Table 1: ESBL designed primers for TEM, AmpC, and KPC genes

gene	F	R	Tm	Amplicon size
	GAGGACCGAAGGAGCTAACC	TTGCCGGGAAGCTAGAGTAA		
	CTCGACCTCGCGACCTATAC	CTGCCACTGGCGGTAGTAGT		
	CAGCTCATTCAAGGGCTTTC	GTCCAGACGGAACGTGGTAT		

Table2: The concentrations and components used in the PCR reaction.

Components	Concentration
Master Mix (1x)	12.5 µl (1x)
F Primer(0.1-1 µm)	1 µl (10 µm)
R Primer(0.1-1 µm)	1 µl (10 µm)
Template DNA	1 µl (20pg)
Sterille Deionized Water	9.5 µl
Total Volume	25 µl

Table3: PCR reactions in the Corbett thermocycler.

Cycle PCR	Temperature	Time (second)	Number Cycle
Primary denaturation			1
Second denaturation		30	35
Annealing		45	35
Extension		40	35
Final Extension		300	1

Table4: Antibiotic resistance pattern of *Escherichia coli* isolates

Antibiotic	Resistant(%)	Intermediate (%)	Sensitive (%)
Ofloxacin(OFX)	98	0	2
Amikacin(AN)	42	32	26
Piperacillin (PIP)	100	0	0
Ciprofloxacin (CP)	98	0	2
Gentamicin(GM)	80	4	16
Cefotaxime(CTX)	100	0	0
Nitrofurantoin(FM)	50	10	40
Imipenem(IMI)	40	4	52
Meropenem(MER)	32	2	64
Norfloxacin (NOR)	98	0	2

Table5: Frequency of antibiotic resistance genes ESBL in *Escherichia coli* isolates

Genes	Resistant frequency%
TEM	58%
KPC	18%
AmpC	84%

Figures

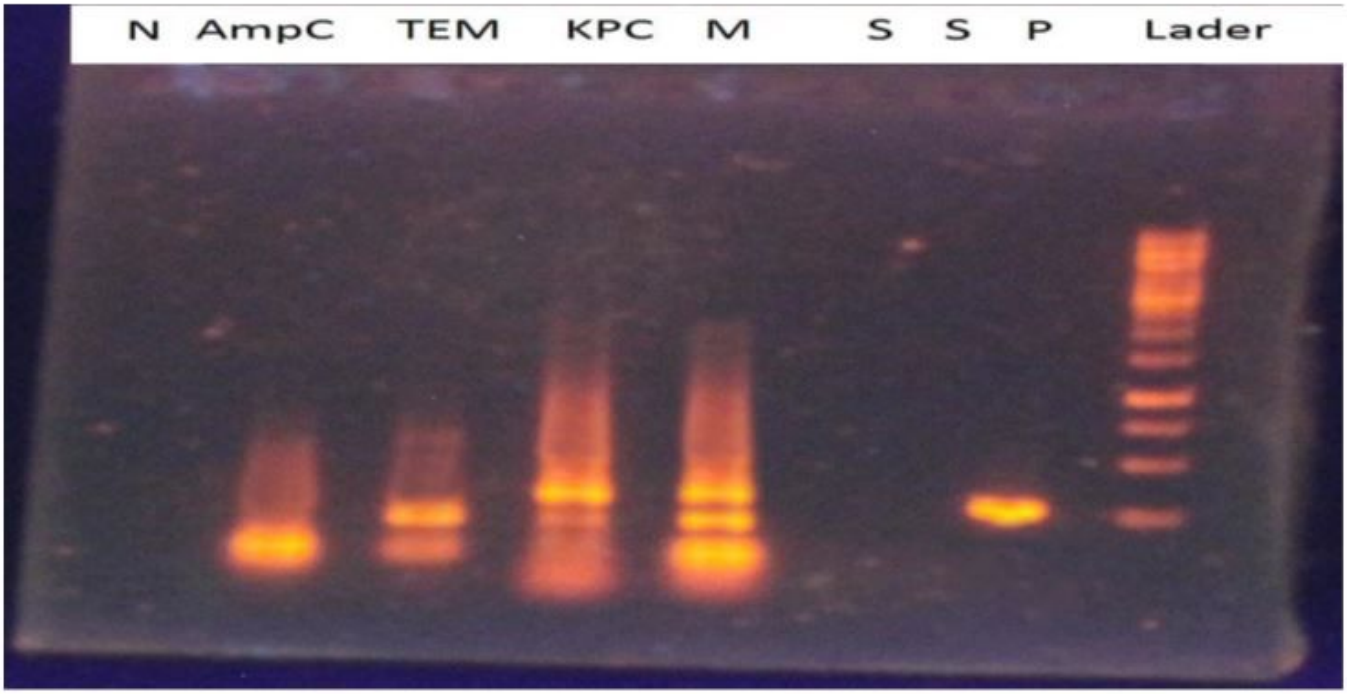

Figure 1

Results of Multiplex PCR for ESBL genes on gel agarose 1%