Original Article :

Identification of some microorganisms directly from clinical sample by multiplex PCR

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Abstract

Background and Aims:

Infectious diseases are affecting people more often than before and that is the main cause of morbidity and mortality in developed and developing countries. Taking the necessary measures by diagnosing earlier and using appropriate antibiotics made significant contributions to the control of the infections. In our study we aimed to develop a method for the diagnosis of some of pathogen gram-negative bacteria by using multiplex PCR (Polymerase Chain Reaction) from a positive blood culture bottles.

Methods:

Extraction method was applied from the blood cultures in which growth was detected. After monoplex PCR experiments were performed and images were obtained, multiplex PCR experiments were performed. Considering the product sizes and primers for multiplex PCR, a quadruple bacterial set was prepared by matching Acinetobacter baumannii, Escherichia coli, Serratia marcescens and Pseudomonas aeruginosa. Experiment conditions and tube concentration were adjusted by making serial trial experiments.

Results:

Multiplex-PCR-based methods have the advantage of defining multiple pathogens in a single PCR reaction. These are used for identifying respiratory infections, bacteraemia, and meningitis which are common bacterial and viral diseases. Type-specific genes and molecular techniques including identification of infectious diseases with nucleic acid amplification are promising tools for the verification of accurate diagnosis.The feasibility of this assay in routine diagnostic testing was evaluated using 146 positive blood cultures and 40 negative blood culture samples.

Conclusions:

Early diagnosis and intervention with effective antimicrobial therapy is important in reducing mortality that is associated with bloodstream infections.

Keywords:

Blood culture, identification, microorganism, PCR.

Introduction

Infectious diseases are among the most common diseases affecting human beings and are among the main causes of morbidity and mortality in developed and developing countries [1, 2]. Infectious diseases can progress quickly and endanger patients' lives.

According to a study obtained from 15 health centers in Turkey and including hospital infection data ;30-72% of hospital infections were caused by gram negative bacteria[3].

These bacteria are the most important causes of hospital and community acquired infections 4 including nosocomial bloodstream infections, especially when the source of bloodstream infections is urinary, respiratory or gastrointestinal tract[5]. Gram-negative bacteria play an important role in the development of bacteremia and sepsis in terms of both their endotoxin production properties and their resistance development[6]. Bloodstream infections are one of the most common invasive infections and can cause mortality and morbidity[7,8].

Therefore, timely implementation of effective treatment in sepsis patients is crucial for a patient's prognosis. However, in recent years, the effectiveness of empirical treatments has seen a decline due to the spread of resistant bacteria[9]. Prolonged use of broad-spectrum antibiotics in empirical treatments has also contributed to antibiotic resistance worldwide[10,11].Early diagnosis and treatment of bloodstream infections are very effective in reducing mortality rates.

Blood culture is an important diagnostic method used to isolate microorganisms that cause sepsis and bacteremia and to initiate appropriate antimicrobial therapy[12-14]. Detection of microorganisms in a patient's blood is extremely important not only for diagnosis but also for the treatment and prognosis of the patien[15]. However, traditionally, identification of pathogens causing bloodstream infection in blood culture and application of antibiotic susceptibility tests is a time-consuming method as it involves multiple incubation steps.

It is crucial that microorganisms isolated in blood cultures should be diagnosed and reported to the clinician in a short time, and antibiotic treatment should be initiated as soon as possible[16]. Speeding up laboratory examinations, informing clinicians about the initial possible results and helping guide the treatment process are amongst the other important actions[17].

Mortality rate of patients with positive blood cultures has been reported to be 12 times higher than that of those without growth in blood cultures [16]. This shows the

urgent need for a rapid and reliable diagnostic method which would allow clinicians make faster and more informed treatment choices for these patients.

With the recent development of new methods and advances in the diagnosis of sepsis, it has become possible for clinicians to obtain faster and more reliable results than they did with conventional methods[18,19]. In particular, identification and antibiotic susceptibility testing directly from a positive blood culture bottle has the potential to further reduce the time from empirical therapy to optimal therapy.

More recently, molecular techniques have been developed to provide faster diagnosis of bacterial infection by detecting bacterial DNA (Deoxyribonucleic acid) in blood. It should be noted that molecular methods complement traditional culture, antigen and antibody-based methods for the epidemiological analysis, identification and identification of infectious microorganisms. Early diagnosis of bacterial infections has a significant impact on the effectiveness of a patient treatment process. A faster and more sensitive method for bacterial DNA and bacterial species differentiation and identification will allow earlier treatment with a specific antibiotic. Moreover, rapid bacterial identification could effectively lead to saving of hospital resources and reduced hospital stays and thereby costs[20].

The aim of our study was to develop a method for the diagnosis of some gram-negative bacteria, which are most commonly could be isolated directly from the clinical sample (blood culture bottle).

Method

Bacterial strains

The strains selected as positive controls in our study were Acinetobacter baumannii (ATCC 19606)) (American Type Culture Collection, Escherichia coli (ATCC 25922), Serratia marcescens (ATCC 13880) and Pseudomonas aeruginosa (ATCC 27853). Bacteria stored in lyophilized form at -20°C were passaged on 5% sheep blood agar and (EMB) Eosin methylene blue agar and were grown in an oven at 37°C. The samples were passaged twice to obtain pure bacterial strains.

Primers

The chosen primer set targets a highly conserved area.

A. baumannii

(5’CATGGACATAAGCCTGT TC-3’, 3’CCCGAGGCATAGACTGTA-5’),

E.coli

(5’ TCA CCC GCT TGC GTG GC 3’, 3’ GGAACTGGAACTCACAAAC 5’),

P. aeruginosa

(5’ GGGGGATCTTCGGACCTC) 3’, 3’ TCCTTAGAGTGCCCCACCCG 5’),

S. marcescens
(5'GGGAGCTTGCTCCCCGG3', 3'AACGTCAATTGATGAACGTATTAAGT 5').

Colony PCR and Blood Culture Obtaining
A visible piece was taken from the colony to be tested with the help of a loop and dissolved in approximately 50 µl (Microliter) of sterile water. Plasmids were removed from the exploded cells by keeping them in boiling water for 10 minutes. Bacterial residues were precipitated by a brief centrifugation. PCR reaction was established by taking 0.5-1 µl from the upper aqueous portion. The formation of the expected bands was determined after agarose gel electrophoresis.

Then, in our study, an experimental blood culture medium was created. For this, Karakoç et al. (2009)'s experiments were modified and followed 21. The study was carried out in two stages. In the first stage, microorganisms (ATCC strains) incubated for 24 hours on 5% sheep blood agar and EMB agar to obtain the logarithmic phase of growth were suspended in LB (Luria-Bertani) broth using the appropriate 0.5 McFarland (1x108)22 standard.

In the second stage, the suspensions were diluted to obtain 106 microorganisms per mL (Mililiter) before inoculation into blood culture bottles. After the prepared suspension was added to the bottle, 1-5 ml of blood was inoculated into blood culture bottles and was grown in an oven at 37°C. Hemoculture samples were evaluated in manual biphasic blood culture systems showing growth signal. Turbidity in the bottle and growth on the agar surface were considered positive growth. Subculture and staining results with the experimentally inoculated microorganism into the blood culture bottle were examined for possible contamination. To test the accuracy, the samples in the blood culture bottles were passaged to 5% sheep blood agar and EMB agar and grown in an oven at 37°C.

DNA extraction
The DNA extraction method of Angeline (2006), one of the blood cultures with positive growth, was modified and applied in our study23. 1 ml of blood culture was taken with sterile ependorfe and centrifuged at 4000 rpm (Revolutions per Minute) for 5 minutes to precipitate erythrocytes.

The supernatant was taken separately and centrifuged at 15000 rpm for 3 minutes. The supernatant was discarded. The pellet was washed 1-2 times with sterile water. 150 µl of TE (Tris-EDTA) (pH:8) was added to the pellet. It was vortexed. It was boiled at 100ºC for 15 minutes. It was immediately brought to -20ºC. After waiting for 15 minutes, spin was done at 10000

rpm for 10-12 seconds. The resulting sample was used as a template in PCR.

Monoplex and Multiplex PCR

First, monoplex PCR experiments were performed and images were obtained, and then multiplex PCR experiments were performed. For multiplex PCR, a set of 4 was prepared considering the product sizes and primers (Table 1).

Table 1. Bacteria ATCC strains and clinical isolates utilized in this study

Species	Clinical sample isolation or ATCC no.
Acinetobacter baumannii	ATCC 19606
Escherichia coli	ATCC 25922
Serratia marcescens	Clinical sample isolation
Pseudomonas aeruginosa	ATCC 27853

A. baumannii, E. coli, S. marcescens and P. aeruginosa, which are frequently isolated in blood cultures, were matched (Table 2). As a result of optimization studies, experimental conditions and tube concentration were adjusted.

Table 2. List of oligonucleotide primers used for conventional multiplex PCR amplification

Target Gene	Nucleotide sequence (5′ to 3′)	Base-pair (bp)	Species
Int1	CAGTGGACATAAGCCTGT TC	160	A baumannii
	CCCGAGGCATAGACTGTA		
trpD	TCA CCC GCT TGC GTG GC	296	E. coli
	GGAACTGGAACTCACAAAC		
16 S rDNA	GGGGGATCTTCGGACCTCA	956	P. aeruginosa
	TCCTTAGAGTGCCCACCCG		
16 S rDNA	GGGAGCTTGCTCCCCGG	452	S. marcescens
	AACGTCAATTGATGAACGTATTAAGT		

Gel Electrophoresis

PCR products were visualized by running in 1% agarose gel. To this end, 1 g of agarose was dissolved in 100 ml of 1X TAE (Tris-asetat-EDTA) buffer using a microwave oven. Completely dissolved agarose was cooled to 45-50ºC and 1.5 µl (10 µg/ml) ethidium bromide was added. It was mixed well and poured into a horizontal electrophoresis cassette with a thickness of 5 mm, and then it was left to cool. Combs were removed from the frozen gel and wells were formed. 1/5 of the PCR products were taken and mixed with 6X loading dye, which was loaded into the wells. The gel was run at 110 milliamperes (mA) in TAE buffer (1X Tris-Acetate-EDTA (Ethylene diamine tetra acetic acid)), and then it was taken on the transilluminator and photographed. The molecular weight of the amplified DNA was determined by the molecular weight standard (100 bp (Base-pair) plus) and its accuracy was determined

Results

In PCR, monoplex PCR was performed by using DNA obtained from colony and blood culture by boiling method as a template. DNA obtained by bacterial culture from ATCC strains and genomic DNA isolation was used as a template in monoplex PCR, using different primer, MgCl2 (Magnezyum klorür) and dNTP (Deoxynucleotide trifosfat) concentrations, as well as different reaction conditions such as number of cycles, primer attachment temperature (annealing temperature) and duration. The most ideal conditions were determined as 36 cycles and 50°C bonding temperature. The study went on to perform colony PCR. Gram-stained strains with gram-negative growth detected by inoculation of various clinical samples were used as templates in PCR. After colony PCR, the identification from direct blood culture was performed. Biphasic blood culture bottles were examined for the presence of gram-negative bacteria by performing gram staining after the positive signal. The samples, in which the presence of gram-negative bacteria was detected, were separated as bacillus and cocci or coccobacillus this time. They were extracted by boiling method and used as a mold in PCR.

The study continued with 146 blood culture positive clinical samples with gram negative bacteria in gram staining and blood culture negative clinical samples. In the optimization of multiplex-PCR, different primer, MgCl2, dNTP concentrations and different reaction conditions such as cycle number, primer attachment temperature and time were used. The most appropriate conditions were again determined as 36 cycles and 50°C bonding temperature. Desired bands were obtained in all of the clinical samples studied. The confirmation was carried out by passage into the medium. Negative samples were found to be negative.

The bands obtained as a result of PCR experiments were checked for expected product size. Fermentas 100 bp plus was used as the molecular weight standard. Images obtained by multiplex PCR experiments are presented below:

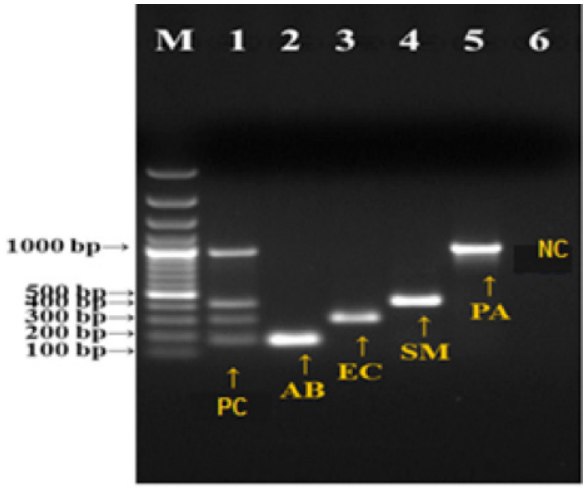


Figure 1. UV transluminator image of multiplex PCR products with primers specific for PA (Pseudomonas aeruginosa), SM (Serratia marcescens), EC (Escherichia coli), AB (Acinetobacter baumannii) from direct blood culture after electrophoresis on agarose gel (1%) (M. Molecular weight standard, 1. Multiplex PCR (PC) 2-3-4 -5. DNA templates obtained by extraction from direct blood culture (PA. P. aeruginosa (956 bp), SM. S. marcescens (452 bp), EC. E. coli (296 bp)), AB. A. baumanni i(160 bp), 6. Negative control (NC)

In this study, first, experimental blood culture positivity studies were followed with a pilot study on twenty clinical samples. Then, 146 positive and 40 negative samples were collected and examined. The results of blood culture and multiplex PCR test method were compared. According to the results of all tests applied, all positive samples were found to be positive and all negative samples were found to be negative.

Discussion

Infectious diseases are diseases that progress quickly and can endanger the life of a patient a short time. It is therefore important to speed up laboratory investigations as much as possible. Effective management of infectious diseases depends on the correct and rapid identification of the causative microorganism.

Invasion of the circulatory system by microorganisms is one of the most serious conditions in infectious diseases[17,24] and is reported to be one of the main causes of morbidity and mortality[24,25]. Thus, it requires a complete and adequate treatment strategy[26].

The detection of microorganisms present in positive blood culture bottles reduces empirical antibiotic therapy adaptation, thereby reducing patient exposure to ineffective or unnecessary antibiotics[26].

Cultivation is the gold standard[27-31] for identifying microorganisms present in the bloodstream, however, this technique is quite slow because it takes one or more days to obtain results[32,33]. Molecular-based studies such as PCR offer solutions to studies such as culture[34]. In some molecular-based studies, the identification of microorganisms by PCR requires a period of five hours[35, 36]. The findings from our study show shorter duration and reduced costs compared to these studies, which is an advantage.

The study period of Trung et al. is similar to that in our study, but a limitation of this study is that it did not yield results at low DNA levels[37]. Our study has advantages such as low cost, specific primers, requiring less time such as 3-4 hours, and identification of the four gram-negative bacteria most frequently isolated in clinical samples in a PCR tube, based on multiplex PCR-based work.

In addition, the isolation of the DNA of microorganisms with the boiling method we used in the research here has the least detailed difficulty. Compared to other methods[36], it requires fewer steps and materials, does not contain toxic materials, requires low skill levels. Also, since all process takes place in the same tube in our study, the amount of waste and the possibility of contamination is minimized. The

chemicals used do not require special use, let alone the biohazard use system.

In our future work, In addition to our specified bacterial identification kit, we aim to further develop our kit by determining different antibiotic resistances in these organisms with the help of multiplex PCR.

Conclusion

The PCR multiplex technique shows great promise for the rapid and accurate diagnosis of microorganisms in infectious diseases. Our study highlighted the advantages of the PCR multiplex technique, including its low cost, shorter processing time, and ability to identify commonly isolated Gram-negative bacteria. Additionally, the simplified DNA isolation method used in our research offers ease of use, minimizes waste and contamination, and does not require specialized equipment or biohazard protocols. Future work in developing the PCR kit to include antibiotic resistance profiling will further enhance its utility in clinical settings.

Conflict of interest

No conflict of interest is declared.

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