Original Article

Identification of the most common pathogenic bacteria in patients with suspected sepsis by multiplex PCR

Mohammad Reza Arabestani[1] , Hossein Fazzeli[2], Bahram Nasr Esfahani[2]

[1] *Department of Microbiology, Hamadan university of Medical Sciences, Hamadan, Iran*
[2] *Department of Microbiology, Isfahan University of Medical Sciences, Isfahan, Iran*

Abstract

Introduction: *Staphylococcus aureus*, coagulase-negative staphylococci, *Enterococcus* spp., *Enterobacteriaceae*, *Pseudomonas aeruginosa,* and *Acinetobacter baumanii* have been found to be the most prevalent bacteremia-causing bacteria in patients with septicemia. Early detection of bloodstream infection (BSI) is crucial in the clinical setting. A multiplex PCR method for identification of these agents in clinical samples has been developed in parallel by conventional microbiological methods.

Methodology: The target genes selected for each of the organisms were very specific for designing primers. Design of primers was done using Mega4, Allel ID6, Oligo6, and Oligo analyzer software. The test comprises a universal PCR from the 16S rDNA gene and multiplex PCR from the *rpoB, gyrA, sss,* and chromosome X (as an internal control).

Results: The sensitivity and specificity for universal PCR and multiplex PCR in comparison with BC were 83.87% and 91.58%, and 74.19% and 91.58%, respectively. The positive predictive value (PPV) and the negative predictive value (NPV) for these two PCRs were 76.47% and 94.57%, and 74.19% and 91.58%, respectively. PCR failed to identify bacteria which were found conventionally in only 3.96% and 6.34% of the cases by universal and multiplex PCR (mostly bacteria not included in the PCR cassette). In 6.34% of the cases, multiplex PCR afforded identification of bacteria, but BC showed no bacteria in the sample.

Conclusions: The multiplex PCR approach facilitates the detection of bacteremia in blood samples within a few hours. Rapid detection of bacteria by multiplex PCR appears to be a valuable tool, allowing earlier pathogen-adopted antimicrobial therapy in critically ill patients.

Key words: Blood culture; Multiplex PCR; Septicemia

J Infect Dev Ctries 2014; 8(4):461-468. doi:10.3855/jidc.3856

(Received 07 June 2013 – Accepted 06 October 2013)

Introduction

Sepsis is a leading cause of morbidity and mortality in hospitalized patients worldwide. More than 750,000 cases of sepsis are reported each year in the United States [1]. The diagnosis of sepsis is difficult, because clinical signs of sepsis often overlap with other non-infectious cases of systemic inflammation. These signs include tachycardia, leukocytosis, tachypnea, and pyrexia, which are collectively termed a systemic inflammatory response syndrome (SIRS) [2-4]. Blood stream infections (BSIs) are usually diagnosed by performing a series of blood cultures [5]. Unfortunately, this technique is slow, usually requiring one or more days to produce a positive result. Microbiological and epidemiological data clearly indicate that only a limited number of bacteria are responsible for the majority of all BSIs in the intensive care unit (ICU). PCR assays developed for the specific detection of pathogens in the blood were described as early as 1993 [6-7]. Further development led to broad-spectrum PCR assay, allowing for more universal detection of microorganisms [8-9]. PCR amplification of microbial genes, followed by detection of amplified products by gel electrophoresis or real-time PCR monitoring using fluorescent dyes or target-directed fluorescent probes, is a quick process allowing pathogen detection within a few hours [10]. Identification of microorganisms can be performed by PCR algorithms, taxon-specific oligonucleotide microarray, or sequencing amplification [11]. Molecular technologies for rapid multiplex detection of microorganisms in patients with sepsis only recently have been made available. Molecular studies for microbial identification target species-specific genes and conserved bacterial DNA sequences, such as 16S rDNA, 23S rDNA, and 16S-23S rDNA interspace regions for amplification [12-14]. In this study, we developed a multiplex PCR method in combination with internal control (IC) for the molecular identification and definition of the

frequent pathogens found in whole blood samples. This technique is based on synchronized amplification of distinct segments of target DNA by employment of two or more primer pairs in a single reaction tube, which, in turn, usually means reduced costs and time requirements. Multiplex PCR results were compared with blood culture as the gold standard for identifying prevalent bacteria.

Methodology

Clinical specimens, microbial detection and identification

Blood samples were collected from hospitalized patients at the medical center located in Isfahan University. Blood for culture was obtained from venipuncture. Twenty mL of blood was inoculated into a pair of aerobic and anaerobic BACTEC plus/F BC bottles (Becton Dickinson, Heidelberg, Germany). For children, 3-5 mL of blood was inoculated into BACTEC Peds BC bottles (Becton Dickinson, Heidelberg, Germany). Immediately after blood was drowning for BC, 2 mL of whole blood was collected in sterile EDTA K2E tubes for the Mastercycler Personal (Eppendorf, Hamburg, Germany). Cultures were incubated in the Bactec 9050 microbial detection system. Cultures found to contain bacteria growth in the bottles were regarded as positive cultures and further processed for identification by conventional microbial methods.

Oligonucleotide primers selection

The target genes for primer design included the 16S rDNA for universal PCR, the *rpoB* of *Enterobacteriaceae,* the chromosome X of Drosophila melanogaster as an internal control, the *rpoB* of *Staphylococcus* spp., *Enterococcus* spp., the *sss* of *Pseudomonas aeruginosa,* and the *gyrA* of *Acinetobacter baumanii,* which were deposited in NCBI, DDBJ, and EMBL databases and are shown in Table 1. The target genes selected for each of the organisms was very specific and the primers were designed using Mega 4, Allel ID 6, Oligo 6, and Oligo analyzer software for checking out multiple alignments, primer design, annealing temperature, and multiplex condition, respectively. All the primers designed were BLAST by nucleotide and primer blast tools (National Center for Biotechnology Information) before they were synthesized by Faza Biotech, Iran.

Bacteria strains and species

Reference strains of organisms such as, *A. baumannii* (ATCC 19606), *P. aeruginosa* (ATCC 27853), *E. faecalis* (ATCC 29212), *S. aureus (*ATCC 29213), *E. coli* (ATCC 25922), *Enterobacte raerogenes* (ATCC13044), *Proteus mirabilis* (ATCC 7002), *K. pneumoniae* (ATCC 13882), *Salmonella enterica* (ATCC 35640), and *Shigella boydii* (ATCC 9207) were purchased from the reference laboratory of Iran and the Pasteur Institute of Iran.

Genomic DNA extraction

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Briefly, bacteria were removed from the culture plate with an inoculation loop and suspended in 180 µL of buffer ATL by vigorous stirring. Twenty µL of proteinase k was added; the solution was mixed by vortexing and was incubated at 56ºC for one to three hours. DNA was bound to the silica membrane and washing was carried out as described in the QIAamp protocol. A pure genome was eluted from the membrane in 100 µL of TE buffer. Qualitative and quantitative assessment of DNA preparation was performed through agarose gel and spectrophotometeric measurements. Purified DNA was stored at -20°C until used for PCR amplification.

Table 1. List of oligonucleotide primers used for conventional multiplex PCR amplification

Organism	Gene name	Forward primer	Reverse primer	Product size (bp)
Drosophila melanogaster	X chromosome	AGCATTCAAATCCTTCATACTG	ATGTTGGTGTAATCTGACTCG	684 bp
Staphylococcus spp.	*rpoB*	CAGGAGAAGTTAAAGAACAAGAAG	GTGAACGAACTAATTGAGATACG	118 bp
Enterococcus spp.	*rpoB*	AGAGAGTAAGGTCCGATTGAAC	GGTTGTTTCCCGTATTATGC	370 bp
16S rDNA	*16S*	AGAGTTTGATCMTGGCTCAG	GYTACCTTGTTACGACTT	1505 bp
Pseudomonsa eruginosa	*sss*	GCCTCTACCAGTACCTGCTAC	AATAGAACAGCTCCAGCAGG	189bp
Acinetobacte rbaumannii	*gyrA*	CACCAATCACACGCAATG	GTATTCCAACCGATATTCACC	246 bp
Enterobacteriaceae	*rpoB*	CAGGTCGTCACGGTAACAAG	GTGGTTCAGTTTCAGCATGTAC	512 bp

Determination of minimal analytical sensitivity and specificity of bacteria and IC

The PCR products of 16S rDNA, *rpoB, sss, gyrA,* and IC were cut out from the agarose gel, purified by the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and ligated with the pTZ57R/T (as TA vector). The ligation mixture was transformed into *E. coli* DH5α strain and the recombinants were selected on LB agar containing ampicillin (100 µg/mL). Recombinant plasmid DNA was purified by the standard method and subjected to further analyses. A series of tenfold dilutions of the PCR products containing plasmid, ranging from 1 to 10^8 copies/reaction or serial dilutions of DNA extracted from human blood samples in vitro spiked by the five prototype strains were prepared and stored at -20°C until used. Specificity was tested with DNA extracted from the 17 bacterial species reported in Table 2.

Colony polymerase chain reaction amplification

For colony PCR amplification, a recombinant colony (white colony on LB agar) of each organism was selected. Each colony placed in tubes containing Mix 2X premix Bio Inc., (Takara Bio, Otsu, Shiga, Japan). A different primer and molecular water were added to each tube to a final volume of 25 µL and amplified by the PCR system Mastercycler Personal (Eppendorf, Hamburg, Germany). The colony PCR was done and the correct sequence of each insert was verified by sequence analysis (Faza Biotech, Tehran, Iran).

PCR and multiplex PCR amplification

The conventional multiplex PCR assay was carried out in two steps. The first step consisted of universal PCR that contained 16S rDNA and internal control primers. The second step consisted of specific PCR which included two parallel reactions (Gram positive or Gram negative); Gram positive included IC, 16S r-DNA, *E. faecalis, S. aureus,* and Gram negative included IC, 16S r-DNA, *Enterobacteriaceae, P. aeruginosa,* and *A. baumannii,* which had the identical annealing temperature for the detection of bacteremia, respectively. PCR conditions were optimized according to the manual of the emerald Amp MATHSP CR Master Mix 2X premix Bio Inc., (Takara Bio, Otsu, Shiga, Japan). The optimal condition for annealing temperature was 60ºC. As a template, 5 µL of extracted DNA was added, resulting in a total volume of 25 µL. Amplification started with a cycle of 4 minutes at 94ºC, followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 60ºC for 30 seconds, extension at 72ºC for 1 minute, and a subsequent final extension at 72ºC for 10 minutes. Amplification and detection were carried out using Mastercycler Personal (Eppendorf, Hamburg, Germany). Multiplex PCR conditions were the same as PCR system conditions.

Gel electrophoresis

The amplicons obtained were submitted to electerophoresis onto 1% agarose gel in Tris-Boric EDTA (TBE) buffer, stained by ethidiume bromide (0.5 µg/mL) for UV light analysis, and digitized (UVIDOC-CF08.XD).

Sequence analysis

PCR products were purified and sequenced by Faza Biotech (Tehran, Iran) in order to confirm the sequence authenticity of the PCR products.

Table 2. Bacteria ATCC strains and clinical isolates utilized in this study

Species	Clinical sample isolation or ATCC no.
Bacillus cereus	ATCC10702
Vibrio cholerae	ATCC14035
Aeromonashydrophila	University of Isfahan culture collection
Campylobacter jejuni	ATCC35918
Mycobacterium tuberculosis	ATCC25177
Neisseria meningitides	ATCC 13091
Pseudomonas aeruginosa	ATCC10145
Legionella pneumophila	ATCC43662
Mycobacterium fortuitum	ATCC23010
Moraxella catarrhalis	ATCC8193
Pseudomonas fluorescens	University of Isfahan culture collection
Streptococcus pneumoniae	ATCC33400
Streptococcus agalactiae	ATCC13813
Stenotrophomonasmaltophilia	University of Isfahan culture collection
Helicobacter pylori	ATCC26695
Mycoplasma hominis	ATCC23114
Mycoplasma arginini	ATCC23838

Table 3. Comparison of universal PCR results to blood culture results

			Universal PCR		Total	Measure of agreement Kappa	P value
			Negative	Positive			
Blood culture results	Negative	Count	87	8	95		
		% within blood culture result	91.6%	8.4%	100.0%		
	Positive	Count	5	26	31		
		% within blood culture result	16.1%	83.9%	100.0%		
Total		Count	92	34	126		
		% within blood culture result	73.0%	27.0%	100.0%	.731[a]	.000[b]

a: .000 to 8.218
b: < 0.05

Table 4. Comparison of specific PCR results to blood culture results

			Specific PCR		Total	Measure of agreement Kappa	P value
			Negative	Positive			
Blood culture results	Negative	Count	87	8	95		
		% within blood culture result	91.6%	8.4%	100.0%		
	Positive	Count	8	23	31		
		% within blood culture result	25.8%	74.2%	100.0%		
Total		Count	95	31	126		
		% within blood culture result	75.4%	24.6%	100.0%	.658[a]	.000[b]

a: .000 to 7.383
b: < 0.05

Table 5. Isolated pathogens with PCR and blood culture

Pathogens	Number of cases detected		
	PCR only	BC only	PCR and BC
S. aureus	3	1	4
S. epidermidis	0	2	3
S. viridanc	0	0	3
E. faecalis	2	1	1
E. coli	0	0	5
Alcaligenes faecalis	0	0	1
S. marccescens	0	0	2
K. pneumoniae	0	0	2
Enterobacteriaceae	3	0	0
E. aerogenes	0	0	2
A. baumannii	0	0	1
A. lowffii	0	1	0
P. aeruginosa	0	0	2
Total	**8**	**5**	**26**

Table 6. Distribution of isolated bacteria with blood culture

			Blood culture result Kind_Bakeri cross tabulation											
			Kind_Bakeri											
			0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Total**
Blood culture results	Negative	Count	87	3	2	0	0	0	0	0	3	0	0	95
		% within blood culture result	91.6%	3.2%	2.1%	0.0%	0.0%	0.0%	0.0%	0.0%	3.2%	0.0%	0.0%	100.0%
	Positive	Count	8	4	2	1	2	5	2	3	0	2	2	31
		% within blood culture result	25.8%	12.9%	6.5%	3.2%	6.5%	16.1%	6.5%	9.7%	0.0%	6.5%	6.5%	100.0%
Total		Count	95	7	4	1	2	5	2	3	3	2	2	126
		% within blood culture result	75.4%	5.6%	3.2%	0.8%	1.6%	4.0%	1.6%	2.4%	2.4%	1.6%	1.6%	100.0%

1: *S. aureus,* 2: *E. faecalis,* 3: *A. baumannii,* 4: *E. aerogenes,* 5: *E. coli,* 6: *K. pneumoniae,*
7: *S. epidermidis,* 8: *Enterobacteriaceae* 9: *S. marccescens,* 10: *P. aeruginosa*

Results

DNA extraction

The DNA of all bacterial species (for details, see microorganisms section) was successfully extracted directly from bacterial culture. The mean OD values and concentration of bacterial DNA (ng/µL) obtained with the QIAamp DNA Blood Mini Kit were 1.9 ± 0.07 and 180 ± 1.1, respectively.

Sensitivity and specificity of the multiplex PCR assay

The lower detection limit for Gram positive was 10^2 copies per reaction or 100 CFU/mL for both *E. faecalis* and *S. aureus*, whereas the lower detection limit for *A. baumannii, P. aeruginosa,* and *E. coli* were 10 copies or 50 CFU/mL, respectively. The correct amplification of the IC showed that the whole workflow, starting from the extraction step, was performed in a reliable way.

Detection of pathogens in clinical samples

A total of 126 samples from patients suspected of having bacteremia were taken for blood culture, and 31 samples were found to be positive. The universal PCR was performed for 126 clinical samples, and 34 samples were found to be positive. To identify the bacterial isolate, multiplex PCR was performed on these 34 isolates, of which 31 showed amplification.

PCR results in BC-positive samples

Compared to BC results (as the gold standard), the diagnostic sensitivity and specificity of the universal PCR and specific PCR were 83.87% and 91.58%, and 74.19% and 91.58%, respectively. Of the 31 BC-positive patients, 26 and 23 were also universal PCRpositive and specific PCR positive. The concordance of universal PCR and BC for both positive and negative samples was (26 + 87)/126 and for specific PCR was (23 + 87)/126 samples (Tables 3 and 4). Species and genera detected in the 39 samples by BC and PCR are shown in Tables 5 and 6.

The five samples that were positive by BC but negative by PCR included two BC-positive with *Staphylococcus epidermidis*, one *S. aureus*, one *Enterococcus faecalis,* and one *Acinetobacter lowffii*; moreover, the eight samples that were positive by PCR but negative by BC included three PCR positive with *S. aureus*, two *E. faecalis,* and three *Enterobacteriaceae*. In addition, polymicrobial growth was observed in three samples (2.38%; 3/126).

Discussion

Conventional diagnostic methods for BSIs require several days to complete, and both false positive [15,16] and false negative results [17,18] have been reported. Delayed diagnosis correlates strongly with the high mortality of BSIs [19,20]. Rapid detection of bacteremia is one of the most important tools in the clinical diagnosis of BSIs. Thus, there is an urgent need for a rapid and reliable diagnostic method that will allow clinicians to make faster and better-informed treatment choices for BSI patients. Molecular techniques have being developed to achieve the diagnosis of bacterial infection more quickly by detecting bacterial DNA in blood [21,22]. For all molecular pathogen identification methods, an efficient isolation from the clinical sample that results in microbial DNA template of high purity is important to achieve accurate results [23]. The majority of the molecular assays are designed specifically for the detection of only one single organism, thus providing a high level of sensitivity and specificity [24-26]. Recently, various broad-range PCR or multiplex PCR were applied in order to screen and detect the presence of multiple organisms in clinical samples [27-29]. The major limitations of these methods are their cost-to-efficacy ratio and the fact that most of these techniques are based on time-consuming procedures for the final identification of the microorganism, such as sequencing, restriction fragment length polymorphism, or hybridization with germ specific probes [30].

In the present study, the diagnosis of bacterial sepsis in the suspected patients by universal PCR and multiplex PCR was 83.87% and 91.58%, and 74.19% and 91.58%, respectively. The positive predictive value (PPV) and the negative predictive value (NPV) for these two PCR methods were 76.47% and 94.57%, and 74.19% and 91.58%, respectively. Of 126 samples taken from patients with septicemia, a pathogenic bacterium was detected in the blood culture of 24.6% of the cases; with PCR, the detection of bacteria was 27%.

Eight patients tested positive for PCR but had negative blood cultures. These patients may have received antimicrobial substances before blood sampling. This suggests that the bacterial species detected by PCR may have been nonviable and thus not detectable by culture. In 5 of 31 BC-positive patients, the PCR results were negative. This may have been caused by a low concentration of bacteria in the blood samples.

In this study, polymicrobial infections were detected in 2.38% (3/126) of patients. Unfortunately, IC DNA in the PCR assay was not amplified in 3.17% (4/126) of samples; therefore, the PCR test gave no information. This was due to either inhibition of the PCR reaction or inappropriate sample preparation [31].

Timely identification of the causative pathogens is important for adequate antimicrobial therapy of septic patients. The PCR can be completed within five hours. The mean time of incubation until positive signaling by the automated BC system was 24 hours.

In conclusion, we evaluated a new conventional multiplex PCR test system which detected 16S rDNA gene by PCR and identified the most common pathogenic bacteria by specific multiplex PCR from the whole blood of patients with suspected bloodstream infections. The results obtained by the PCR test correlated well with those from BC. The PCR test offers several advantages, notably higher diagnostic sensitivity by detection of nonviable pathogen cells and a shorter total assay time. Thus, multiplex PCR should provide a useful supplemental method in the diagnosis of sepsis.

Acknowledgements

The authors are grateful to Vice Chancellor of Research of Isfahan University of Medical Sciences for the financial support for this study.

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Corresponding author
Mohammad Reza Arabestani
Department of Microbiology, Faculty of Medicine
Hamadan University of Medical Sciences, Hamadan, Iran
Phone: +98-9188662009
Fax: +98-8118380762
Email: mohammad.arabestani@gmail.com

Conflict of interests: No conflict of interests is declared.