Polish Journal of Microbiology
2004, Vol. 53, No 3, 145–149

Usefulness of the PCR Technique for Bacterial DNA Detection in Blood of the Patients after "Opened Heart" Operations

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Received in revised form 5 July 2004

Abstract

To confirm the sensitivity of the polymerase chain reaction (PCR) technique (versus blood cultures) and to gain a better understanding of the incidence of true- and false-positive results when using this technique, one hundred randomly chosen patients treated operationally because of valve defects were examined. In our studies we found that PCR techniques using universal primers complementary to the bacterial 16S rDNA showed promise as being more sensitive than conventional blood culture (BC) techniques. From the time that a blood culture is positive, conventional methods of culture and antibiotic susceptibility testing require at least 64 h for the detection of infection or colonization. Rapid identification of bacteria from blood using PCR technique accelerates the microbiological diagnosis.

Key words: PCR, blood infections, DNA

Introduction

Cardiosurgical operations connected with an extracorporeal blood circulation (EBC), because of the large extent of the injury, disturbance of the immunological system and damage of anatomic barriers create the conditions, which are remarkably convenient for development of infection based complications. Conduction of the preventive and therapeutic antibiotic treatment as well as selective decontamination of the alimentary tract can make the diagnosis more difficult and time consuming.

During the last decade there has been increasing use of nucleic acid based techniques, mainly polymerase chain reaction (PCR), for the identification of infection in clinical samples. In cardiosurgical patients the likely causative bacterium is often not known-necessitating amplification of a section of bacterial DNA that is common to all bacteria (the so-called „universal" sequences). There has been a significant amount of work demonstrating that amplification of sections of the 16S rDNA gene has been found to be both sensitive and specific for the detection of bacterial DNA from almost all known species of bacteria (Greisen *et al*., 1994; Steinman *et al*., 1997; Hitti *et al*., 1997; Jantos *et al*., 1998; Seward and Towner, 2000; Jordan and Durso, 2000). The 16S rDNA gene codes for the 16S ribosomal and component is found in bacteria only – and is thus not affected by contamination with mammalian DNA.

The aims of our studies were: (1) evaluation the sensitivity of the PCR technique of the bacterial DNA detection in blood samples and (2) its comparison with classical bacterial examination of the blood during the period close to the 'opened heart' operations.

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Experimental

Materials and Methods

Clinical methods. The data consisted of 900 blood samples obtained from 100 randomly chosen patients from Clinic of Cardiac Surgery at Medical University of Gdańsk (Poland) over the period April 1998 to November 2000. The patients were treated operationally because of valve defects. An extended set of clinical observations supplemented by bacteriological tests was obtained. The 47 women (range age 31–68 mean 53 years) and 53 men (range age 31–68 mean 53 years) were examined. The surgical procedures are shown in the Table I. Mean aortic cross-clamping was 80 minute (from 34 to 184) and total extracorporeal perfusion time 119 minutes (range 54 – 298).

Table I
The surgical procedures of a 100 randomly chosen patients from Clinic of Cardiac Surgery at Medical University of Gdańsk (Poland)

Procedure	Number of patients (100)
AVR	31
AVR + resection of lung tumour	1
MVR	35
MVR + AVR	10
AVR + CABG	13
MVR + CABG	7
Operation mode Bentall	1
Operation mode Bentall + CABG	1
Suture perivalvular leak + TVP	1

AVR – aortic valve replacement, MVR – mitral valve replacement,
TVP – tricuspid valve plastic, CABG – coronary artery by-pass grafting

All patients were administered routinely by antibiotic – Cefamandol 1.0 iv. 1 hour before procedure, during and each 6 hour till the fourth postoperative day. Sixteen patients were treated prophylactically by Vancocin because of hospital's *S. aureus* in their nose and skin before operation.

The blood samples were taken, in a sterile manner, from patients and then sent for PCR and blood cultures (BC) diagnosis. The blood drawing was carried out due to the following scheme: (1) 24 h before the operation, (2) in time of wound closing during operation, and (3) 4 h, (4) 24 h, (5) 2 days, (6) 3 days, (7) 4 days, (8) 5 days, and (9) 7 days after the operation. A total of 900 blood samples from 100 randomly chosen patients were analysed by PCR for the 16S rRNA gene, and results were compared to the blood culture results.

Blood cultures. The blood cultures were processed in the manner routine to the microbiological department of our hospital (BacT/Alert, Organon Teknika). The FAN type blood culture bottles were inoculated with 5 ml of blood (two bottles for each time of test, one for oxygenic and second for unoxygenic condition) from patients and were incubated at 36.5°C in the BACTEC 9240 automated continuous monitoring system for 7 days.

DNA isolation. One ml of EDTA chelated blood was added to 9 ml of LB or 2YT medium and cultured 4 h with aggregation at 37°C. After incubation, 200 µl of the cultures were taken to Eppendorf tubes and 1ml of sterilised water was added. The samples were centrifuged and pellets were poured with 1 ml of 1mM NaOH. The solutions were centrifuged and the pellets were poured with 1ml of sterilized water and centrifuged. The pellets were then resuspended in 100 µl of water and 10 µl of lysostafin (2 mg/ml) and 20 µl lysosyme (50 mg/ml) was added. The solutions were incubated 10 min at 37°C and then 20 µl of proteinase K (20 mg/ml) was added. The incubation was continued at 37°C for further 10 min. Thereafter, the enzymes were inactivated by incubation at 75°C for 10 min. The solutions were then centrifuged and the supernatants containing the DNA were used for PCR.

PCR. PCR amplification of 16 S rDNA was performed as described by Widjojoatmodjo *et al.* (1995) using forward primer 5'-GAGGAAGGTGGGGATGACGT and reverse primer – 5'-AGGCCCGGGAACGTATTCAC. These primers target highly conserved regions of the 16 S rDNA and are universal for amplification of the bacterial DNA. PCR amplification was performed with a DNA Thermal Cycler 480 (Perkin-Elmer) by using PCR reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 0.1% Triton X-100) containing each of the deoxynucleoside triphosphates at a concentration of 200 µM, each of the primers at a concentration of 1 µM, 0.1 µg target DNA and 1 U *Taq* DNA polymerase (Shark2, DNA-Gdańsk II s.c., Poland) in a total volume of 50 µl. A total of 35 cycles of amplification were performed with template DNA denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and primer extension at 72°C for 2 min. PCR specific products (207 bp) were separated electrophoretically on a 2% agarose gel (Sigma) using 1xTris-borate EDTA running buffer at a field strength of 8V/cm. The DNA was visualised on an ultraviolet transilluminator following ethidium bromide staining and photographed.

Positive DNA samples (from 70 patients) with primers target highly conserved regions of the 16 S rDNA were further analysed for identification of the genomic species of bacteria belonging to *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* using specific primers for PCR. The *S. epidermidis* specific primers: Se705-1 (5' ATC AAA AAG TTG GCG AAC CTT TTC A) and Se705-2 (5' CAA AAG AGC GTG GAG AAA AGT ATC A) and amplification conditions used were

the same as described in Martineau *et al.* (1996). For *S. aureus* PCR identification, nuc1 (5' GCG ATT GAT GGT GAT ACG GTT) and nuc2 (5' AGC CAA GCC TTG ACG AAC TAA AGC), and amplification conditions used were the same as described in Barski *et al.* (1996). A multiplex PCR test using two pairs of specific primers: PS1 (5' ATG AAC AAC GTT CTG AAA TTC TCT GCT) and PS2 (5' CTT GCG GGC TGG CTT TTT CCA G), and PAL1 (5' ATG GAA ATG CTG AAA TTC GGC) and PAL2 (5' CTT CTT CAG CTC GAC GCG ACG) was carried out for *P. aeruginosa* detection according to De Vos *et al*. (1997).

Results

A total of 900 blood samples from 100 randomly chosen patients were analysed by PCR for the 16S rRNA gene, and results were compared to the blood culture results. Table II illustrates the comparison between the PCR results and those obtained using BACTEC 9240 automated continuous monitoring system and reveals a high level of detection in case of PCR in comparison to blood culture. However, the vast majority of blood samples lacked detectable levels of bacteria by both culture and PCR analyses. Of the 102 PCR positive (from 31 patients) for detection of bacterial DNA, 34 were positive (from 25 patients) for blood culture. The positively rates for blood specimens analysed by culture and PCR were 3.8% and 11.3% positive specimens from a total of 900 tested, respectively. Such low degree of detection using blood culture method is probably caused by prevent antibiotic treatment for all patients (the growth of bacteria in conventional processing has been inhibited by antibiotics). For 21 patients (29 blood samples) the PCR and blood culture results were positive (Table II). When assessing concordance of BC and PCR, 5 samples were PCR negative and BC positive, and 75 samples were PCR positive and BC negative. Most of the positive results could be attributed to colonization. Only temporary bacteraemia were detected among the patients after the cardiosurgical operations connected with extracorporal blood circulation.

Table II
Comparison of blood culture (BC) and polymerase chain reaction (PCR) results

Method	Number of patients (100)	Number of samples (900)
BC positive	25	34
PCR positive	31	102
BC and PCR positive	21	29

Table III illustrates the results of bacteriological tests of the patient's blood by the classical (BC) and PCR methods in time of the blood drawing form patients. Table IV illustrates results for five representative patients that tested positive in both culture and PCR methods. The application of PCR method may lead to the quicker (24–72 hours compared with the standard method) identification of the presence of bacteria in the patient's blood.

We have also attempted to perform specific PCR reactions on the positive blood samples to identify the causative bacterial species. Positive DNA samples (from 70 patients) using PCR method were further analysed for identification of the genomic species of bacteria belonging to *S. epidermidis*, *S. aureus* and *P. aeruginosa* using specific primers for PCR. The most frequently *S. epidermidis* was detected (59 positive out of 73 blood samples tested) (Table V). *S. aureus* was detected in 5 samples and *P. aeruginosa* was not

Table III
The results of bacteriological tests of the patient's blood obtained by the classical (BC) and PCR methods

Method	Time of the blood drawing from patients								
	24 h before operation	operation – in the time of wound closing	4 h after operation	24 h after operation	48 h after operation	96 h after operation	72 h after operation	120 h after operation	168 h after operation
BC (number of positive patients)	4	6	2	1	3	5	6	5	2
PCR (number of positive patients)	7	8	15	9	13	17	11	13	11

Table IV
The results of bacteriological tests obtained by the classical (BC) and PCR methods for the representative patients

No of patient	Method	Time of the blood drawing from patients								
		24 h before operation	operation – in the time of wound closing	4 h after operation	24 h after operation	48 h after operation	96 h after operation	72 h after operation	120 h after operation	168 h after operation
1	BC	–	–	–	–	–	–	–	+	–
	PCR	–	–	–	–	+	+	+	+	–
2	BC	–	–	–	–	–	–	–	+	–
	PCR	–	–	+	+	+	+	–	+	–
3	BC	–	–	–	–	–	–	–	+	+
	PCR	–	–	+	–	+	+	+	+	+
4	BC	–	–	–	–	+	–	–	–	–
	PCR	–	+	+	+	+	+	+	–	–
5	BC	–	–	–	–	–	+	–	–	–
	PCR	–	–	–	+	+	+	+	+	+

Table V
Specific PCR identification of the bacteria from positive blood samples (from 70 patients)

Bacteria	Number of positive samples (patients)
Staphylococcus epidermidis	59 (27)
Staphylococcus aureus	5 (2)
Pseudomonas aeruginosa	0 (0)

detected at all. *S. aureus* was detected in two patients and *S. epidermidis* in 27 patients. The PCR examination was with 100% agreement to the classical diagnostics.

The approximate time required to complete the PCR assay described here is roughly 8 h. This includes blood incubation sample preparation, DNA amplification and detection by gel electrophoresis. Attempts are under way to reduce still further the entire assay time, without sacrificing assay sensitivity. A PCR assay would allow early detection and faster clinical decision making. The use of PCR technology is likely to be significantly more expensive than the use of conventional culture methods. An assay such as the one described here may be useful in patients with known risk factors for infection. However, the clinical and economic benefits to the provision of this information remain to be elucidated.

Discussion

This study was designed as a feasibility study, not as an outcome-based study, to compare the utility of a 16S rRNA PCR assay to that of the BACTEC 9240 system for detecting bacteria in blood obtained from patients during the period close to the 'opened heart' operations.

Rapid detection and identification of bacteria in blood is crucial in patient management because the mortality rate associated with infections in the bloodstream is very high (Jones and Lowes, 1996; Moreno *et al*., 1999; Bossink *et al*., 1999). Among the various methods currently used in clinical laboratories for detection of bacterial infections, culture is the most sensitive one. However, culture requires at least 48 h of incubation. Additional time is needed to perform biochemical or immunological tests to identify the bacteria. The universal PCR method will provide physicians with results at least 1 day earlier than conventional methods.

The main goal of this study was to develop an alternative method for detection of bacteria in blood. Since the 16S rRNA genes of almost all common bacterial pathogens found in body fluids have been sequenced, it is possible to design PCR primers capable of amplifying all eubacteria based on the conservative nature of the 16S rRNA genes (Steinman *et al*., 1997; Garrouste-Orgeas *et al*., 2000). In our studies we

found that PCR techniques using these universal 16S primer sequences showed promise as being more sensitive than conventional blood culture (BC) techniques. Although blood culturing will not be completely replaced by a nucleic acid amplification technology anytime soon, as pure isolates remain essential for antimicrobial drug susceptibility testing, PCR does appear to be an excellent diagnostic test choice for a rapid means of ruling out bacterial infection in certain patient populations. From the time that a blood culture is positive, conventional methods of culture and antibiotic susceptibility testing require at least 64 h for the detection of infection and the provision of antibiotic susceptibility testing results. Rapid identification of bacteria from blood cultures using PCR method accelerates the microbiological diagnosis.

The main results of our study were that generic PCR is able to detect the presence of bacterial DNA in many BC-negative samples. However, there are not false positive results because after some time BC-positive samples were identified from patient with temporary bacteraemia. The extreme sensitivity of PCR makes the technique very susceptible to environmental contamination during the period close to the 'opened heart' operations. Infection with more than one organism is difficult to identify. If the PCR product consists of DNA amplified from more than one species of bacterium, the same size PCR products are obtained. In the future it may be possible to use alternative methods of species identification to overcome this problem – such as by multiplexing species-specific probes or utilizing the different melting points of different PCR products.

In conclusion, this study demonstrates that the universal 16S rDNA PCR is more sensitive than blood culture (BC) in detecting bacterial DNA in patients examined – and may be especially useful in patients who have received prior antibiotics. Temporary bacteraemia were detected among the patients after the cardiosurgical operations connected with extracorporal blood circulation. The application of PCR method may lead to the quicker (24–72 hours compared with the standard method) identification of the presence of bacteria in the patient's blood.

Acknowledgements. The work was supported by the State Committee for Scientific Research Grant No. 4 P 05 C 07814.

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