DIAGNOSIS OF BACTEREMIA USING UNIVERSAL PCR IN FEBRILE ILL CHILDREN

S. Afsharpaiman[1], S. Mamishi[*2], B. Pourakbari[2], A. Siyadati[2], P. Tabatabaee [2] and G. Khotaee[2]

1) Department of Pediatric, School of Medicine, Baghiyatallah University of Medical Sciences, Tehran, Iran

2) Department of Pediatric Infectious Diseases, Children's Medical Center, School of Medicine, Medical Sciences/University of Tehran, Tehran, Iran.

Abstract- Early diagnosis of bacteremia and its complications is the most important part of care and management of the febrile patients. However, a majority of patients who appear to be clinically septic have negative blood culture. The use of polymerase chain reaction (PCR) techniques has allowed identifying the pathogenic organisms rapidly and accurately. The objective of this study was to investigate the prevalence of bacteremia in febrile pediatric patients, comparing universal PCR and conventional blood culture. One hundred febrile children (45 males, 55 females) with suspected septicemia were evaluated. A total of 100 paired blood samples were collected from children to analyze for bacterial detection using universal PCR and culture. Twelve patients were blood culture positive. The most common pathogens isolated from blood culture were *Staphylococcus aureus*, coagulase negative staphylococci (CoNS) and *Pseudomonas aeruginosa*. The PCR were positive in 19 patients. The comparison revealed sensitively, specificity and accuracy of 91.67, 90.91 and 91%, respectively, for PCR. The present study shows that the use of PCR is more sensitive than the use of conventional blood techniques for the detection of bacterium pathogens based on patients' clinical context.

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INTRODUCTION

Rapid diagnosis of bacterial infections can result in optimal and early treatment of febrile children admitted for suspected septicemia, and important information about patients' prognosis and appropriate choice of antibiotic regime can be provided. Consequently, the morbidity and mortality of the patients will decrease. Also, it is not possible to distinguish between bacterial and viral infections in ill patients with fever only based on clinical presentation. If bacteremia could be documented in this setting during earlier hours, unnecessary broad-spectrum antibiotic therapy is avoided and the resistance rates to antimicrobial agents are reduced. Therefore, the duration of patient's admission in hospital and in turn the health care costs will decrease (1-3).

Currently, conventional blood culturing is considered to be the gold standard for diagnosing bacterial sepsis. However, this technique have some limitations, including 1) it takes a long time for growth of an organism (24-48 hours); 2) it can have unacceptably low sensitivities in many clinical situations (40%) and its sensitivity might be less if the patients have had prior antibiotic treatment (4).

In recent studies, the utility of molecular techniques such us universal PCR has been showed to be effective in identifying bacterium pathogens in

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*** Corresponding Author:**
S. Mamishi, Department of Pediatric Infectious Disease, Children Medical Center, School of Medicine, Medical Sciences/University of Tehran, Tehran, Iran
Tel: +98 21 66428996, Fax: +98 66428996
E-mail: smamishi@sina.tums.ac.ir

septic patients, because it is possible to detect small amount of pathogenic particle (DNA, RNA) in blood. Also, this technique can be performed in less time (almost 6 hours), and DNA amplification does not rely on the viability or growth of bacteria even in the presence of antibiotics (5, 6). The universal PCR is based on detection of bacterial particles shared in all bacteria genome. These universal primers have been designed to amplify DNA sequence, usually selecting the 16S ribosomal DNA (rDNA) region as a target (7). However, universal PCR can identify only the presence of bacteria, and for further differentiate between different bacterial species more complex stages are required (8, 9).

Little clinical work has been published regarding use of universal PCR as a screenings test to detect the possible presence of a wide range of bacterial species in septic children. This study was designed to evaluate efficacy of universal PCR technique of blood samples in the diagnosis of septicemia in febrile children compared to conventional blood culture. If an amplification assay could reliably rule out and/or document pediatric sepsis in less time with more sensitivity, this approach would permit shorter hospital stays and significantly reduce medical costs, and emotional burdens of the children and their families.

MATERIALS AND METHODS

This is a descriptive cross-sectional study on 100 consecutive febrile children (45 males, 55 females) admitted to Infectious Disease Ward in Children Medical Center during two-year period from 2004 to 2006.

Patients were considered to be febrile if they had axillary temperature 38.5ºC or more. The patients were excluded whether they had clinical presentations suggesting viral infections such as coryza, conjunctivitis, underlying disease (*e.g.*, malignancies, neutropenia and other immunodeficiency) or they did not have consent to attend in the survey. This study followed in accordance with Ethical Committee approval of Tehran University of Medical Science, and based on the declarations of Helsinki guidelines for medical researches. The written informed consent was obtained from the patients or their parents. This survey has reported only the data related to the objectives, and other patients' information has been conserved. The blood samples required for analysis by blood culture and PCR techniques were obtained accompanying with other necessary sampling during diagnostic evaluation. Therefore, no mental and physical burdens and additional costs were undergone by the patients.

The selected pediatric population had clinical evidence of probable bacterial sepsis with or without infections foci from whom blood sample was being drawn for the culture. No additional blood was collected from the children for the purposes of PCR analyzes. Blood specimens for culture and PCR were taken in a sterile style according to standard hospital protocol. Between 3.0 and 4.0 ml of whole blood was added per blood culture bottle. The bottles were incubated at 37 ºC immediately upon receipt in the microbiology laboratory and inspected visually every morning. If culture was positive the organism was identified using Gram staining and subculturing to agar plates using conventional methods. The positive growth and related isolated organisms was recorded at 48 h to 72 h.

The PCR analysis was done at a separate microbiology laboratory in the Infectious Disease Ward, with the microbiologist blind to the clinical details and the results of the blood cultures. Universal PCR may have false positive results, because shared region detected by this method exists in contaminated microorganism. So, all cares was taken to reduce the possibility of background contamination, both by carrying out manipulations in an aseptic manner and by using only sterilized consumables and solutions. Precautions taken to avoid laboratory contamination included physical separation of the pre-PCR area used for sample preparation, DNA extraction and preparation of the reaction mixture from the post-PCR areas. The staff working in the post-PCR areas did not participate in handling of the samples or PCR reagents before amplification. Biosafety hoods and positive-displacement pipettes or barriers tips were used when handling specimens and reagents to avoid carryover and amplicon contamination.

Extraction of bacterial DNA from blood specimens was performed as follows. The EDTA Vacutainer was centrifuged at 400×g to separate plasma from blood cells. The cleared plasma was removed completely, and 10.0 mL of red blood cell lysis solution (0.155M NH_4Cl, 0.005 M $MgCl_2$, 10 mM $NaHCO_3$ pH 7.2) was added to remaining cell pellets. It was mixed slowly and incubated for 5 min at 37 ºC in room air.

The supernatant layer was discarded in a detergent solution (Savlon). Once more, 10.0 ml of the red blood cell lysis solution was added to suspended cells to dissolve the pellate and the sample was centrifuged as described above. The supernatant was removed, the remaining cell pellet was dissolved in phosphate- buffered saline (pH 7.2), and stored at -20 ºC. This stage allowed separating leukocytes from whole blood specimen.

The next step was consisted of white blood cell lysis and DNA extraction from leukocytes. The resuspended specimen was centrifuged at 12000×g for 10 min, the supernatant was discarded, and to the cell pellet 500 µL of leukocyte lysis solution (0.01 M Tris- HCl, 0.05 M KCl 0.45%, Nonidet P40 Tween 20 0.45%, 0.005M $MgCl_2$, proteinase K 12 µg/mL) was added. The sample was incubated at 60 ºC for 24 hr, proteinase K being activated. The proteinase K within the sample was inactivated by incubating at 95ºC for 15 min. Next, equaled phenol (pH 8) was added to the same volume of Lysate solution, and centrifuged at 8000×g for 5 min after exposure above sucrose for 10 min. The supernatant layer was transferred to another 1.5 mL micro tube. To the separated specimen, the same volume of $CHCl_3$ was added, and the solution was centrifuged as described above.

The supernatant was transferred to another micro tube using sampler. The concentration of sodium acetate was achieved up to 0.3 M in the sample by adding 3 M sodium acetate. Next, ethanol was added, the specimen was centrifuged at 12000 × g for 5 min after incubating at -20 ºC for 2 hr. The DNA precipitate was washed in 100 mL 70% ethanol, and then centrifuged at 12000×g for 5 min. The supernatant was discarded and the precipitated DNA was allowed to be dried. To dissolve the sediment, 50 µL sterile water was added and incubated at 37 ºC for 30 min. After these stages, the samples would be prepared for universal PCR amplification.

The sequences of the primers used were as follows: U1: 5′- CCAGCAGCCGCGGTAATACG-3′; and U2:5′- ATCGG (C/T) TACCTTCTTACGATTC-3′ to detect all the bacterial DNA. The primers amplify 997 pairs base of 16S ribosomal RNA (rRNA). Each prepared specimen was added to PCR mix consisted of 0.1-1µg DNA, 2.5 mM $MgCl_2$, 0.4 mM dNTP, 20 picomole primers, 1.5 units Taq DNA polymerase, and 5µL 10× PCR buffer. The sample volume was achieved up to 50 mL by sterile water.

The PCR phases were performed as follows: denaturation at 94 ºC for 30 sec, annealing at 55 ºC for 45 sec, and extension at 72 ºC at 30 sec. The predenaturation stages were performed for 5 min before being subjected to 30 rounds of amplification. The PCR product was confirmed by 1.5% agarose gel electrophoresis and visualization with ethidium bromide using ultraviolet transilluminator. Simultaneously, sterilized sterile water and extracted DNA from standard species were applied as negatives and positive controls, respectively.

The PCR product on agarose gel considered to be positive if the visualized band was in accordance to weight marker of DNA (100 bp) of original specimen. The negative and positive controlled must be negative and positive, respectively, unless the PCR tests were repeated.

The sensitivity, specificity and accuracy of the PCR were computed utilizing the blood culture result as the gold standard for bacteremia.

Clinical characteristics of each patient enrolled in the study were entered into a computerized database. The data was analyzed using statistical software SPSS version 11.5. The quantitative variables and categorical data were reported by mean ± standard deviation (SD) and relative frequency, respectively. The Chi^2 and Fisher's exact test was used for categorical data. *P* value of less than 0.05 was accepted at the level of significance.

RESULTS

The age of patients was from one month to 10 years (mean ± S.D. was 26.31 ± 29.96 months). Sixty five pediatric patients were 3 to 36 months old. The mean temperature of patients was 38.98 + 0.57 ºC (ranged from 38.5 to 40.7). Prior antibiotic treatment had been prescribed orally and parenterally for 45% and 12% of patients, respectively.

Of 100 specimens with suspected septicemia, 19 were positive by universal PCR, while 12 specimens were positive by conventional blood culture. The positive rate of PCR was significantly higher than blood culture ($P < 0.001$) (Table 1). Of the positive PCR specimens, 11 were positive for blood culture, and it was negative only for one positive blood culture of coagulase negative staphylococci (CoNS).

The most common pathogens isolated from blood culture were *Staphylococcus aureus* (3 specimens), CoNS (3 specimens) and *Pseudomonas aeruginosa* (2 specimens).

Table 1. Comparison between polymerase chain reaction and blood culture results*†

Blood culture	PCR		
	Negative	Positive	Total
Negative	80	8	88
Positive	1	11	12
Total	81	19	100

Abbreviation: PCR, polymerase chain reaction.
* Data are given as number.
† $P < 0.001$, Chi^2 and Fisher's exact test.

The sensitivity, specify and accuracy of PCR based on blood culture result was 91.67, 90.91 and 91%, respectively. The most common clinical diagnosis in the patients was pyelonephritis (24 episodes), pneumonia (22 episodes), meningitis (7 episodes) and gastroenteritis (7 episodes). The clinical diagnosis was shown in Table 2 by results of blood cultures and PCR tests.

Table 2. Clinical diagnosis as origin of suspected blood stream infections and fever by results of polymerase chain reaction blood cultures*

Origin of infection/fever	Blood culture					
	Negative			Positive		
	Negative PCR	Positive PCR	Total	Negative PCR	Positive PCR	Total
Fever and seizure	2	0	2			
Pneumonia	19	1	20	0	2	2
Meningitis	5	0	5	0	2	2
Pericarditis	1	0	1			
Preorbit cellulites	1	0	1			
Pyelonephritis	21	1	22	0	2	2
Sepsis	17	4	21	1	0	1
Endocarditis				0	1	1
Septic arthritis	3	0	3	0	2	2
Gastroenteritis	4	1	5			
Osteomyelitis				0	1	1
Abscess	3	0	3			
Brucellosis	1	0	1			
Pharyngitis	1	0	1			
FUO	2	0	2			
Septic shock	0	1	1			
Salmonellosis				0	1	1
Total	80	8	88	1	11	12

Abbreviation: FUO, fever of unknown origin.
* Data are given as number.

DISCUSSION

This study was designed to compare the utility of a 16S rRNA PCR assay to that of the conventional culture for detecting bacteria in blood obtained from febrile children suspected of having bacterial sepsis prospectively. The comparison of 100 consecutive paired blood samples revealed a high level of agreement between the two methodologies, with sensitivity, specificity, and positive, negative predictive values and accuracy of 91.67, 90.91, 57.89, 98.76 and 91% respectively, for PCR. The high negative predictive value that was calculated for the PCR assay compared to that of culture is indicative of the assay's usefulness in accurately ruling out the diagnosis of bacterial sepsis in the uninfected febrile children admitted to the infectious diseases ward for such an evaluation.

There are numerous surveys of PCR-based assays for detecting bacteria in blood, including *Streptococcus pneumoniae* DNA from whole blood (10) and coagulase-negative *Staphylococcus* spp. from blood culture bottles (3). A different PCR-based assay that detected *Candida* spp. DNA directly from 26 of 27 blood samples obtained from neonates with culture-proven candidemia was developed (11). Another study illustrated the close agreement between PCR and bacterial culture in 15 of 16 culture-positive amniotic fluid samples obtained from women in premature labor (12). In another study, among the 33 newborn infants classified as being at risk for early-onset sepsis, was shown the PCR was able to detect the 16S rRNA gene in all four of the culture-proven sepsis cases, as well as in two samples with negative culture results. A PCR assay using primers which recognize an 861-bp fragment of the 16S rRNA gene was suggested for use in triaging bacterial sepsis (13). That study revealed the successful amplification of the rRNA gene from 12 different species of bacteria, including gram-negative and gram-positive organisms, without amplifying human genomic DNA. In another survey, the rate of bacterial detection was 25 and 22 for the use of universal PCR and blood culture (14). In that study, it was probable that six patients had bacteremia with negative blood culture and two patients were false positive PCR based on clinical context. In a study suspected neonatal septicemia, 27 episodes (4.9%) were PCR positive, while BACTEC system was detected 25 (4.5%) bacterial sepsis (12). In another study, PCR was positive in 92% of documented positive culture with detection of six new species (15). In a study of suspected Staphylococcal bacteremia in neonates, PCR targeting 16S rRNA and blood culture were positive for 13 and 9 episodes, respectively (16). PCR assay was shown to detect pneumococcal bacteremia in 12 patients out of 21 positive blood culture of *Streptococcus pneumoniae* and 206 specimens out of 459 negative blood cultures (17). In another study, PCR amplification and microarray hybridization in 16S rRNA gene was positive in 17 specimens (9.88%) out of 172 suspected bacterial sepsis, while positive blood culture was yielded in 8 specimens (4.6%).

More recently, a clinical model for predicting outcomes was developed for young infants which includes laboratory and diagnostic markers (18). A similarly based algorithm containing a PCR-based assay for detecting the 16S rRNA gene in blood specimens might be used as an effective diagnostic tool in rapidly identifying uninfected term infants. The approximate time required to test neonatal blood for bacterial 16S rRNA gene is roughly 9 h. This includes 5 h of incubation, followed by 1.5 h for sample preparation, 2 h for 28 cycles of DNA amplification, and 0.5 h for gel electrophoresis of the amplified PCR master mix (19).

Several previous studies established the usefulness of PCR in diagnosis of septicemia, targeting universal bacterial and fungal genes were shown in Table 3. The use of PCR to amplify the region of 16S rDNA in leukocyte appeared to reliably detect the presence of bacteria in the blood-stream in a significant number of patients who were missed using conventional blood culture techniques. Depending on the technique used to extract the DNA and the population studied, the yield could be PCR could have different sensitivity and time requirement to detect an organism (14, 9). We speculate that in the PCR-positive, blood culture-negative group, PCR detected bacteremic episodes of organisms that may or may not have been nonviable. However, irrespective of viability, they were still

Table 3. Reported studies of the use polymerase chain reaction in diagnosis of septicemia*

Ref. No.	Organism	Sample size†	Sensitivity	Specificity	PPV	NPV	Accuracy
Current Study	Bacterium	100	91.67 (11/12)‡	90.91 (80/88) ‡	57.89 (11/19) ‡	98.76 (80/81) ‡	91 (91/100) ‡
20	Staphylococcus	124	69.2	100	100	98	-
10	Bacterium	61	78	100	100	83	-
21	Fungus	70	77	77	43	93.6	-
22	Candida	61	95	95	90	97	-
20	Bacterium	33	100	93	67	100	-
17	Bacterium	101	87	65	-	-	-
18	Bacterium	548	96	99.4	88.9	99.8	-
13	Meningococcus	4113	88.4	100	-	-	-
23	Meningococcus	-	83-100	87-100	-	-	-
24	Streptococcus	114	55	100	-	-	-
16	Staphylococcus	215	69.2	100	100	98	-
17	Pneumococcus	480	57	55	-	-	-
9	Bacterium	172	100	97.85	-	-	97.9

Abbreviations: PPV, positive predictive value; NPV, negative predictive value.
*Data are given as percent unless specified otherwise.
†, number.
‡ number detected/total.

present in sufficient numbers in the bloodstream to trigger the clinical septic state. The question arises whether the bacteria detected by PCR and/or blood cultures are false positives or true positives. Because there is no true "gold-standard" test, we adopted the usual approach of interpreting the bacterial detection (PCR and blood culture) data in the context of the overall clinical picture and progression of the disease. Although this is somewhat subjective, there appears to be no alternative method of deciding the meaning of the microbiological and PCR results. In current study, it seems that the major positive PCR with negative blood culture were true positive, as the six patients had have prior antibiotic treatment, the PCR technique was performed under sterile conditions, and also the clinically sepsis (4 cases), septic shock, pneumonia, pyelonephritis and gastroenteritis (each one case) were suggested for them (Table 4).

Because of the extreme sensitivity of the PCR technique, any contamination occurring during collection of the blood specimen or in the laboratory processing will lead to false positives. The routine use of positive and negative controls is necessary to reliably rollout laboratory contamination. Contamination resulting from poor sample collection is difficult to exclude except based on the usual clinical criteria that are used to interpret the results of blood culture. We did not attempt a formal statistical comparison of PCR and blood culture because no true gold standard exists by which to establish the true sensitivity and specificity of the test. In addition, the main deficiency is that nonviable bacterial fragments are probably cleared from the circulation relatively quickly and thus may be missed by only one or two samples. This has been demonstrated clearly in conventional blood culture techniques, in which the bacteremias may be transient and unrelated to pyrexial episodes.

Table 4. Clinical and demographic characteristics of patients with positive PCR and negative blood culture

Age	Sex	T ºC	Clinical diagnosis	Antibiotic therapy
13	M	38.5	sepsis	PO
32	F	38.5	pyelonephritis	PO
22	F	39	sepsis	PO
9	M	38.5	gastroenteritis	PO
13	M	38.5	sepsis	No
6	M	40.5	pneumonia	IV/IM
13	F	39.5	sepsis	PO
6	M	38.5	septic shock	No

Abbreviations: T, temperature; M, male; F, female; PO, per oral; IV, intravenous; IM, intramuscular

We speculate that if a screen of four or six blood samples were taken over a 24-hr period in all patients with sepsis, recurrent bacteremic episodes would be detected in most cases. Failure to do this was a deficiency in our study. As similar to many studies, another limitation of our survey was universal PCR can identify whether bacteria is present, and for further differentiate between different bacterial species no more hybridization stages were performed. Further investigation is required to distinguish among multiple bacterial strains in clinical samples. To our knowledge, the cost-effectiveness of the molecular techniques such as PCR has not been evaluated for helping management of febrile patient suspected septicemia. Also, a meta-analysis systematic review needs to determine the role of PCR methods in diagnostic approach. Although it has been shown that PCR techniques can improve diagnosis of infectious diseases they may be used as screening, adjunctive or documenting diagnosis based on the accessible laboratory settings. However, blood culture remains as gold standard technique for diagnosis of bacteremia in clinical practice. Although our study was not set up logistically for rapid analysis, PCR can potentially give a result sooner than blood culture techniques, leading to the institution of early appropriate antimicrobial therapy.

Conflict of interests

The authors declare that they have no competing interests.

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