Intensive Care Med (2001) 27: 1269–1273
DOI 10.1007/s001340100981

ORIGINAL

James Sleigh
Ray Cursons
Mary La Pine

Detection of bacteraemia in critically ill patients using 16S rDNA polymerase chain reaction and DNA sequencing

Received: 2 November 2000
Final revision received: 13 March 2001
Accepted: 23 April 2001
Published online: 3 July 2001
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The work was carried out in the Department of Intensive Care, Waikato Hospital and Department of Biological Sciences, Waikato University, Hamilton, New Zealand.

We acknowledge the financial assistance of the Waikato Medical Research Foundation.

J. Sleigh (✉) · M. La Pine
Department of Intensive Care,
Waikato Hospital, Pembroke Street,
Hamilton, New Zealand
E-mail: sleighj@hwl.co.nz
Phone: +64-7-8398899
Fax: +64-7-8398761

R. Cursons
Dept Biological Sciences,
Waikato University, Hamilton,
New Zealand

Abstract *Objective*: 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.
Design: Observational study.
Setting: Fourteen-bed, level 3 intensive care unit.
Patients: Hundred twenty-six critically ill adult patients. Hundred ninety-seven blood culture and PCR samples taken as clinically indicated for suspected sepsis, according to routine ICU protocol.
Measurements and results: The PCR product (16SrDNA: 341F–1195R) was sequenced and compared with a database of known species (Genebank) to identify the bacterial nucleic acid. The PCR or blood culture result was classified as a true-positive if there was other microbiological or clinical supporting evidence.

Keywords Polymerase chain reaction · Sepsis · Intensive care

Introduction

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 septic patients. In ICU 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 [1, 2, 3, 4, 5, 6]. 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. In a previous study we found that PCR techniques using these 'generic' or 'universal' 16S sequences showed promise as being more sensitive than conventional blood culture (BC) techniques [7]. However, the clinical interpretations of the results from the study were

limited by the fact that we were not able to determine the species of bacteria present.

In this present study, therefore, we have attempted to perform nucleic acid sequencing on the PCR products and then compared the sequence with an electronic database of genetic sequences (Genebank) to identify the causative bacterial species. The aims were to (1) confirm the sensitivity of the PCR technique (versus BCs) and (2) gain a better understanding of the incidence of true- and false-positive results when using this technique.

Methods

Clinical methods

We report on an observational study in which we have reviewed the results from blood samples that were taken, in a sterile manner, from adult ICU patients and then sent for PCR and BC. The study was approved by the regional ethics committee. The samples were taken as clinically indicated for suspected sepsis according to our routine ICU protocol. A peripheral blood sample was taken when the patient was suspected of having a septic episode. This was defined as the development of a new fever (> 38.5 °C), development of a new organ failure or significant organ functional deterioration. If indwelling lines were suspected as a cause for the sepsis, additional samples were taken from the suspicious line.

The analysis of the PCR was carried out, at a laboratory distant to the ICU, by one of the authors (RTC), who was blinded to the clinical diagnosis and the results of the BCs. At the time of taking the blood samples, the severity of sepsis of the patient was quantified using the temperature, white cell count and modified SOFA score [8]. The SOFA score differed slightly from that published as we excluded the bilirubin component to the score. We did this because, in a large study, Moreno et al. found that no independent contribution could be associated with the hepatic score [9]. The blood cultures were processed in the manner routine to the microbiological department of our hospital (BACTEC 9000 system, Becton Dickinson Microbiological Systems, Sparks, Maryland).

Polymerase chain reaction technique

The possibility of residual laboratory bacterial DNA contamination in all reagents was eliminated using type II restriction enzymes (Alu1 and Rsa1). This was confirmed using a negative control in all runs. All chemicals were of molecular biology grade and the filter sterilised (not autoclaved, which will lyse many bacteria). The laboratory process was as follows.

Extraction of the bacterial DNA from leucocytes (WBC) and removal of polymerase inhibitors

Four hundred microlitres of EDTA chelated blood was added to 900 μl of red blood cell lysis solution. The red blood cells were lysed by NH_4Cl and the WBC (and any bacteria present in the plasma) separated from the rest of the sample by centrifugation at 15 kG/5 min. Heme (a potent polymerase inhibitor) was reduced in the residual (100 μl) WBC/bacterial pellet using 15 μl of H_2O_2 for 5 min at room temperature. The pellet of cells was then resuspended and 300 μl of lysing solution (0.1 M TRIS pH 9.0; 50 mm EDTA; 1 % sodium dodecyl sulphate (SDS); 0.1 M NaCl) added. This solution was incubated at 75 °C/15 min; frozen at –70 °C/15 min; heated at 75 °C/5 min and then at 95 °C/10 min. Thereafter, 300 μl of 5 M LiCl was added to salt-out SDS and proteins, followed by 700 μl $CHCl_3$. The biphasic solution was emulsified and mixed for 15 min at room temperature. The solution was then centrifuged at 10 kG/10 min and the aqueous solution containing the DNA mixed with an equal volume of isopropanol. DNA was precipitated for 1 h, centrifuged at 16 kG/15 min, washed in 70 % ethanol and finally resuspended in 100 μl of trisEDTA (TE) (10 mM TRIS; 1 mM EDTA, pH 8.0).

DNA amplification cycles

For PCR all reagents (buffers, goldtaq enzyme, primers) were predigested for 2 h at 37 °C with Alu1/Rsa1 (0.2 U of each) restriction enzymes. The restriction enzymes were then heat-killed at 65 °C/20 min, 5 μl of DNA template was added and then amplified using 94 °C/5 min to activate the polymerase enzyme. This was followed by [94 °C/20 s; 55 °C/20 s; 72 °C/45 s] × 50 cycles. PCR products were then analysed by electrophoresis using a 2 % agarose gel and trisborateEDTA (TBE) buffer followed by staining with ethidium bromide.

Extraction and sequencing of polymerase chain reaction products

Samples containing a band at the correct position were precipitated with an equal volume of 25 % polyethylene glycol 8000/0.6 M sodium acetate/10 mM $MgCl_2$, washed twice in 100 % ethanol and resuspended in TE to give 10 ng/μl. Of this template DNA, 1.5 μl was then cycle sequenced using the sequencing primer. The primer sequences were:

1. Forward: 341F/ CCTACGGGAGGCAGCAG
2. Reverse: 1195R/ GACGTCRTCCNCDCCTTCCTC where R = (A/G), D = (A/G/T) and N = (A/C/G/T)
3. Sequencing primer: 519F/ CAGCAGCCGCGGTAATAC

The initial primers are designed to capture the *conserved* sections (crosses in Fig. 1) of the 16S gene – i.e. sections that are found in *all* bacteria. This makes the initial PCR very sensitive. The sequencing primer is used to identify the subregion of the gene – consisting of both variable and conserved sections of the gene. This is then input into the sequencer for maximum specificity. Positive (beta-microglobulin) controls were included to detect the presence of PCR inhibition.

Comparison of the sequenced polmerase chain reaction product with the database to determine the species of bacterium

The PCR product was then sequenced and the product compared with the Genebank database to determine the bacterial species (http://www.ncbi.nlm.nih.gov/Entrez/). In almost all cases the number of agreements in base-pairs exceeded 400 (and > 98 % agreement). However, because of the limited length of the variable region in the 16S gene that is available for amplification and sequencing, in about a quarter of the sequences more than one closely related species had identical base-pair agreement.

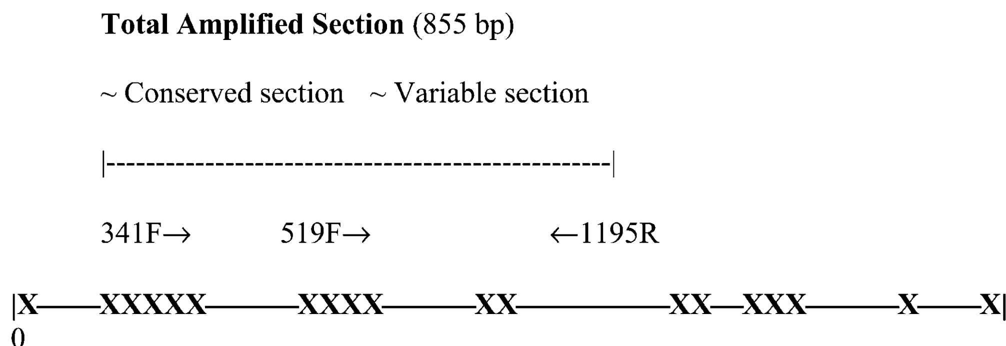

Fig. 1 A diagram of the 16S rDNA gene. (*line segments* (–) are divergent sections, and *crosses* (X) are conserved sections)

Table 1 Comparison of blood culture *(BC)* and polymerase chain reaction *(PCR)* results. The numbers considered to be true-positives are shown in brackets (*+ve* positive, *–ve* negative)

		PCR		
		–ve	+ve	TOTAL
Blood culture	–ve	121	48 (25)	169
	+ve	13 (8)	15 (13)	28 (21)
TOTAL		134	63 (38)	197

Table 2 Comparison of clinical parameters [mean(SD)] for the blood culture *(BC)* and polymerase chain reaction *(PCR)* groups (*WBC* white blood cells, *+ve* positive, *–ve* negative)

	BC		PCR	
	–ve	+ve	–ve	+ve
Temperature	38.4 (1.3)	38.7 (1.2)	38.6 (1.3)	38.1 (1.4)
WBC	14.0 (8.1)	13.6 (6.4)	14.5 (8.5)	12.2 (7.0)
SOFA score	8.2 (3.8)	7.8 (3.8)	8.3 (4.0)	8.2 (3.6)

Analysis

The positive and negative rates between the BC and PCR were compared using a two-way contingency table. We then classified the positive (BC or PCR) results as *true-positives* if there clearly existed other supporting clinical or microbiological evidence that the isolated organism was causative of the disease. This evidence could be either: (1) multiple positive blood results (either PCR and/or BC) from several different samples taken over a period in time (making sampling contamination unlikely), (2) other (non-blood) confirmatory microbiological specimens (e.g. meningococcus obtained from cerebrospinal fluid (CSF) or (3) clear clinical or radiological evidence (e.g. lobar consolidation would confirm that the *Streptococcus pneumoniae* found in the blood culture was a true-positive). If there was no supporting evidence as to the significance of the isolated organism, the result was classified as *indeterminate*.

Results

The data consisted of 197 samples obtained from 126 patients over the period 3rd March, 2000, to 10th July, 2000. The comparison of the BC and PCR results are shown in Table 1.

When assessing concordance of BC and PCR, of the 13 samples 'missed' by the PCR (PCR negative and BC positive) – 11 grew coagulase negative *Staphylococci* and two grew *Staphylococcus aureus*. Of the 48 samples "missed" by the BC (PCR positive and BC negative), 17 were sequenced as *Staphylococci*, 10 as *Streptococci*, 2 as *Meningococci* and 14 were not sequenced. The remainder were identified as various gram-negative bacteriaciae (*Escherichia coli* (2), *Stenotrophomonas maltophilia*, *Yersinia enterocolitica*, *Burkholderia cepacia*).

Of all 28 BC positive results, 21 (75 %) were classified as true-positives. Most of the rest could be attributed to probable sampling contamination or colonisation (e.g. samples taken from old central venous lines). Of all 63 PCR positive results, 38 (60 %) were classified as true-positives. Of the remaining 25 indeterminate positives, 17 were not successfully sequenced due to the presence of multiple organisms (causing multiple concurrent nucleotide sequences) or insufficient PCR product (< 15 ng). Of those classified as indeterminate that were successfully sequenced, the bacteria identified were not known to be pathogenic (e.g. *Aquaspirillium*, *Planococcus*, *Flavobacterium*) or were coagulase negative *Staphylococci* from indwelling lines with concomitant negative results from true peripheral blood samples. Of the 38 PCR results classified as true-positives of this template DNA, 10 were classified using the 'multiple positive specimens' criterion alone, 5 using the 'other microbiological specimens' criterion alone and 6 using the 'clinical or radiological evidence' criterion alone. Seventeen involved a combination of more than one criterion group.

When comparing the SOFA score, WBC and temperature between the BC positive and negative groups or the PCR positive and negative groups, there was no significant difference (Mann-Whitney U test, Table 2)

Table 3 Polymerase chain reaction (true) positive patients with corresponding negative blood culture post-antibiotics (*B* Burkholderia, *S* Staphylococcus, *E* Escherichia, *Str* Streptococcus, *Y* Yersinia, *N* Neisseria, *CNS* coagulase negative Staphylococcus, *P* Pseudomonas, *Time*: refers to the time between the institution of antibiotics and the PCR sample was taken)

Patient	Diagnosis	Outcome	Other evidence	Antibiotics	Time	PCR result
1	Pyelonephritis /septic shock	Died	Urine and pre-antibiotic BC: *E. coli*	Augmentin/gentamicin	13 h	*E. coli*
2	Post abortion/ARDS	Lived	Clinical: foul lochia at operation	Cefotetan/gentamicin	6 h	*Str. mitis*
3	Chronic renal failure/ septic shock	Died	Endotracheal *P. aeruginosa*	Meropenem	> 24 h	*B. cepacia*
4	Meningitis/septic shock	Lived	CSF: *N. meningitidis*	Penicillin	7 h	*N. meningitidis*
5	Multiple trauma	Lived	Next day BC and PCR +ve	Flucloxacillin	> 24 h	CNS
6	Fractured jaw and abscess	Lived	*Str. anginosus* from jaw wound	Cefuroxime/metronidazole	> 24 h	*Str. anginosus*
7	Streptococcal shock	Died	Pre-antibiotic BC: *Str pyogenes*	Ceftriaxone/gentamicin	12 h	*Str. pyogenes*
8	Staphylococcal shock	Died	Pre-antibiotic BC: *S. aureus*	Cefuroxime/gentamicin	8 h	*S. aureus*
9	Multiple trauma	Lived	Clinical: cellulitis and abscess	Flucloxacillin/gentamicin	> 24 h	*S. aureus*
10	Pancreatitis and sepsis	Died	Pre-antibiotic BC: *E. coli*	Meropenem	18 h	*E. coli*
11	Guillane-Barre syndrome	Lived	Multiple +ve BC later (CNS)	Vancomycin	> 24 h	CNS
12	Cellulitis, abscess and septic shock	Lived	Pre-antibiotic BC: *S. aureus*	Cefuroxime/meropenem	6 h	*S. aureus*
13	Multiple trauma and blood reaction	Lived	Pyrexia/shock: blood transfusion	Augmentin	4 h	*Y. enterocolitica*
14	Meningococcal shock	Lived	Pre-antibiotic BC: *N. meningitidis*	Ceftriaxone	6 h	*N. meningitidis*

Because our hospital is the tertiary referral unit for a large district, many of our patients had BCs taken at outlying hospitals or in our emergency department, immediately followed by intravenous administration of broad spectrum antibiotics. Often these initial (pre-antibiotic) BCs were positive, but the repeat BCs taken some time later on admission to our ICU were negative – whereas the concomitant PCR specimen *at this time was positive*. The clinical details of this group of patients are shown in Table3. These were patients who were felt to be true PCR positives, but had negative BCs due to antibiotic activity in the sample.

Discussion

The main results of our study were that generic PCR, followed by identification of the bacteria through nucleic acid sequencing, is able to detect the presence of bacterial DNA in many BC-negative samples. When correlated with other clinical and microbiological information, about two thirds of these PCR-positive results probably identify the true causative pathogenic bacteria (Tables 1 and 3).

About one third of the PCR results were classified as indeterminate and probably were, indeed, false-positives. There are at least four explanations for these observations. (1) We intentionally erred on the side of caution – being over-conservative in classifying true-positives. For example, correctly or incorrectly, we completely excluded *Planococcus* as a cause of sepsis a priori. (2) The extreme sensitivity of PCR makes the technique very susceptible to environmental contamination during the process of blood collection. Although we were able to control adequately for laboratory contamination by strict use of negative controls, it is not possible fully to prevent/detect contamination by any bacterial DNA in the process before this point. (3) It is conceivable that 'asymptomatic' bacteraemias, which result in phagocytosis of bacterial DNA by leucocytes, are not uncommon in intensive care patients who have many invasive monitoring devices [10]. (4) Infection with more than one organism is difficult to sequence. If the PCR product consists of DNA amplified from more than one species of bacterium, the sequencer detects more than one type of nucleotide base at

many sites and thus cannot produce a reliable sequence to enable identification of the species. In contrast, if the PCR product was derived from a single species there was usually very high agreement (> 98 %) with the Genebank. 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 utilising the different melting points of different PCR products. It is likely that PCR techniques could be significantly faster than conventional BC [11].

The choice and length of the amplified region influences the ability to differentiate between closely related micro-organisms. Using the 16S sequences some bacteria are indistinguishable (e.g. *Burkolderia cepacia* and *Pseudomonas aeruginosa* in patient 3, in Table 3). The use of the, more variable 16S-23S intergenic region may prove to be better in this regard, and deserves further investigation.

As has been observed in many studies, the development of organ failure (as quantified by the SOFA score) does not correlate well either with positive blood cultures or a positive PCR [12, 13, 14]. Our data support the idea that much of the pathology of the sepsis syndrome may be due to host responses.

Because there is *no gold standard* with which to compare the results of our PCR technique, there is inevitably an element of subjective clinical judgement in our false-positive/false-negative classification. Information about the presence of bacteria forms only one part of the overall clinical picture. We have attempted to accumulate data and applied clinical common sense in its interpretation. This was the basis of our classification – which is, in essence, the same as that commonly used to establish the false-positive rate for conventional BCs. In the future we believe it will be necessary to include information about the host response – such as cytokine mRNA expression.

The cases shown in Table 3 highlight the utility of the PCR in detecting the presence of bacterial nucleic acid in the leucocytes; in situations where the growth of bacteria in conventional processing has been inhibited by antibiotics. Our results are very similar to those described in a recently published experimental study [15]. In this study mice were inoculated with *Streptoccocus pneumoniae* and then treated with antibiotics. *S. pneumoniae* DNA was detected in the leucocytes of most of the animals for at least 48 h after the start of antibiotic treatment.

This study demonstrates that: (1) the universal 16S rDNA PCR is about twice as sensitive as BC in detecting bacteraemias in ICU patients – and may be especially useful in patients who have received prior antibiotics. (2) There is still a sizeable proportion of patients who are clinically septic, but without detectable bacterial DNA in their blood. (3) There is probably a significant false-positive rate when using PCR techniques, although the status of many bacterial species that have hitherto been believed to be non-pathogenic is open to question.

References

1. Jordan JA, Durso MB (2000) Comparison of 16S rDNA gene PCR and BACTEC 9240 of detection of neonatal bacteremia. J Clin Microbiol 38 (7):2574–2578
2. Greisen K, Loeffelholz M, Purohit A (1994) PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J Clin Microbiol 32: 335–351
3. Steinman CR, Muralidhar B, Nuovo GJ (1997) Domain-directed polymerase chain reaction capable of distinguishing bacterial from host DNA at the single-cell level: characterization of a systematic method to investigate putative bacterial infection in idiopathic disease. Analyt Biochem 244: 328–339
4. Hitti J, Riley DE, Krohn MA, Hillier SL, Agnew KJ, Krieger JN, Eschenbach DA (1997) Broad-spectrum bacterial rDNA polymerase chain reaction assay for detecting amniotic fluid infection among women in premature labor. Clin Infect Dis 24 (6):1228–1232
5. Jantos CA, Roggendorf R, Wuppermann FN, Hegemann JH (1998) Rapid detection of Chlamydia pneumoniae by PCR-enzyme immunoassay. J Clin Microbiol 36 (7):1890–1894
6. Seward RJ, Towner KJ (2000) Evaluation of a PCR-immunoassay technique for detection of Neisseria meningitidis in cerebrospinal fluid and peripheral blood. J Med Microbiol 49 (5):451–456
7. Cursons RT, Jeyerajah E, Sleigh JW (1999) The use of polymerase chain reaction to detect septicemia in critically ill patients [see comments]. Crit Care Med 27 (5):937–940
8. Vincent JL, Mendonca A, Cantraine F, Moreno R (1998) Use of the SOFA score to assess the incidence of organ dysfunction/failure in intensive care units: results of a multicenter, prospective study. Crit Care Med 26 (11): 1793–1800
9. Moreno RVJ, Matos R, Mendonca A, Cantraine F, Thijs L, Takala J, Sprung C, Antonelli M, Bruining H, Willatts S (1999) The use of maximum SOFA score to quantify organ dysfunction/failure in intensive care. Results of a prospective, multicentre study. Working Group on Sepsis-related Problems of the ESICM. Intensive Care Med 25 (7): 686–696
10. Garrouste-Orgeas M, Chevret S, Mainardi JL, Timsit JF, Misset B, Carlet J (2000) A one-year prospective study of nosocomial bacteraemia in ICU and non-ICU patients and its impact on patient outcome. J Hosp Infect 44 (3): 206–213
11. Laforgia N, Coppola B, Carbone R, Grassi A, Mautone A, Iolascon A (1997) Rapid detection of neonatal sepsis using polymerase chain reaction. Acta Paediatr 86 (10):1097–1099
12. Bone RC (1992) Toward an epidemiology and natural history of SIRS (systemic inflammatory response syndrome) [see comments]. JAMA 268 (24):3452–3455
13. Bossink AW, Groeneveld AB, Thijs LG (1999) Prediction of microbial infection and mortality in medical patients with fever: plasma procalcitonin, neutrophilic elastase-alpha1-antitrypsin and lactoferrin compared with clinical variables. Clin Infect Dis 29 (2):398–407
14. Jones GR, Lowes JA (1996) The systemic inflammatory response syndrome as a predictor of bacteraemia and outcome from sepsis. QJM 89 (7):515–522
15. Ng W, Olsen K, Lutsar I, Wubbel L, et al. (2000) Buffy coat PCR for the diagnosis of experimental pneumococcal pneumonia. APMIS 108: 729–733