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

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 (Genbank) 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 get 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 (AluI and Rsal). 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 NH₄Cl and the WBC (and any bacteria present in the plasma) separated from the rest of the sample by centrifugation at 15 K×g for 5 min. Heme (a potent polymerase inhibitor) was reduced in the residual (100 μl) WBC/Bacterial pellet using 15 μl of H₂O₂ 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 CHCl₃. The biphasic solution was emulsified and mixed for 15 min at room temperature. The solution was then centrifuged at 10 K×g/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 K×g/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, goldaq enzyme, primers) were pre-digested for 2 h at 37°C with AluI/RsaI (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 mM sodium acetate/10 mM MgCl₂, 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/ GACGCTCCACCTTCTCCCT where R = (A/G), D = (A/G/T) and N = (A/C/G/T)
3. Sequencing primer: 519F/ CAGACGTCGCGTAATAC

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 polymerase 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.