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DETECTION OF BACTERIAL DNA BY PCR AND REVERSE HYBRIDIZATION IN THE 16s rRNA GENE*

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Abstract: The clinical diagnosis of sepsis is difficult, particularly in neonates. To devise a rapid and reliable method for identifing bacteria in blood and cerebrospinal fluid (CSF), we developed a pair of primers according to the gene encoding 16 s rRNA, found in all bacteria. DNA fragments from different bacterial species and from clinical samples were detected with polymerase chain reaction (PCR), and with reverse hybridization using a universal bacterial probe, a gram-positive probe and a gram-negative probe. Our results showed that a 371 bp DNA fragment was amplified from 20 different bacterial species. No signal was observed when human DNA and viruses were used as templates. The sensitivity could be improved to 10^{-12}g. All 26 culture-positive clinical samples (22 blood samples and 4 CSF samples), were positive with PCR. The gram-negative and gram-positive probe hybridized to clinical samples and to known bacterial controls, as predicted by Gram's stain characteristics. Our results suggest that the method of PCR and reverse hybridization is rapid, sensitive and specific in detecting bacterial infections. This finding may be significant in the clinical diagnosis of sepsis in neonates.

Key words: 16s, rDNA, rRNA PCR, bacterial infection, hybridization, neonate
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INTRODUCTION

Evaluation of the patient with symptoms of sepsis requires a combination of clinical acumen and laboratory support. The clinical diagnosis of bacterial infection may be difficult in certain patients, such as neonates (Baker et al., 1990). When sepsis is suspected, many patients, especially neonates, are hospitalized for a period of observation and for administration of intravenous antibiotics before culture results are available. In many cases, cultures are negative (Jones et al., 1993). Thus, a rapid, reliable method for identifing bacteria in blood and other body fluids could dramatically reduce hospitalizations and medical costs.

Although polymerase chain reaction (PCR) has been used to detect pathogens, most PCR detection systems are not suitable for the detection of pathogens in sterile clinical sites, such as blood and cerebrospinal fluid (CSF), because of the large number of possible pathogens. However, certain DNA sequences are present in all bacteria and are remarkably similar among microbes (Angert et al., 1993). In this paper, we describe the use of PCR plus reverse hybridization in detecting and identifing bacteria. Specifically, we have used conserved 16s rRNA gene primers, gram-positive and gram-negative probes, in assessing bacterial infection and in discriminating between gram-positive and gram-negative bacteria in 26 culture-positive clinical samples.

MATERIALS AND METHODS

Bacterial Strain and Clinical Specimens

We used 7 gram-positive bacterial strains and 13 gram-negative strains as positive controls in this study (Table 1). For negative controls we used total human DNA, cytomegalovirus (CMV), hepatitis B virus (HBV) and the Epstein-Barr virus (EBV). We collected 22 blood specimens and 4 CSF samples from patients with

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Table 1 Results of PCR amplification and reverse hybridization with 20 types of bactoria

Organism(strain) *		PCR Amplification	Probe		
			Universal	Gram +	Gram −
Gram-positive(n = 7)					
Staphylococcus aureus	(SS279)	+	+	+	−
Staphylococcus epidermidis	(SS303)	+	+	+	−
Streptococcus pneumoniae	(ATCC33400)	+	+	+	−
Streptococcus salivarius	(ATCC13419)	+	+	+	−
Streptococcus sanguis	(ATCC10556)	+	+	+	−
Bacillus subtilis	(ATCC6051)	+	+	+	−
Micrococcus varians	(SS148)	+	+	+	−
Gram-negative (*n* = 13)					
Escherichia coli	(ATCC11775)	+	+	−	+
Enterbacter aerogenes	(ATCC13048)	+	+	−	+
Proteus mirabilis	(SS1003)	+	+	−	+
Klebsiella pneumonia	(ATCC13883)	+	+	−	+
Citrobacter freundii	(ATCC8090)	+	+	−	+
Pseudomonas aeruginos	(ATCC10145)	+	+	−	+
Haemophilus influenzae	(ATCC33391)	+	+	−	+
Serratia marcescens	(SS987)	+	+	−	+
Shigella dysenteriae	(SS777)	+	+	−	+
Salmonella typhimurium	(SS913)	+	+	−	+
Salmonella typhi	(SS920)	+	+	−	+
Acinetobacter Iwoffi	(SS801)	+	+	−	+
Aeromonas hydrophila	(ATCC7966)	+	+	−	+

* ATCC strains were obtained from the American Type Culture Collection. Strains designated SS were obtained from the clinical collection, Children's Hospital, Zhejiang University, Hangzhou, PR China.

suspected bacterial infection. Bacteria were detected by culture of blood and CSF.

DNA isolation

Bacteria used for positive controls were cultured overnight at 37 ℃, scraped and lysed in a mixture of 0.03% sodium dodecyl sulfate、1% Tween 20, and 1% Nonidet p-40, for 5 min at 95 ℃. The sample was centrifuged for 10 s, and 5 ul of the supernatant was used for PCR amplification. DNA was isolated from blood samples by lysing red blood cell with five volumes of lysis buffer (0.3 mol/L sucrose, 10 mmol/L Tris-HCl pH7.5, 5 mmol/L $MgCl_2$, and 1% Triton - 100). Leukocytes were pelleted, resuspended in proteins K buffer (0.7% SDS, 75 mmol/L NaCl, 25 mmol/L EDTA at pH 8.0 and 0.1 μg/μl proteinase K) and digested over night at 37 ℃. Lysates were extracted with phenol and chloroform and precipitated with ethanol. After centrifugation, the nucleic acid was suspended in distilled water for PCR amplification. CSF samples were centrifuged for 5min in a microphage. Then, the supernatant was poured off and the last drops were removed with paper towels. The pellet was resuspended and the steps outlined above were followed.

Synthesis of biotinylated primers

We converted tetraethylene glycol to the monophthalimido derivative by reaction with phthalimide in the presence of triphenylphosphine and diisopropyl azodicarboxylate. The monophthalimide was converted to the corresponding β-cyanoethyl diisopropylamino phosphoramidite by standard protocols. The resulting phthalimido amidite was added to the 5' ends of the primers during the final cycle of automated DNA synthesis by using standard coupling conditions. During normal deprotection of the DNA (concentrated aqueous ammonia for 5hr at 55 ℃), the phthalimido group was converted to a primary amine, which was subsequently acylated with an appropriate biotin active ester. NHS-LC-biotin was selected because of its water solubility and lack of steric hindrance. The biotinylation

was performed on crude, deprotected oligonucleotide, and the mixture was purified by a combination of gel filtration and reverse-phase HPLC.

Tailing of probes

A universal bacterial probe, a gram-positive probe and a gram-negative probe were synthesized on a DNA synthesizer (model 8700, Bioserarch) with β-cyanoethyl N, N-diisopropyl phosphoramidite nucleosides according to the manufacturer's instructions. The probe (200 pmol) was tailed for 60 minutes at 37 ℃ in a buffer of 100 μl of 100 mmol/L potassium cacodylate/25 mmol/L Tris·HCl/1 mmol/L $CoCl_2$/0.2 mmol/L dithiothreitol, pH 7.6, with 100 nmol deoxyribonucleoside triphosphate (dTT) and 60 units (50pmol) of terminal deoxyribonucleotidyltransferase (TdT) (Promega, Madison, WI). The tailing reactions was stopped by addition of 100 μl of 10 mmol/L EDTA.

Preparation of nylon membranes

The tailed probes were diluted in 100 μl of TE(10 mmol/L Tris·HCl/0.1 mmol/L EDTA, pH 8.0) and applied to a nylon membrane(Zeta-probe) with a spotting manifold (BioDot, Bio-Rad). The damp nylon membrane was placed on TE-soaked paper pads and irradiated for 10 min at 254 nm with UV light and then washed in 200 ml of 5 SSPE(1 × SSPE is 180 mmol/L NaCl/10 mmol/L NaH_2PO_4/1 mmol/L EDTA, pH 7.2) with 0.5% SDS for 30 min at 55 ℃ to remove unbound probes.

DNA amplification

The sequences of primers are shown in Table 2. The PCR mixture (50 μl) contained 50mmol/L Tris-HCl(pH 9.0), 50 mmol/LKCl, 7 mmol/L $MgCl_2$, 2 mg/ml bovine serum albumin, 16 mmol/L $(NH_4)_2SO_4$, 100 μmol/L (each) primer, and 1.0 U of Taq polymerase(Boehringer, Mannheim, Germany). The PCR mixture was incubated with 0.5 μg of DNase I, which is active on double-stranded DNA (dsDNA, Boehringer, Mannheim, Germany) for 15 min at room temperature.

The DNase was inactivatied by incubating the mixture for 10min at 95 ℃, Then, 5 μl of the cell lysate, which contained the target DNA, was added to the PCR mixtrue. PCR was performed for 32 cycles of 1 min at 94 ℃, 15s at 72 ℃, and 45 s at 55 ℃ on a DNA thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). After amplification, the samples were incubated an additional 5min at 72 ℃.

Table 2 Sequences and location of primers and probes

Name	Sequences(5'-3')	Location
Primer 1	TGCGGTTGGATCACCTCCT	1521 - 1539
Primer 2	TCCCCACCTTCCTCCAGTT	1187 - 1169
Universal probe	CGGTGAATACGTTCCCGGGCCTTGTAC	1368 - 1394
Gram-positive probe	GACGTCAAATCATCATGCCCCTTATGTC	1189 - 1216
Gram-negative probe	GACGTAAGGGCCATGATGACTTGACGTC	1189 - 1216

Reverse hybridization and detection of amplified DNA

Each nylon membrane with three bound probes was placed in 4ml of hybridization solution containing 5 × SSPE, 0.5% SDS and 400ng of streptavidin-horseradish peroxidase conjugate (See Quence, Eastman Kodak). The PCR-amplified DNA (10 ul) was denatured by adding an equal volume of 400 mmol/L NaOH and 10 mmol/L EDTA immediately to the hybridization solution, which was then incubated at 55℃ for 30 min. The nylon membranes were briefly rinsed twice in 2 × SSPE/0.1% SDS at room temperature and washed once in 2 × SSPE/0.1% SDS at 55 ℃ for 10 min, finally, briefly rinsed twice in 2 × PBS (1 × PBS: 137 mmol/L NaCl/2.7 mmol/L KCl/ 8mmol/L Na_2HPO_4/1.5 mmol/L KH_2PO_4, pH 7.4) at room temperature. The color was developed by incubating the nylon membranes in 25 - 50 ml of red leuco dye at room temperature for 5 - 10 min.

RESULTS

Specificity of primers and sensitivity of the PCR assay

To verify that the primers used in this study were specific for bacteria (20 species), human

DNA、CMV、HBV and EBV were tested. Amplified products of 371 bp were detected from bacterial DNA, but not from human DNA、CMV、HBV and EBV. Therefore, our primers did not cross react with DNA from human and viruses, and were highly specific for bacteria. To examine the lower limit for detection of bacteria, PCR was performed on serial 10 – fold dilutions of DNA from E.coli. We could detect a band with template amounts as low as 1 pg (Fig.1), which is equal to the amount of DNA contained in approximately 3 organisms(Shirai et al.,1991).

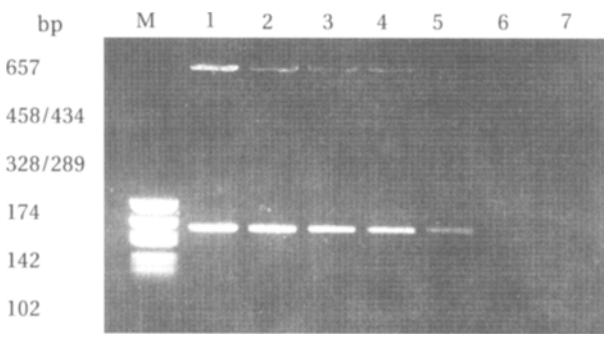

Fig.1 Sensitivity of PCR. Serial dilutions of DNA from *E coli* were tested with 32 cycle PCR. Lanes 1 to 7, 10 ng, 1ng, 100 pg, 10 pg, 1pg, 100 fg, 10 fg of DNA from *E. coli*, respectively; lane M, ds DNA molecular size markers

Reverse hybrization efficiency of tailed probes

Of the 20 types bacteria tested a tested, all hybridized with the universal probe (Table 1). Both gram positive probe and gram negative probe were specific. DNA from all 7 types of gram positive bacteria hybridized to the gram positive probe, but not to the gram negative probe. Similarly, DNA from the 13 gram negative species hybridized only to the gram negative probe.

Testing of clinical specimens

The amplified products of the expected size (371bp) were detected from all 26 clinical samples tested in our reverse hybridization assay (Table 3). No amplified products were seen when 30 blood samples from healthy children were tested.

DISCUSSION

Although PCR amplification of bacterial DNA is rapid and sensitive, most current methods are too specific to be used for initially evaluating the possibility of bacterial infection when the identity of the organism is not known. The specific sequence of the 16s ribosomal RNA (16S rRNA) gene was used recently to detect, identify, and classify bacteria (Riffard et al., 1998. Mccabe et al., 1995. Saruta et al.,

Table 3 Culture, PCR and reverse hybridization results obtained with 22 blood samples and 4 CSF samples

Organism*	PCR Amplification	Probe		
		Universal	Gram+	Gram–
Blood (n = 22)				
Staphylococcus epidermidis (n = 5)	+	+	+	–
Corynebacterium aquaticum (*n* = 2)	+	+	+	–
Streptococcus pyogenes (*n* = 1)	+	+	+	–
Enterbacter aerogenes (*n* = 1)	+	+	–	+
Haemophilus influenzae (*n* = 1)	+	+	–	+
Klebsiella pneumonia (*n* = 3)	+	+	–	+
Escherichia coli (*n* = 1)	+	+	–	+
Enterobacter agglomerans (*n* = 1)	+	+	–	+
Proteus mirabilis (*n* = 1)	+	+	–	+
Staphylococcus aureus (*n* = 5)	+	+	+	–
Enterococcus faecalis (*n* = 1)	+	+	+	–
CSF (*n* = 4)				
Staphylococcus epidermidis (*n* = 2)	+	+	+	–
Staphlococcus saprophyticus (*n* = 1)	+	+	+	–
Enterbacter aerogenes (*n* = 1)	+	+	–	+

* Organism identified on culture

1995). This sequence was chosen for several reasons. Every bacterial 16s rRNA gene consists of approximately 1,500 nucleotides and includes several highly conserved regions(Relman et al., 1993). Universal or broad range primers, can be designed from the conserved region of 16s rRNA gene sequences. When used in combination with the species-specific primers or probes from the species-specific region of 16s rRNA gene sequences, universal primers can identify pathogens taxonomically. In this study, we developed a pair of primers against all types of bacteria by analysing the 16s rRNA gene with the discs of SEQNCE, PRIMERS and PCR DESN. We amplified a 371 bp DNA fragment from 20 different types of bacteria. Our primers did not cross-react with human or viral DNA. Furthermore, our amplification method was sensitive; serially diluted DNA from E. coli was seen on an ethidium bromide stained gel with template amounts as low as 1 pg, which is the amount of DNA seen in about three organisms. In addition, we developed three probes: a universal probe for all bacteria, a gram-positive probe, and a gram-negative probe. We were able to differentiate gram-positive and gram-negative organisms using our probes. The unversal probe recognized amplified DNA from all bactcrial species tested. These results indicate that our method had excellent specificity and sensitivity.

Effective treatment of bacterial infections often requires rapid and accurate detection and identification of bacteria in sterile body fluids, such as blood and CSF. The most sensitive method currently used to detect bacteria is culture, which requires at least an eight-hour incubation and biochemical tests to identify the bacterium. More time may be required to identify slow growing organisms or organisms present in low numbers. Because mortality associated with untreated bacteremia or bacterial meningitis is high, empiric or prophylactic antibiotic therapy may be prescribed. This practice can reduce the chance of a positive culture at a later time. In this study, we used PCR and reverse hybridization on clinical samples to help overcome the temporal disadvantage of culture. After PCR, we detected the 371 bp DNA fragment in all 26 culture-positive clinical samples. The gram-positive and gram-negative probe hybridized appropriately to all clinical samples, as indicated by gram's stain characteristics. We described our assay as a reverse hybridization because the probes (universal、gram-positive、gram-negative), and not the PCR product , were immobilized on a solid support (a nylon membrane). This format would allow for multiple probes to be used on a single blood or CSF sample. Amplification and analysis of bacterial DNA in blood or in CSF could be completed in six hours or less, which is significantly less than the time required for culture. Because the regions of the 16s rRNA gene sequences are highly conserved among all bacteria, PCR amplification will permit identification of both common and uncommon pathogens. Less common pathogens often require unusual culture conditions, so our method would improve not only the speed, but also the spectrum of clinical bacteriological examinations.

In conclusion, we have used the technique of PCR plus reverse hybridization to detect and identify bacteria in clinical samples. This method provides useful information for bacterial identification and takes only 6 hours to obtain a diagnosis.

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