

# Detection of Bacterial Pathogens in Cerebrospinal Fluid using Restriction Fragment Length Polymorphism

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## Abstract

**Background:** Polymerase chain reaction (PCR) is useful for rapid microbial detection in body fluids with low microbial load. It is easier to use universal or broad range primers for the amplification of conserved stretches of DNA common to all bacteria like 16S rRNA gene, followed by restriction fragment length polymorphism (RFLP) of PCR products.

**Methods:** Forty samples of cerebrospinal fluid were collected. After DNA extraction, universal or broad range PCR was performed using two universal primers U1-5'-CCAGCAGCCGCGGTAATACG-3', corresponding to nucleotides 518 to 537 of the *Escherichia coli* 16S rRNA gene, and U2 - 5'-ATCGG(C/T)TACCTTGTTACGACTTC-3', corresponding to nucleotides 1513 to 1491 of the same gene. The PCR product was subjected to digestion by endonucleases- *HaeIII*, *Mn11*, *BstB1* and *Alu1*. Restriction pattern obtained was compared with that of standard organisms to identify the pathogen. The results were compared with conventional methods.

**Result:** Universal PCR could detect pathogens in 20% samples within 13-18 hours as compared to 16% by conventional methods. The analytical sensitivity was 10 Gram negative and 250 Gram positive organisms per 200 µl sample. Overall sensitivity was 83.3% and specificity was 91.2%.

**Conclusion:** Universal PCR followed by RFLP of PCR product is a good alternative to conventional diagnosis of bacterial pathogens.

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**Key Words :** Body fluids; Polymerase chain reaction; Restriction fragment length polymorphism

## Introduction

Conventional methods of bacterial pathogen detection are time consuming and an optimal etiological concentration of 10<sup>5</sup> cfu/ml is often required to achieve a reliable positive result. An extensive study over a period of 27 years revealed that culture might miss the diagnosis of bacterial meningitis in at least 13% of cases [1,2]. Regardless of the type of organism in the CSF; the percentage of positive microscopic results is only 25% with < 10<sup>3</sup> cfu/ml and 60% in the range of 10<sup>3</sup> to 10<sup>5</sup> cfu/ml [3]. Nucleic acid amplification technology using polymerase chain reaction (PCR) has opened new possibilities for rapid microbial detection and characterization. Many primer sets have been developed to detect species-specific sequences in simple PCRs [4-6]. However the use of species-specific primers is impractical for routine analysis of clinical samples that may contain several different pathogens. Therefore it is easier to use universal or broad range primers for the amplification of conserved stretches of DNA followed by sequence analysis of the PCR products [7], or by restriction fragment length polymorphism (RFLP) [8].

Since the 16S rRNA genes are common and conserved in all bacteria, it is possible to design PCR primers capable of amplifying this DNA [8]. The universal or broad range PCR followed by RFLP is a new and effective method of detection of bacterial pathogens in normally sterile body fluids. Most of the “culture-resistant”, fastidious, inactivated or slow-growing bacteria can be identified within 18 hours with the use of one restriction enzyme and the rest with the use of another three enzymes.

## Material and Methods

Forty CSF samples were collected during the study period from patients suspected of having meningitis. All samples were subjected to the conventional methods of pathogen identification. An aliquot (200 µl) was processed for DNA extraction with QIAamp DNA Mini Kit (QIAGEN Inc., Chatsworth, Calif) as per the protocol provided by the manufacturer. The eluted DNA was stored at -20°C.

One pair of primers, designated U1 and U2, with sequences conserved among all bacteria under the study was selected. The sequence of primer U1 was 5'-CCAGCAGCCGCGGTAATACG-3', corresponding to

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nucleotides 518 to 537 of the *E. coli* 16S rRNA gene, and that of U2 was 5'-ATCGG(C/T)TACCTTGTT ACGACTTC-3', corresponding to nucleotides 1513 to 1491 of the same gene. PCR performed with these two primers is referred to as the universal PCR or broad range PCR in this study.

A reaction mixture containing approximately 5µl of template DNA, 5ul of 10x PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.5 mM MgCl<sub>2</sub>; 0.001% gelatin), a 0.2 uM concentration of each PCR primer, a 0.2 mM concentration of each deoxynucleoside triphosphate and 2.5 U of *Taq* DNA polymerase in a total volume of 50 ul was prepared. After a 10 minute denaturation at 94°C, the reaction mixture was run through 35 cycles of denaturation for one minute at 94°C, annealing for one minute at 55°C and extension for two minutes at 72°C, followed by a final extension for 10 minutes at 72°C. Ten microlitres of PCR product was electrophoresed on a 1% agarose gel with ethidium bromide and bands were visualized by ultraviolet (UV) transillumination, to determine the size of the product. Both positive and negative controls were run with each PCR run.

Ten microlitres of the purified PCR product was digested with 10 U of *Hae*III in presence of 2µl of appropriate buffer in a total volume of 20µl. Digestion was carried out at 37°C for three hours in a shaker water bath. The reaction was stopped by heating the reaction mixture at 65°C for 20 minutes. Restriction pattern was studied with electrophoresis using a 5% non-denaturing polyacrylamide gel (using a 30% acrylamide, 0.8% bis acrylamide stock) in TBE buffer. A 50ml mixture was prepared per gel and polymerization was initiated with addition of ammonium persulphate and TEMED. The gel was run at constant speed of 5-8volts/cm of the gel for 90 minutes. Gels were then stained in 0.5X TBE containing 0.7% (W/V) ethidium bromide for 30 minutes prior to visualization in an UV transilluminator.

## Result

Total of 40 CSF samples were collected. Out of these only six were positive by conventional culture while eight were positive by PCR. All parameters for meningitis were correlated with the culture and PCR results (Table 1).

When the PCR products were digested with *Hae*III, all PCR products, except for those of *Staphylococcus aureus* and *Staphylococcus epidermidis*, were digested into several

fragments. *Streptococcus pyogenes* produced four bands at 460 bp, 330 bp, 135 bp, and 95 bp. *Streptococcus pneumoniae* and *Enterococcus faecium* produced identical patterns of bands at 460bp and 140 bp which could be further differentiated using *Alu* I digestion. *Escherichia coli* and *Klebsiella pneumoniae* produced similar six bands at 220bp, 210bp, 190bp, 160bp, 130bp, and 65bp (Fig.1). To differentiate them digestion by *Bst*BI was used whereby *Escherichia coli* product was not digested by *Bst*BI, while *Klebsiella pneumoniae* was digested into two fragments (876 and 120 bp) (Fig. 3). *Pseudomonas aeruginosa* produced a different pattern with bands at 390bp, 220bp, 175bp, 135bp, and 40bp (Fig.1). *Mycobacterium tuberculosis* also produced a different pattern with bands at 390bp, 195bp, 150bp, 105bp, and 65bp (Fig. 1). *Haemophilus influenzae* gave a unique pattern, with bands at 380bp, 270bp, 175bp and 135bp while *Neisseria meningitidis* gave at 710bp and 285bp (Fig 1).

The PCR products from *Staphylococcus aureus* and *Staphylococcus epidermidis* gave rise to different patterns when they were digested with *M*nlI. *Staphylococcus aureus* gave bands at 600bp, 485bp, 340bp, 250bp, 140bp, 110bp, 95bp, 25bp and *Staphylococcus epidermidis* produced bands at 485bp, 250bp, 110bp, 95bp, and 25bp (Fig. 2).

Out of 40 CSF samples, the study yielded nine positive results for bacterial presence by culture/PCR. Out of these six were positive by conventional culture and eight were positive by PCR, the details of which are given in Table 1.

## Discussion

Body fluids such as CSF, present a diagnostic challenge due to small sample volume and paucity of slow growing or uncultivable organisms that results in lower sensitivity. This study was carried out with the objectives to establish an alternative method of identifying bacterial pathogens in body fluids by means of broad range PCR followed by RFLP, and to compare it with conventional method of culture and identification.

Unlike culture, most molecular assays are designed specifically for one organism only. Broad-range assays, based on 16s rRNA genes, are designed to overcome this limitation. By using PCR primers that are targeted at conserved regions of 16S rRNA genes, it is possible

**Table 1**  
Laboratory parameters of patients with bacterial meningitis

Sex	Age	Bacterium identified by culture/PCR	Sugar (mg/dl) (10-30)	Protein (mg/dl) (12-60)	WBC (cells/µl) (<5)	CSF culture	CSF PCR
M	5	<i>Escherichia coli</i>	10	210	1,000	+	+
M	13	<i>Pseudomonas aeruginosa</i>	15	295	2,400	+	+
F	32	<i>Staph aureus</i>	34	90	220	+	+
M	38	<i>Mycobacterium tuberculosis</i>	76	24	260	-	+
F	26	<i>Staphylococcus epidermidis</i>	60	42	4	+	-
M	70	<i>Streptococcus pneumoniae</i>	10	760	2,600	-	+
M	25	<i>Neisseria meningitidis</i>	15	180	270	-	+
F	06	<i>Haemophilus influenzae</i>	27	150	560	+	+
M	45	<i>Streptococcus pneumoniae</i>	24	185	1,220	+	+

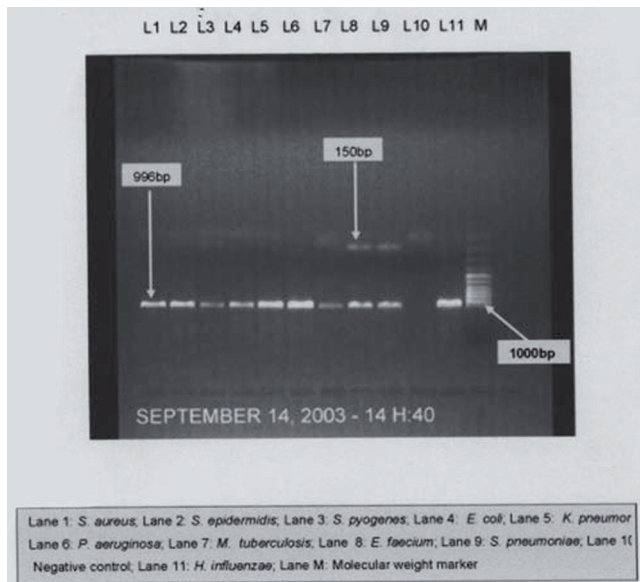


Fig. 1 : PCR of control strains with universal primers

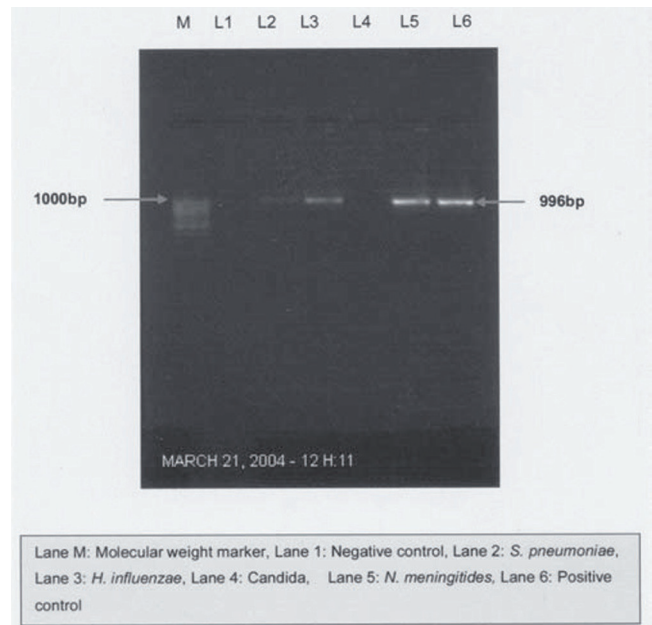


Fig. 3 : PCR with universal primers

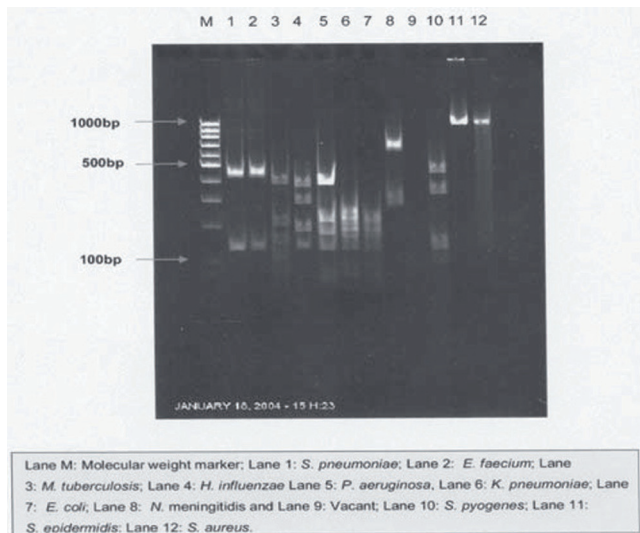


Fig. 2 : HaeIII digestion patterns of universal PCR products

to design broad-range PCRs capable of detecting DNA of almost any bacterial species. The identification of the bacterium is done by nucleotide sequencing of the PCR product [9] or by restriction endonuclease digestion [8].

Broad range PCR products from different bacteria had different restriction patterns, while PCR products from different isolates of the same bacteria had similar restriction pattern. These results formed the basis of identification of bacteria in this study.

Lu et al [8], have designed universal primers U1 and U2 for amplification of the bacteria found in CSF, which are then identified by restriction enzyme digestion on the amplified PCR product. Single set of universal primers for PCR followed by sequencing for identification of bacteria has been used by other workers also [10-14]. In this study, we detected bacteria with

only one set of PCR primers and used restriction enzyme analysis, instead of species-specific probes or sequencing, to identify bacteria.

Out of 40 CSF samples, six (15%) were positive by culture and eight (20%) were positive by PCR. One specimen that was negative by PCR, had grown *Staphylococcus epidermidis* in culture, but the microscopy and biochemical tests were not in favour of bacterial infection (Table 1). Lu et al [8], had a culture positivity of 8.6% (13/150) and PCR positivity of 10%.

Biochemical and cytological studies were carried out on the CSF specimens to corroborate the findings of culture and PCR. Out of the nine positive samples, there were two isolates of *Streptococcus pneumoniae* and one isolate each of *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Neisseria meningitides*, *Mycobacterium tuberculosis* and *Haemophilus influenzae*. The CSF glucose level varied from 10-76 mg/dl, protein levels from 24 to 760 mg/dl and WBC levels from 4-2,600 cells/ $\mu$ l. The biochemical and cytological parameters of the specimen that grew *Staphylococcus epidermidis* were within normal limits. Hence this isolate was considered to be a culture contamination. Three CSF specimens were negative by culture and positive by PCR.

History revealed that two of these patients were treated with antibiotics previously and one was tubercular in origin, which correlates well with other studies that show CSF sterilization following antibiotic use [11]. Other studies have also shown the efficacy of broad range PCR in cases where cultures are rendered sterile by antibiotic administration [10,12,13].

The universal PCR had a lower detection limit of 10



Gram-negative bacteria (e.g., *Escherichia coli*) and 250 Gram-positive bacteria (e.g., *Staphylococcus aureus*) as shown by Lu et al. [8]. Carroll et al [15], reported a sensitivity of the nested PCR between 10 fg and 1 pg of bacterial DNA, depending on the species tested, which was equivalent to between 24 and 4 live bacteria spiked in 100 µl water [15]. When mechanical disruption was used as the method of cell lysis, the sensitivity of the PCR was estimated to be 10-100 cfu per reaction for both *Escherichia coli* and *Staphylococcus aureus* [9]. Kiausegger et al [16], reported a sensitivity of 10 cfu of *E coli* per PCR for the Gram-negative-specific PCR and 10 cfu for Gram-positive-specific PCR using *Staphylococcus aureus*. Schuurman et al [17], had a lower detection limit of 100 and 200 for Gram positive and Gram negative organisms respectively. Our protocol had sensitivity below 10 cfu/ml, which is considered as the infection threshold in 85% cases of meningitis [3].

Our sample size was small to arrive at a significant statistical correlation. However comparing the study with culture as the gold standard; five out of six bacterial culture CSF specimens were positive by the universal PCR in our study. The sensitivity of PCR in our study was 83.3% and specificity 91.2%. The positive predictive value is 62.5%, and the negative is 96.8%. The lower positive predictive value is partly due to inefficiency of culture. Lu et al [8], reported a sensitivity of 92.3%. Richardson et al [18], have described a PCR assay for the diagnosis of meningococcal meningitis, which had a sensitivity of 97% as compared to a CSF culture sensitivity of 55%. Schuurman et al [17], found broad range PCR was more useful in meningitis with sensitivity of 86%, specificity of 97%, positive predictive value of 80% and negative predictive value of 98% as compared to conventional culture. Xu et al [19], recommended use of highly resolving polyacrylamide matrix for separation of amplicons and digestion fragments.

Thus, universal PCR followed by RFLP is an alternative, rapid, simple and effective method for detection of bacterial pathogens in CSF.

### Conflicts of Interest

None identified

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