

Basic Science in Medicine

DETECTION OF BACTERIA BY AMPLIFYING THE 16S rRNA GENE WITH UNIVERSAL PRIMERS AND RFLP

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ABSTRACT

Background: There is a conserved portion in the 16S rRNA gene of bacteria which can be amplified by the universal PCR method. This fragment is 996 bp in length. In this method, only one set of universal primers is used for the amplification of the conserved region of the 16S rRNA gene, in common bacterial pathogens. Therefore, using the universal PCR method, these bacteria are detectable only by one set of primers; then for detection of the bacteria, the PCR products are digested by the restriction endonucleases. Since the restriction patterns of bacteria (RFLP) are expected to be different from each other, on that basis we can identify the bacteria.

Methods: The conserved fragments of the 16S rRNA genes of the following bacteria were amplified by the universal PCR method: *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*. The PCR products were digested by BsuRI (Hae III) restriction endonucleases and were electrophoresed on agarose gel.

Results: The restriction patterns of these bacteria were different. Thirty isolated *E. coli* and 28 isolated *S. pyogenes* from clinical samples were studied by this method. The size of PCR products and RFLP patterns of every bacterium were the same as standard strains. In comparison with culture method, the sensitivity of the universal PCR is 92.3 %. The sensitivity of this method was determined up to about 11 and 190 bacteria for gram negatives and gram positives respectively.

Conclusion: These studies suggest that the universal PCR method accompanied with RFLP is a very useful and rapid method, for detection and identification of bacteria in body fluids.

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Keywords: Universal-PCR & Primer, 16S rRNA gene, Bacteria, Detection, RFLP.

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INTRODUCTION

In emergency cases such as meningitis (that can be cerebral, spinal or cerebrospinal), even seconds are vital for diagnosis of infectious agents. Therefore rapid diagnosis of bacteria in body fluids especially cerebrospinal fluids (CSF) is vital, because the mortality rate associated with infections in the blood stream or central nervous system is very high^{10,14,15}. For detection of bacterial infections in clinical laboratories, culture is the most sensitive method. But culture requires at least 8 hours of incubation time. On the other hand additional time is needed to perform biochemical or immunological tests to identify the bacteria. The 16S rRNA genes of almost all common bacterial pathogens found in body fluids have been sequenced.^{2,8,16} With one set of designed universal primers that can amplify this gene in all bacteria based on the conserved fragment of the 16S rRNA genes,^{4,11} we can detect and identify bacteria by restriction enzyme digestion of universal PCR products in 4.5 hours. Since RFLP patterns of the universal PCR products from different bacterial species are different, the conserved fragment of the 16S rRNA genes (996 bp) could be differentiated by this method.

MATERIAL AND METHODS

Bacterial strains

The following bacteria were used as controls: *Pseudomonas aeruginosa* ATCC 25668, *Neisseria gonorrhoeae* CDC 98, *Streptococcus pyogenes* ATCC 19615 and *Escherichia coli* ATCC 25922. Instead of customary methods for extraction of genomic DNA and required material, we used only sterile distilled water.

Preparation of samples for PCR analysis

The supernatant of the bacterial solution, after lysing by boiling, were used for PCR. Approximately 105 CFU of bacteria were washed in 1mL of sterile distilled water and then pelleted by centrifugation at 13,000 g for 5 min. Each pellet was suspended in 100 microliters of sterile distilled water and then boiled in a water bath for 30 min to release the DNA. The cell debris was removed by centrifugation at 13,000 g for 5 min, and the supernatant was saved for PCR.

Clinical specimens

From May 2003 to December 2003, 70 CSF specimens were obtained from 70 different patients in Tehran hospitals. These specimens were examined for the presence of bacteria by both culture and the universal PCR developed in this study. Twenty-six of the 70 CSF specimens were positive by bacterial culture. CSF specimens from

patients with bacterial meningitis usually have a decreased sugar concentration and an increased protein concentration. Although every CSF specimen had an elevated white blood cell count, sugar and protein levels were not completely consistent with the presence or absence of bacteria determined by culture or PCR.

PCR amplification

A reaction mixture containing approximately 50 ng of template DNA, 10X PCR buffer, a 20 pmol concentration of each PCR primer, a 2.5 mM concentration of dNTPs and 2.5 U of Taq DNA polymerase in a total volume of 50 microliters was prepared. After a 10 min denaturation at 94°C, the reaction mixture was run through 35 cycles of denaturation for 20 seconds at 94°C, annealing for 20 seconds at 58°C, and extension for 1 min at 72°C, followed by a final extension for 10 min at 72°C. Ten microliters of PCR product was electrophoresed on 1% agarose gel to determine the size of the product. Both negative and reagent controls were included in each PCR run. The reagent control consisted of all PCR components except for the template DNA. If either control becomes positive, the entire PCR was repeated. Restriction enzyme analysis was also performed on PCR products to detect contamination. If the digestion patterns from different PCR products were the same, the sample was suspected to have been contaminated. The PCR was repeated. To set up a PCR method capable of amplifying all bacteria, nucleotide sequences of the 16S rRNA genes of common pathogenic bacteria were compared. One pair of primers, designated U1 and U2, with sequences conserved among all of these bacteria was selected. The sequence of forward primer is 5'-CCAGCAGCCGCGGTAATACG-3', corresponding to nucleotides 518 to 537 of the *E. coli* 16S rRNA gene, and that of reverse is 5'-ATCGG(C/T)TACCTTGTTACGACTTC-3', corresponding to nucleotides 1513 to 1491 of the same gene.¹ The PCR performed with these two primers is referred to as the universal PCR in this research.

Restriction endonuclease digestion

Eight microliters of each PCR product was digested with BsuRI (Hae III) enzyme in appropriate restriction enzyme buffer (R-buffer) in a total volume of 25 microliters. After incubation for 2 hours at the recommended temperature, the digested DNA was electrophoresed on 2% agarose gel.

RESULTS

DNA from some American Type Culture Collection Control and CDC bacteria were examined by the univer-

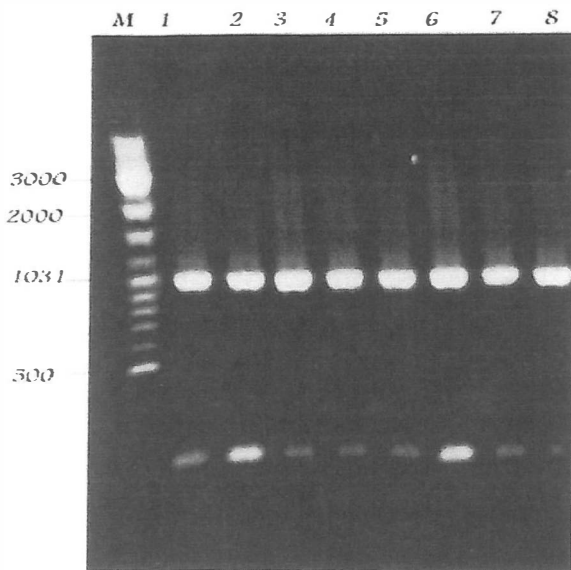


Fig. 1. Electrophoresis on 1% agarose gel of universal PCR products: Samples in different lanes, that are the same, were PCR products from the following bacteria: lane 1, 2, *P. aeruginosa*; lane 3, 4, *E. coli*; lane 5, 6, *S. pyogenes*; lane 7, 8, *N. gonorrhoeae*. Lane M, Marker, containing molecular size standards (base pairs). The sizes of the molecular size standards are marked on the left of the gel. The size of Universal PCR product is 996 bp. A band of approximately 160 bp is also seen in lanes 1-8; this band may be the result of nonspecific amplifications. Another band of approximately 50 bp may be also present in the mentioned lanes; this band is the primer dimer that formed during the PCR.

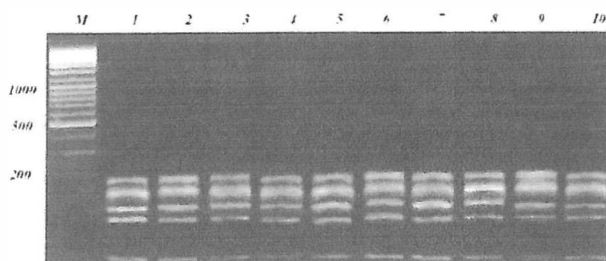


Fig. 3. Assessment of 16S rRNA gene conserved fragment RFLP patterns of *E. coli* different isolates: Lanes 1-10 were BsuRI (Hae III) digested universal PCR products of different isolates that had the same RFLP patterns; Lane M, marker.

sal PCR. All of these DNA samples generated a PCR product of the expected size (996 bp) (Fig. 1). A nonspecific band of approximately 160 bp was also produced.

The PCR products were then digested with BsuRI (Hae III) to determine whether there is a restriction fragment length polymorphism that can be used to identify certain bacteria. For bacterial identification, the Universal PCR products were digested into several fragments

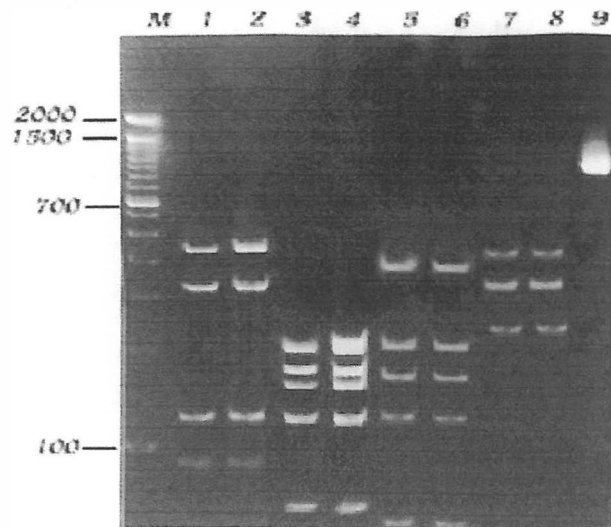


Fig. 2. Hae III digestion patterns of standard bacteria and CSF samples universal PCR products. Samples in different lanes were Hae III-digested Universal PCR products from the following bacteria: lane 1, *S. pyogenes* (1, CSF sample and 2 standard sample); lane 3, 4, *E. coli* (3, CSF sample and 4 standard sample); lane 5, 6 *P. aeruginosa* (5, standard sample and 6, CSF sample); lane 7, 8, *N. gonorrhoeae* (standard samples); Lane 9, PCR product. Lane M, marker, contained molecular size standards (base pairs). The sizes of the molecular size standards are marked on the left of the gel.

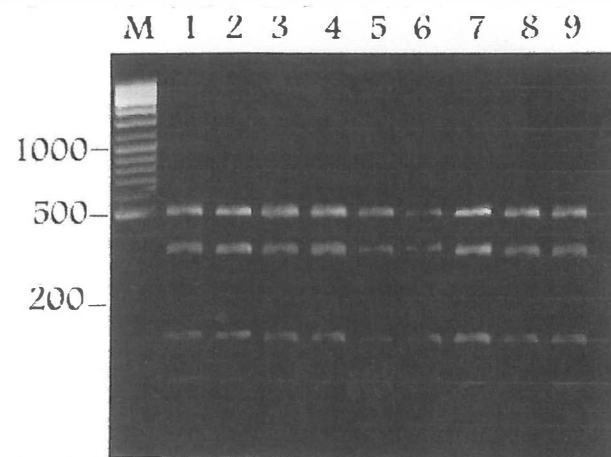


Fig. 4. Assessment of 16S rRNA gene conserved fragment RFLP patterns of *S. pyogenes* different isolates: Lanes 1-9 were BsuRI (Hae III) digested universal PCR products of different isolates that had the same RFLP patterns; Lane M, marker.

by BsuRI (Hae III) restriction enzyme. The universal PCR products of each bacterium had a unique RFLP pattern (Fig. 2) and different clinical isolated samples corresponding to one bacterial species had the same RFLP patterns (Fig. 3, 4).

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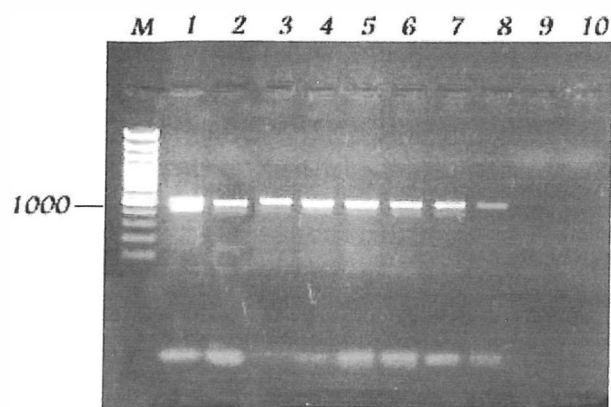


Fig. 5. Sensitivity determination of universal PCR method for detection of the fewest gram negative bacteria (*E. coli*) number in the samples; Lanes 1-10 were universal PCR products of DNA extracted from the last 10 dilutions respectively containing 1523, 761, 380, 190, 95, 47, 23, 11, 5, 2 bacteria. The concentrations of PCR products have been decreased from lanes 1-8, but in lane 9 and 10 there are no PCR products. Thus, at least 11 gram negative bacteria were detectable by this method.

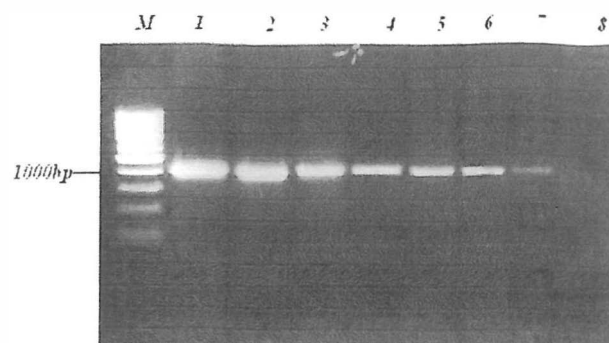


Fig. 6. Sensitivity determination of universal PCR method for detection of the fewest gram positive bacteria (*S. pyogenes*) number in the samples; Lanes 1-8 were universal PCR products of DNA extracted from the last 8 dilutions respectively containing 12191, 6095, 3047, 1523, 761, 380, 190, 95 bacteria. The concentrations of PCR products have been decreased from lane 1-7, but in lane 8 there isn't any PCR product. Thus, at least 190 gram positive bacteria were detectable by this method.

Table I. Estimation of sensitivity and specificity of Universal PCR method; from 70 CSF samples, 24 samples were true positive PCR and 2 samples were false negative PCR; thus the sensitivity is equal to 92.3%; 38 samples were true negative PCR and there wasn't any false positive, thus the specificity is equal to 100%.

Results	
Sensitivity Estimation	Specificity Estimation
A) Total samples for sensitivity: 26	A) Total samples for specificity: 38
B) True Positive samples : 24	B) True Negative samples: 38
C) False Negative samples: 2	C) False Positive samples: 0
Sensitivity = $B\% = A\% - C\% = 92.3\%$	Specificity = $B\% = A\% - C\% = 100\%$

To determine the sensitivity of this universal PCR, *E. coli* ATCC 25922 and *S. pyogenes* ATCC 19615 cultures were serially diluted in a CSF sample that was confirmed to be PCR negative. An aliquot of each dilution was used for DNA extraction, and the purified DNA was used as a template for the PCR. Determined number of *E. coli* and *S. pyogenes*, detectable by universal PCR, was found to be approximately 11 (Fig. 5) and 190 (Fig. 6) respectively.

The Universal PCR method was used for 70 CSF samples, and 26 samples from these were culture positive. In these 26 culture positive samples, 24 samples were PCR positive and two samples were PCR negative, thus the sensitivity of this method is 92.3%.

From 70 specimens, 44 showed negative results by

culture. These were also tested by universal PCR; out of these 44 samples, six were PCR positive. On the other hand, these six patients had symptoms of bacterial meningitis and were cured by antibiotic therapy.

Although specimens from these six patients were culture negative, since they were responsive to antibiotic therapy, we considered these samples as positive. On that basis, for the estimation of the specificity of universal PCR, the true number of culture negative results was considered to be 38 instead of 44; from which the specificity amounts to 100% (Table I).

Two PCR negative samples had high WBC count (12400×10^6 cell/liter, 14200×10^6 cell/liter) and protein level (8950 mg/liter and 7230 mg/liter). The high protein level

may be the cause of a negative PCR, as reported by Jang-Jih Lu et al.¹ In the 44 remaining CSF samples that were culture negative, six samples were PCR positive. These six patients had symptoms of bacterial meningitis and were cured by antibiotic therapy.

DISCUSSION

To develop a rapid and sensitive method for detection and identification of bacteria, particularly when emergency cases such as meningitis are to be diagnosed is very important. In these studies, we designed our system based on conserved sequence of 16S rRNA genes of bacteria;^{1,4,11} this conserved sequence could be amplified by PCR, using universal primers. Subsequently digestion of this region by restriction enzymes showed different restriction patterns with ATCC standard bacteria as well as local isolates of these bacteria from CSF's. These bacteria were received from 70 CSF samples from different hospitals in Tehran.

Since there have been no changes in restriction patterns of local isolates in comparison with ATCC bacteria, it seems that there has been no particular mutation in these local isolates. Therefore, universal PCR followed by RFLP, could be considered as a simple and sensitive method for detection and identification of bacteria.

Quantification and identification of these bacteria from CSF samples could also be performed, using specific probes or sequencing^{2,8,11,16} for this conserved region, following the universal PCR; and detection can be done by a much more sensitive method such as PCR-ELISA.¹⁸ PCR-ELISA may not be cost effective in comparison with restriction digestion, but has many advantages.¹⁸ However, Universal PCR-RFLP is an affordable and accessible method with acceptable sensitivity, which could be used in medical diagnostic laboratories for CSF samples.

Amplification of conserved sequence region in different bacteria by universal PCR technique for DNA sequencing or probe hybridization have been used by Wilson et al., Lane et al., Radstrom et al., Butger et al. and Greisen et al.^{2,4,8,11,16} but all of them used several sets of primers for detection and identification of bacteria. However, in this universal PCR method only one set of universal primers and one restriction endonucleases enzyme were used for bacterial detection instead of using probes or sequencing.

Twenty-four of 26 culture-positive samples were PCR positive, giving a sensitivity equal to 92.30%. Two false-negative PCR results could be due to high protein level (8950 mg/liter and 7230 mg/liter) of the samples. We had no false-positive PCR result but it has been reported¹ that mishandling and contamination of the samples can

cause false-positive PCR results.

Six culture negative samples with meningitis symptoms were found to be PCR-positive. Since a total of 44 samples were culture negative, therefore the number of PCR negative samples were considered to be 38 and accordingly specificity is 100% (Table I). The detectable number of gram negative (e.g. *E.coli*) and gram positive bacteria (e.g. *S. pyogenes*) by universal PCR were found to be 11 and 190 respectively. This gives adequate sensitivity for detection of bacteria from CSF samples.⁹

The overall time required to perform the whole process including DNA extraction, was found to be about 4.5 hours, which is quite good compared to conventional methods, which takes from one to two days for identification of bacteria.

Rapid detection and identification, high sensitivity and specificity and reduced expenses are the significant advantages of this universal PCR method.

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