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Original Article

Molecular Detection of Common Bacterial Pathogens Causing Meningitis

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Abstract

Background: The clinical diagnosis of meningitis is crucial, particularly in children. The early diagnosis and empiric antibiotic treatments have led to a reduction in morbidity and mortality rates. PCR and the enzymatic digestion of 16SrDNA fragment which is produced by universal primers led up fast and sensitive determination. The purpose of this study was to investigate a rapid method for detection of common bacterial pathogens causing meningitis.

Methods: According to the gene encoding 16SrDNA found in all bacteria, a pair of primers was designed. Then the universal PCR was performed for bacterial agents of meningitis (*Streptococcus pneumoniae, Neisseria meningitidis, Haemophilus influenzae,* etc.) by employing broad- range DNA extraction method. The obtained universal PCR products were digested with restriction enzymes (*Hae*III, *Alu*I and *Mnl*I) to identify bacterial species.

Results: By the enzymatic digestion of the universal products of each standard strain of the above bacteria, specific patterns were achieved. These specific patterns may be used for comparison in CSF examination. The analytical sensitivity of the assay was approximately 1.5×10^2 CFU/ml of CSF even in samples with high amount of proteins.

Conclusion: The universal PCR coupled with enzymatic digestion can be used to detect and identify bacterial pathogens in clinical specimens rapidly and accurately. Molecular diagnostic of bacterial meningitis, though expensive and labor-intensive, but is valuable and critical in patient management.

Keyword: *Bacterial diagnosis, Universal PCR, Meningitis*

Introduction

Bacterial meningitis is a serious disease with high morbidity and mortality. To reduce death or permanent neurological sequels as much as possible, a fast and correct diagnosis is of the utmost importance. The annual attack rate in the United States is about 3 cases per 100000 populations (1, 2).

The rates of meningitis (per 100,000) for the major pathogens in 1995 were *S. pneumoniae*, 1.1*; N. meningitidis*, 0.6; *group B Streptococcus*, 0.3; *L. monocytogenes*, 0.2; and *H. influenzae*, 0.2. *Group B streptococcus, N. meningitidis* and *S. pneumoniae* were the predominant pathogens among newborns, children 2 to 18 years old and adults respectively. Pneumococcal meningitis had the highest rate of fatality (1-3). The attack rate in one year children is higher than adults (4, 5). Rapid detection and identification of bacteria in cerebrospinal fluid (CSF) and blood is crucial in disease management in order to decrease the high mortality rate which is associated with infections in the blood stream or central nervous system, especially in developing countries (6). Although the most usual and routine method for diagnosis of CSF infection is the bacterial culture of samples, and then identify the responsible microorganisms (7, 8) but the results of previous studies have shown that culture might loose the diagnosis of bacterial meningitis in at least 13% of cases (9, 10). Microscopic investigation for the CSF is also recommended, however it is not an accurate method. Bacterial concentration in the CSF has a profound effect on the results of microscopy. Regardless of the type of organism in the CSF, the percentage of positive microscopic results is only 25% (11). Results of culture may only be available after 24 or 48 h and sometimes, for instance, when the number of viable organisms

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in the CSF is low, it may take even longer, while This time is not acceptable for such a serious diseases like meningitis. Meanwhile, sometimes following antibiotic therapy in advance, bacteria can not grow on culture media, and the results will be negative in accurately (12, 13).

In order to solve these problems we decided to find a precise and novel method for detection and identification of bacteria.

In recent years, PCR techniques have been developed (14, 15) and increasingly used to amplify and detect microbial DNA in CSF (16, 17). Selection of an appropriate primer pairs is an important factor in PCR technique. Therefore it is restricted factor. This limitation can be solved by using broad range bacterial primers that are designed based on conserved region of 16SrDNA of bacteria. Then it might be followed by applying appropriate restriction enzyme to accomplish digestion patterns of universal PCR products, to identify the bacterial species.

The purpose of current study was to rapid detection of common bacterial pathogens causing meningitis.

Material and Methods

Bacterial strains

The following bacteria were used in order to accomplish standard enzymatic digestion patterns. *Streptococcus pneumoniae* ATCC 49619, *Haemophilus influenzae* ATCC 33930, *Neisseria meningitides* ATCC 10377, *Listeria monocytogenes* ATCC 49594, *Streptococcus agalactiae* ATCC 1020, *Escherichia Coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 7881, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 51299, *S. epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *S. pyogenes* ATCC 19615.

Clinical samples

Ten samples of meningitis cases were collected from three hospitals of Tehran University of Medical Sciences.

DNA extractions

DNA from colony and CSF samples was extracted by DNeasy Blood and tissue kit (QIAGEN-Germany).

Briefly, each CSF sample was divided into two equal parts (2×200 µl). Then it was spin down for 10 min at 9500 RPM (8300 g) and discarded supernatant gently. Next the bacterial pellet was resuspended in little remainders of supernatant. One tube was prepared for gram negative bacteria and next steps were followed through the instruction of kit. Another tube was for gram positive bacteria. At first, enzymatic lysis buffer and lysostaphin was added and incubated at 37 ^{o}C for 30 min. After the incubation, lysozyme was added and incubated it at 37 ^{o}C for 30 min. The next steps were followed through the instruction. Finally, Just 30 µl of AE buffer was added in each tube. These methods helped us to gain DNA from a few amount of bacteria in CSF.

For DNA extraction from standard colony the kit instruction was followed.

PCR amplification

TEHF and TEHR primers were used (Table 1).

Table 1: Details of the primers used in this study

Primer	Sequence (5'---3')	Tm (°C)
TEHF	5'-CAGCAGCCGCGGTAA- 3'	59.4
TEHR	5'- ACGGTTACCTTGTTACGACTT C-3'	58.8

A reaction mixture consisted of 72.7µl ddH_2O, 10 µl PCR buffer 10×(Fermentase, Denmark, Copenhagen), 1.5 mM Mgcl2 (50 mM, Fermentas, Denmark, Copenhagen), 0.2 mM dNTPs (10mM, Fermentase, Denmark, Copenhagen), 0.5 µM of each primers (10 µM, TAG Copenhagen A/S, Denmark, Copenhagen), 2µl DNA template (approximately 50 ng of template DNA) and 1.5 U Taq polymerase (5 U/ml, Fermentase, Denmark, Copenhagen). The whole amount of reaction mixture was 100 µl. The PCR was started with an initial denaturation step at 94 ^{o}C for 4 min. Then, the reaction mixture was run through 30 cycles of denaturation at 94 ^{o}C for 45S, annealing at 60 ^{o}C for 30 S and extension at 72 ^{o}C for 90S. 2µl of each PCR product was electrophoresed on a 1% agarose gel to show the amplification in PCR.

Enzymatic digestion

The fragments which were obtained from the universal PCR were digested by restriction enzyme *Hae*III (fermentas, Denmark, Copenhage). All of the above bacteria produced different patterns except *S. aureus* and *S. epidermidis*, *S. pneumoniae* and *L. monocytogenes*, *E. coli* and *K. pneumoniae* which produced the same patterns. For both staphylococci *Mnl*I (Fermentas, Denmark, Copenhagen) was used and for the rest *Alu*I (Fermentas, Denmark, Copenhagen) was employed. To digest PCR products, a mixture including; 15µl distilled water, 2.5 µl buffer, 1.5 µl enzyme and 6µl of PCR product was performed and incubated at 37 ^{o}C water bath for 2 h.

Polyacrylamide electrophoresis

To show a digestion patterns, 8% polyacrylamide gel was used and run in 22 mA for 1.5 h, and visualized by ethidium bromide under UV light (254 nm).

Results

In order to increase a capability of PCR to amplify all bacteria, nucleotide sequences of the 16SrDNA genes of common pathogenic bacteria were studied. Then a pair of primers, TEHF and TEHR, with sequences which were conserved among all of these bacteria was designed.

PCR was performed with the above primers, which is known as universal PCR. We could extract DNA by expanding method in order to cover all eventual bacteria in CSF samples (Fig. 1).

Then PCR were performed on extracted DNA. The size of fragments obtained from the universal PCR was between 990-997 bp (Fig. 2).

Next these DNA extractions were digested by employing restriction enzyme. The enzymatic digestion patterns by *Hae*III, *Alu*I and *Mnl*I are shown in Figs. 3- 5. They are standard patterns for comparison in universal PCR-enzymatic digestion method with results that fulfilled from digestion by *Hae*III, *Alu*I and *Mnl*I. Therefore we can recognize all bacterial agents in meningitis.

The results of the digestion patterns are as follows:

Digestion by HaeIII

S.pneumoniae (3 fragments), *N. meningitidis* (2 fragments), *H. influenzae* (4 fragments), *S. agalactiae* (4 fragment), *E. coli* (6 fragments), *L. monocytogenes* (3 fragments), *S. aureus* (No Cut), *S. epidermidis* (No Cut), *S. pyogenes* (4 fragments), *E. faecalis* (3 fragments), *K. pneumoniae* (6 fragments), *P.aeruginosa* (4 fragments)

Digestion by AluI

S. pneumoniae (4 fragments), *H. influenzae* (6 fragments), *S. agalactiae* (4 fragments), *E. coli* (4 fragments), *L. monocytogenes* (4 fragments), *S. aureus* (5 fragments), *S. epidermidis* (5 fragments), *S. pyogenes* (4 fragments), *E. faecalis* (4 fragments), *K. pneumoniae* (4 fragments), *P. aeruginosa* (6 fragments).

Digestion by MnlI

S. aureus (5 fragments), *S. epidermidis* (6 fragments).

Based on the results of enzymatic digestions the following diagram (Fig. 6) was designed. According to this diagram it is possible to manage the procedure.

In order to determine whether PCR products from different isolates of one species of bacteria have the same restriction fragment length polymorphism patterns, first, the genome of different strains which are obtained from gene bank were checked. Next, different isolates for each bacterium (at least three isolates for each one) were prepared and then universal PCR and enzymatic digestion were performed respectively. Therefore we found same results from different isolates; it means this method is accurate and reliable.

To determine the sensitivity of this method (expanded DNA extraction and Universal PCR) to diagnose a few bacteria in clinical specimens, a serial dilution of *E. coli* and *S. aureus* were prepared.

In CSF samples (10 specimens) the analytical sensitivity of assay was approximately 1.5×10^{2} CFU/ml. The amounts of protein in the specimens were between 38-390 mg/dl. Even the highest amount of protein in samples (CSF) like 390 mg/dl did not affect on the results.

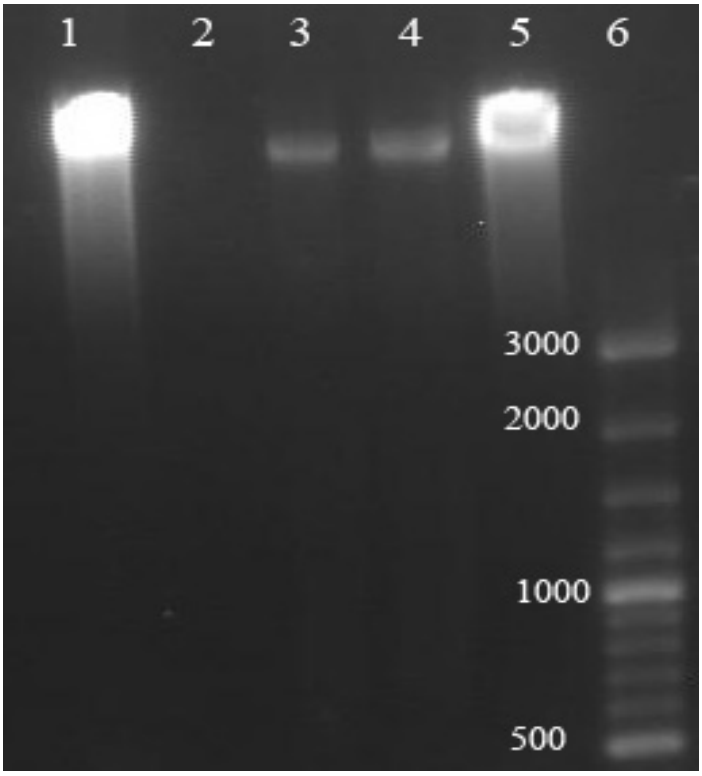

Fig.1: Bacterial genome extraction on 1% agarose gel. Lane1,*E.coli*; Lane2,Negative control; Lane3, *N. meningitidis*; Lane4, *S. aureus*; Lane5, *H. influenzae*; Lane6, Molecular marker (100bp plus)

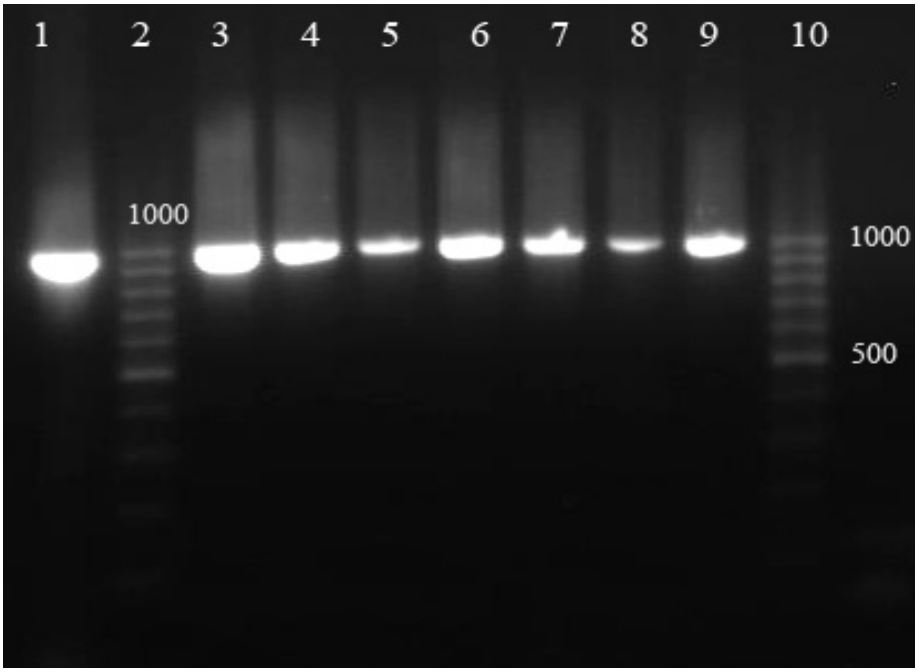

Fig. 2: Universal PCR fragments for various kinds of meningeal pathogens (~1000bp) on 1% agarose gel. Lane1, *E. coli*; Lane2, Molecular marker(100bp plus); Lane3, *N. meningitidis*; Lane4, *S. aureus* ; Lane5, *H. influenzae*; Lane6, *S. pneumoniae*; Lane7, *L. monocytogenes*; Lane8, *S. agalactiae*; Lane9, *S. pyogenes*; Lane10, Molecular marker (100bp plus)

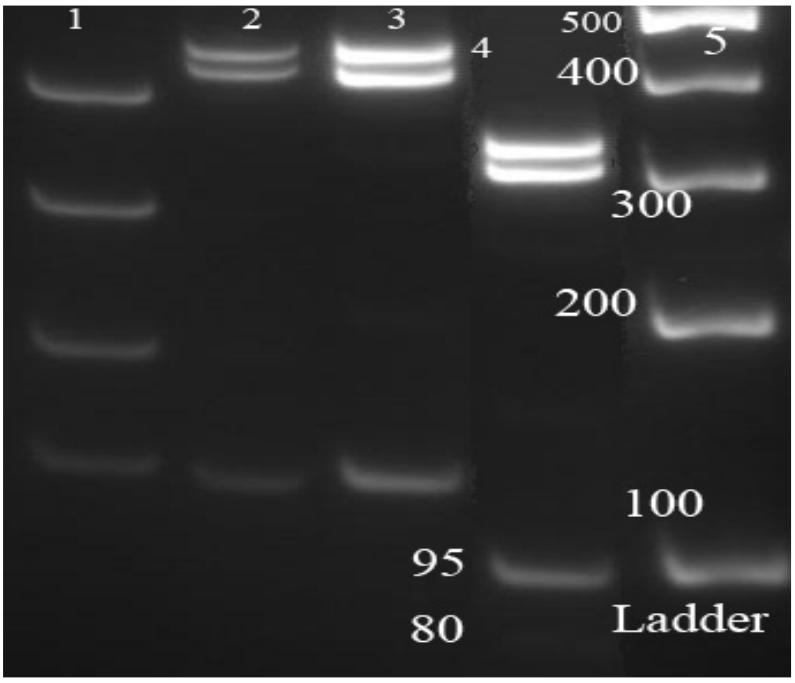

Fig. 3: *Hae*III digestion patterns of universal PCR products on 8% polyacrylamide gel. Samples in different lanes were *Hae*III-digested PCR products from the following bacteria: Lane1, *H. influenzae*; Lane2, *S. pneumoniae*; Lane3, *L. monocytogenes*; Lane4, *S. agalactiae*; Lane5, Molecular marker (100bp plus)

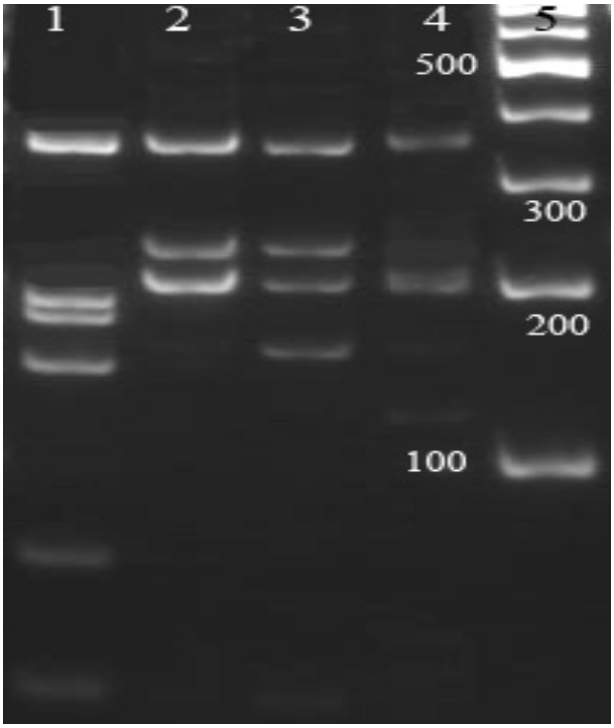

Fig. 4: *Alu*I digestion patterns of universal PCR products on 8% polyacrylamide gel. Samples in different lanes were *Alu*I-digested PCR products from the following bacteria: Lane1, *H. influenzae*; lane2, *S. pneumoniae*; Lane3, *L. monocytogenes*; Lane4, *S. agalactiae*; Lane5, Molecular marker(100bp plus)

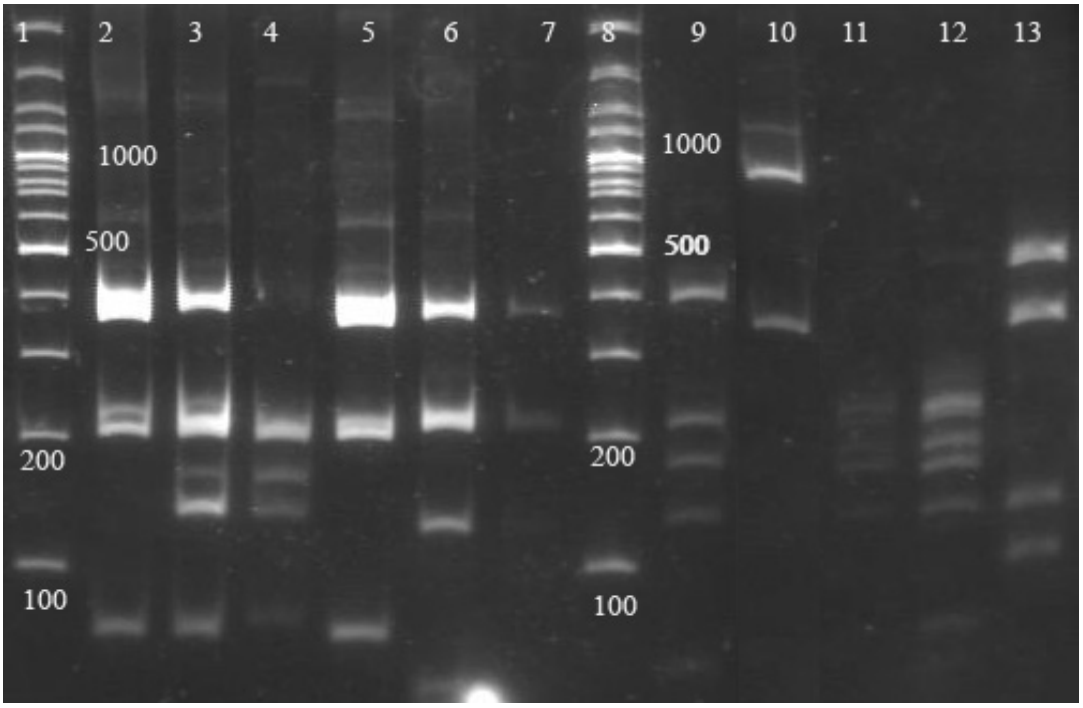

Fig. 5: *Alu*I and *Hae*III digestion patterns of universal PCR products on 8% polyacrylamide gel. Samples in different lanes were *Alu*I/*Hae*III-digested PCR products from the following bacteria: *Alu*I digestion patterns: Lane1, Molecular marker(100bp plus); Lane2, *K. pneumoniae*; Lane3, *E.coli*; Lane4, *P. aeroginosa*; Lane5, *S. saprophyticus*; Lane6, *S. epidermidis*; Lane7, *S. aureus*; Lane8, Molecular marker(100bp plus); *Hae*III digestion patterns: Lane9, *P. aeroginosa*; Lane10, *N. meningitidis*; Lane11, *K. pneumoniae*; Lane12 , *E. coli*; Lane13, *S. pyogenes*

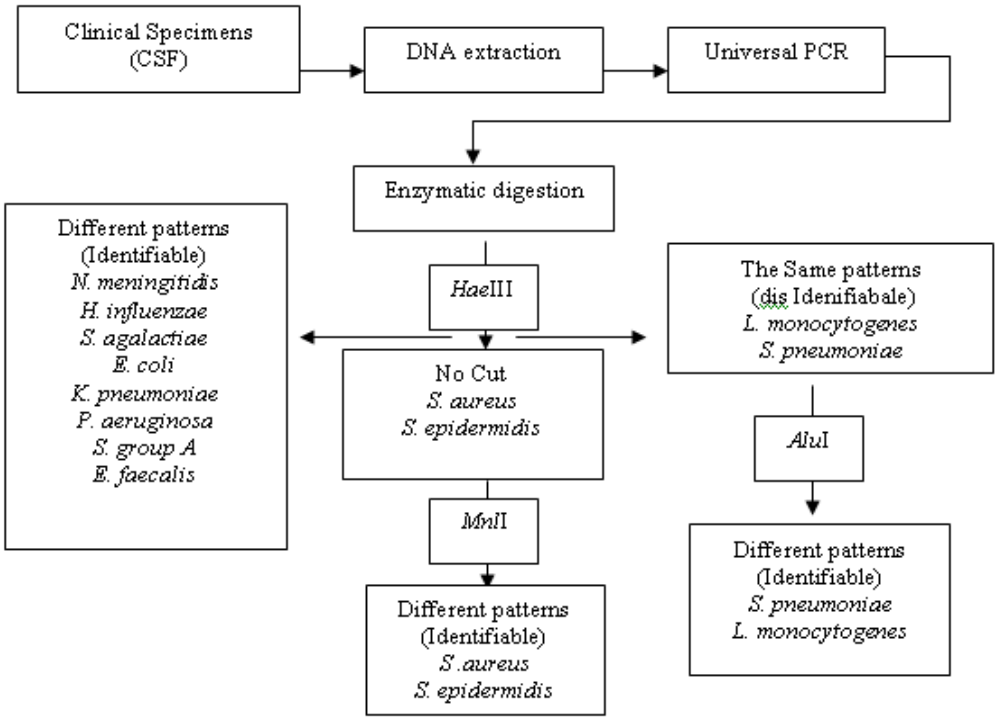

Fig. 6: Flow chart of the universal PCR and RFLP for detection of common bacterial pathogens in CSF

Discussion

Our principle purpose in this study was to develop a rapid and sensitive method to detect and identify bacteria in CSF specimens. In order to achieve this goal, DNA extraction method (DNeasy Blood and tissue kit) was developed. Then a special pair of primers was designed according to conserved sequence of 16SrDNA to cover all of the bacteria. Next, with some restriction enzymes we found specific pattern for each species of bacteria. In addition, universal PCR products from different isolates of the same bacteria were found to have the same restriction pattern. In contrast to the traditional method (culture), the rapid method which is cost effective has been designed. Moreover, this method is practical, sensitive, and has less negatively incorrect results. By employing this method it is simply to detect bacteria in CSF even in suspicious cases that were received prior antibiotic treatment. In comparison with other studies that used universal primers (12, 15, 18), here there are some advantages. Some of these studies just detected the genus of bacteria in specimens (12, 19) while this method continues to identify the species by restriction enzymes. In 2005, Afsharpeiman et al. used universal PCR to diagnose bacteremia. The method's sensitivity and specificity were 91.67 and 90.91, respectively (12). At the same time, Pandit et al. performed an experience about using 16SrRNA universal primers and restriction endonuclease digestion by employing two enzymes *Hae*III and *Mnl*I (18) to identify species of bacteria in specimens of meningitis cases. While according to our investigation using just these enzymes are not enough to identify all of the bacteria which play a role in meningitis. Others used different methods such as sequencing (20, 21), species specific probes like hybridization (22), real time PCR (23) and microarrays (24, 25) to identify bacterial species (26). The employed methods are sophisticated, complex and expensive therefore it is not possible to use them as routine tests for suspected patients with meningitis.

In comparison to current study, there were two studies in which universal primers and restriction enzyme were used to identify bacterial species (13, 18). LU et al. applied five different enzymes for identification the species, but it makes confusing and increase the cost and time, while by our method this could be carried out by using just three enzymes (13).

In routine clinical tests, we need simple method with simple equipments and low costs, which could be carried out by laboratory technician.

The current molecular detection decrease the terrible consequences of negatively incorrect results in CSF examination, therefore physicians can get reliable results with high sensitivity and specificity from CSF examination in less than 10 h, in comparison to culture method which takes 48 h. Moreover, the bacteria were detected in small amounts of CSF (200 µl) and high amounts of protein.

These results suggest that the universal PCR coupled with enzymatic digestion can be used to detect and identify bacterial pathogens in clinical specimens rapidly and accurately. We are currently adapting the standard procedures in our laboratory for accurate detection of bacterial meningitis, even though when antimicrobial therapy has already started at the time of lumber puncture or when cultures remain negative although the suspicion of bacterial meningitis remains.

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The authors declare that they have no conflict of interests.

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