

Comparison of 16S rDNA-PCR Amplification and Culture of Cerebrospinal Fluid for Diagnosis of Bacterial Meningitis

Mohammad-Reza Sarookhani^{1,2*}, PhD; Parviz Ayazi², MD; Safar Alizadeh¹, MSc; Farshad Foroughi¹, MSc; Ahmad Sahmani¹, MSc, and Mohtaram Adineh¹, BSc

1. School of Allied Medicine, Qazvin University of Medical Sciences, Qazvin, IR Iran
2. Cell & Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, IR Iran

Received: Dec 23, 2009; Final Revision: Apr 03, 2010; Accepted: Jun 04, 2010

Abstract

Objective: Early and accurate diagnosis of bacterial meningitis is of critical concern. Optimum and rapid laboratory facilities are not routinely available for detecting the etiologic agents of meningitis. The objective of this study was to compare polymerase chain reaction (PCR) assay with culture for detection of bacteria in central nervous system (CNS) samples from patients suspected to have meningitis.

Methods: One-hundred cerebrospinal fluid (CSF) samples were obtained and divided into two parts. One part of samples was used for standard bacterial culture and gram staining. The remaining was used for DNA extraction. PCR assay was performed with universal primers for 16S rDNA gene of bacteria. Performance characteristics of the test were determined.

Findings: The PCR method was able to detect bacteria in all 36 culture-positive and in 38 of 64 culture-negative cases showing sensitivity and specificity of 100% and 40.6% respectively. Positive predictive value was 48.6% and negative predictive value 100%, however, Kappa coefficient showed the correlation of the 2 methods to be at 0.33.

Conclusion: There are advantages and disadvantages in performance characteristics of the conventional CSF culture and universal CSF 16S rDNA PCR. Therefore, it is recommended to use both methods in clinical practice, particularly in suspicious contaminated samples, with presumable presence of fastidious or slow growing bacteria because of antibiotic consumption.

Iranian Journal of Pediatrics, Volume 20 (Number 4), December 2010, Pages: 471-475

Key Words: Meningitis, Bacterial; rDNA; PCR; Central nervous system; Infection

Introduction

Infections of the central nervous system (CNS) are serious and potentially life threatening.

These infections may be caused by bacteria, fungi, viruses, or parasites. Specific etiologic diagnosis is detected by laboratory testing and

* Corresponding Author;

Address: School of public health, Qazvin university of medical sciences, Bahonar Blvd, Qazvin, IR Iran

E-mail: sarokhani2002@yahoo.com

© 2010 by Pediatrics Center of Excellence, Children's Medical Center, Tehran University of Medical Sciences, All rights reserved.

positive laboratory findings are communicated directly to the clinician^[1,2]. Acute meningitis is commonly caused by bacteria or viruses. Patients with acute meningitis usually have fever, headache, vomiting, photophobia and altered mental status^[1,2].

The likely etiologic agents of bacterial meningitis depend on the age of the patient. The current standard for the diagnosis of bacterial meningitis is microscopic examination and subsequent culture of cerebrospinal fluid (CSF)^[1,2]. However, this approach might have some disadvantages with regard to desired rapidity and sensitivity.

Results of culture may only be available after 24 to 48h and sometimes, for instance, when the number of viable organisms in the CSF is low, it may take even longer. Moreover, the sensitivity of microscopic examination and culture of CSF can be debated, especially after the start of antibiotic treatment and meningitis due to fastidious or slow-growing or anaerobic microorganisms^[3-8]

In the last decade, PCR-based assays have become available to provide an early and accurate diagnosis of bacterial meningitis. Most of these assays target 16S rDNA gene, which is highly conserved in all eubacterials^[9-12].

Here we report comparison of the results of 16S rDNA gene (transcribes as 16S rRNA) amplification method to the results of conventional microbiologic methods for the examination of CSF to evaluate each assay and introduce the best practical approach for the diagnosis of bacterial meningitis.

Subjects and Methods

From March 2007 to March 2008, all CSF samples with high cellular content from patients suspected to have bacterial meningitis admitted to hospitals of Qazvin University of Medical Sciences (QUMS) were collected. Regardless of clinical manifestations, up to 100 samples referred to laboratory for routine microbiological investigation were processed.

Upon arrival of CSF samples in laboratory, after standard bacterial culture and gram staining, the remaining CSF (100-300 μ l) was stored under sterile conditions at -80°C until further processing for DNA extraction and PCR. The permission for using CNS fluid samples for this study was taken from physicians of the patients.

For DNA extraction, 100 μ l of CSF was taken in microcentrifuge tube and heated in a boiling water bath for 20 minutes and centrifuged at 10.000 rpm for 1 minute^[7,13]. The concentration and desired DNA content of each supernatant was checked by Nanodrop (ND 1000, USA) instrument.

The selection of universal primers was based on the published work of Lu and Pandit which are complementary to the conserved region of 16S rDNA. The presence of any kind of bacteria can be detected with these primers regardless their species. The sequence of the primers were: primer U1(F): 5'-CCAGCAGCCGCGTAATACG-3' and primer U2(R):

5'ATCGG(C/T)TACCTTGTTACGA CTTC-3'^[4-6].

These primers were synthesized in ATG Co., Denmark.

The reaction mixture contained 5 μ l of DNA extract, 1 μ l (20 pmol) of each U1 and U2 primers, 0.3 μ l (1.5 U) of Super Taq DNA polymerase, 5 μ l of 10X PCR buffer, 2.5 μ l (2.5 mM) MgCl₂, 0.5 μ l ml of dNTPs (0.4 mM) and 34.7 μ l DW (Final reaction volume: 50 μ l).

All reaction mixture constituents were purchased from Fermentase Co, villnius, Lithuania.

PCR amplification was carried out in a MWG thermocycler (USA). The thermal cycling regimen used was as follows: 3 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 45 sec at 55°C, 30 sec at 72°C, and further for a time extension of 5 min at 72°C. Multiple negative (water) and positive (known 16S rDNA) controls were included in every set of PCR reaction^[2,4,5] PCR products (amplicons) were detected by 1.5% agarose gel electrophoresis.

For detection of analytical sensitivity (cut off) of the assay, serial dilutions from known concentration (adjusted to optical density of 0.5 Mc Farlin bacteriological standardized tube, i.e. 10⁸ CFU or microorganisms/ml) of test strains

including *E. coli* ATCC 25972, *haemophilus influenzae* ATCC 35056 and *streptococcus pneumoniae* ATCC 49617 were prepared and after DNA extraction and measurement of DNA contents by Nanodrop instrument, these different serial copy numbers were used as templates in PCR protocol^[13].

Statistical analyses for determining performance characteristics were done by SPSS software version 13.5.

Findings

The analytical sensitivity (cut off) of the assay for all known strains were approximately 10^2 to 10^3 CFU or copy number/ml (about 10 ng/ μ l) of CSF (Fig. 1). From 100 evaluated samples, 36 were culture positive. The most prevalent isolate was *Staphylococcus aureus*. From 100 samples evaluated, 74 were PCR positive. An example of PCR positive sample (996 bp fragment) is shown in Fig. 2.

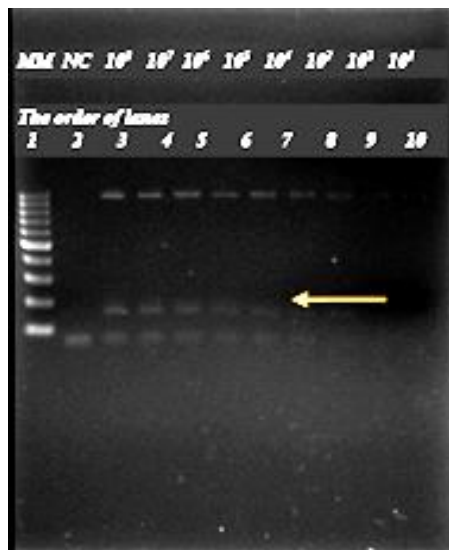


Fig 1: Detection of analytical sensitivity of PCR assay. A dilution series of *E. coli* DNA was analyzed by 16S rDNA PCR and gel electrophoresis. *E. coli* DNA used in the PCR reaction was extracted in amounts from 10^8 (lane 3) to 10^1 (lane10) CFU/ml. DNA extracts (copies) up to 10^2 CFU/ml (about 10 ng/ μ l) produced expected visible (996 bp) band. Negative (lane 2) and 1kb DNA ladder (lane1) included.

Table 1: Comparison of the results of 16S rDNA PCR assay with cultures for detection of suspected bacterial meningitis

PCR	Culture	Positive (n=36)	Negative (n=64)	Total (n=100)
Positive (n=74)		36	38	74
Negative (n=26)		0	26	26

For the evaluation of performance characteristics of the PCR assay, results were compared with CSF cultures as current gold standard. The data are shown in Table 1. According to these data, performance characteristics of the PCR assay were calculated as follows: Sensitivity (false neg.): 100%, specificity (false pos): 40.6%, Positive predictive value (PPV):48.6 and Negative predictive value (NPV):100%. The kappa coefficient obtained as 0.33 by SPSS software calculation.

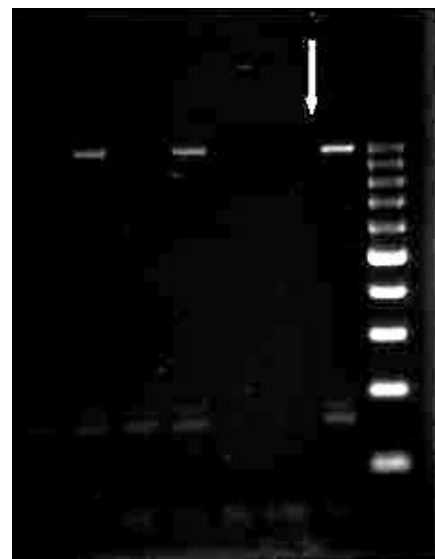


Fig. 2: An example of positive and negative CSF samples detected by 16S rDNA PCR assay. From top to bottom: 1kb ladder (size marker), positive control, negative control, -, +, -, + and- samples. The arrow indicates the band of expected size (996 bp). The first and second negative samples belong to a culture negative patient but the third negative sample belongs to a culture negative patient who has taken antibiotic. The first positive sample belongs to a culture negative patient with antibiotic consumption and the second positive sample belongs to a culture positive patient.

Discussion

In the present study, we evaluated 16S rDNA PCR for diagnosis of bacterial meningitis. Our results showed that the PCR findings were not in good agreement with those obtained from bacteriologic data. Although some performance characteristics like sensitivity (100%) and negative predictive value (100%) were in good correlation with culture results, the specificity (40.6%) and positive predictive value (48.6%) of the assay were low. It means that false positive results of the PCR assay are high or alternatively, some culture results were false negative. The latter condition is more probable. As noted earlier, the consumption of antibiotics or the presence of some special kinds of bacteria could result in such circumstance^[6,7,8].

To elucidate the above mentioned conflict, the correlation of two methods was evaluated by calculating the kappa coefficient which became 0.33. This value indicates low correlation between two methods. Some studies have also described low correlation of sensitivity and specificity of the two methods^[6-8]. Pandit recommended simultaneous use of CSF culture and CSF PCR especially for culture negative samples^[4]. On the other hand, in CSF PCR, antibiotic sensitivity (antibiogram) of the causative bacterial agent is not determined^[7].

The overall culture or PCR positive samples in our study were high because, as stated earlier, we used high cellular content samples for better comparison of the methods.

The most important troubling factor in universal CSF PCR is the contamination of samples during CSF collection, DNA extraction, reaction mixture preparation, amplification and detection procedures^[2,5,6]. Using extra pure reagents and application of real time PCR (RT-PCR), Corless et al improved the results of the PCR assay^[3]. However, in cases where dead or inactive bacteria are present in samples, e.g. in post antibiotic therapy, the results of PCR assay may become falsely positive, i.e. the use of 16S rDNA PCR can not detect active or inactive bacteria.

Conclusion

Regarding the advantages and disadvantage in performance characteristics of the conventional CSF culture and universal CSF 16S rDNA PCR, it is strongly recommended to use both methods in clinical practice, particularly in suspicious contaminated samples, presence of fastidious or slow growing bacteria and antibiotic consumption as well as to get rapid results.

Acknowledgment

This work was financially supported by Qazvin University of Medical Sciences (grant No. 28/20/174). We thank members of microbiology laboratories as well as pediatric clinicians of the academic hospitals of Qazvin University of Medical Sciences for their contributions and permission to use samples for this study.

Conflict of Interest: None

References

1. Numbiar S, Kalavati S. Infection of the central nervous system. In: Mahon C, Manuselis G, Lehman D (eds). Textbook of Diagnostic Microbiology. 3rd ed. Philadelphia: Saunders. 2006; Pp: 853-66.
2. Xu J, Millar BC, Moore JE, et al. Employment of broad-range 16S rRNA PCR to detect aetiological agents of infection from clinical specimens in patients with acute meningitis. Rapid separation of 16S rRNA PCR amplicons without the need of cloning. J Applied Microbiol. 2003;94(2):197-206.
3. Corless CE, Guiver M, Borrow R, et al. Simultaneous detection of Neisseria meningitidis, Haemophilus influenzae, and Streptococcus pneumoniae in suspected cases of meningitis and septicemia using real-time PCR. J Clin Microbiol. 2001;39(4):1553-8.
4. Pandit L, Kumar S, Karunasagar I, et al. Diagnosis of partially treated culture-negative bacterial meningitis using 16S rRNA universal primers and restriction endonuclease digestion. J Med Microbiol. 2005;54 (Pt 6):539-42.

5. Lu J, Perng C., Lee S, Wan C. Use of PCR with universal primers and restriction endonuclease digestion for detection and identification of common bacterial pathogens in cerebrospinal fluid. *J Clin Microbiol.* 2000; 38(6):2076-80.
6. Schurman T, Boer R, Kooistra-smid AM, et al. Prospective study of use of PCR amplification and sequencing meningitis of 16S ribosomal DNA from CSF for diagnosis of bacterial meningitis in a clinical setting. *J Clin Microbiol.* 2004;42(2):734-40.
7. Nakhjavani FA, Bonakdar F, Kalani M.T, et al. Detection of haemophilus influenzae type B in cerebro spinal fluid of suspected children with meningitis by PCR. *Med J Islamic Republic Iran.* 2005;19(2):181-4.
8. Kotilinen P, Jalavi J, Meurman O, et al. Diagnosis of meningococcal meningitis by broad range bacterial PCR with cerebrospinal fluid. *J Clin Microbiol.* 1998;36(8):2205-9.
9. Jaton K, Sahli R, Bille J. Development of polymerase chain reaction assay for detection of *Listeria monocytogenes* in clinical cerebrospinal fluid samples. *J Clin Microbiol.* 1992;30(8):1931-6.
10. Abdel Salam HA. Direct PCR assay for detection of *Neisseria meningitidis* in human cerebrospinal fluid. *Folia Microbiol (Praha).* 1999;44(6):689-94.
11. Lin HW. Use of universal polymerase chain reaction assay and endonuclease digestion for detection of *Neisseria meningitidis*. *J Microbiol Immunol Infect.* 2004;37(6):371-4.
12. Liu Y. Development and evaluation of 16S rDNA microarray for detecting bacterial pathogens in cerebrospinal fluid. *Exp Biol Med.* (Maywood). 2005;230(8):587-91.
13. Plesis M, Smith AM, Klugman KP. Rapid detection of penicillin resistant streptococcus pneumoniae in cerebrospinal fluid by a semi nested PCR strategy. *J Clin Microbiol.* 1998; 36(2):453-7.