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A two-step protocol for the identification of the etiology of bacterial meningitis in cerebrospinal fluid by PCR-amplification of the 16s ribosomal RNA gene

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Objective We assessed the utility of a two-step PCR-based assay for the detection and identification of the etiology of bacterial meningitis directly in 46 CSF specimens.

Method Bacterial DNA was extracted and detected using a set of universal primers that flank a 370-bp sequence on the 16S rRNA gene which was conserved among all bacteria. Identification of bacteria was done using genus/species-specific primers for *Haemophilus* spp., *Streptococcus pneumoniae, and Staphylococcus aureus*, previously used as probes. These primers are complementary to a variable region within the 370-bp conserved sequence. Primers that amplify the Insertion Sequence IS6110 were used for the identification of *Mycobacterium tuberculosis*. DNA extracted from ATCC strains were used as positive controls.

Results Our data have shown that of the 46 CSF specimens collected from patients on admission, initially suspected to have bacterial meningitis. Ten specimens were positive by PCR and these were from patients shown to have bacterial meningitis as determined by the clinical picture (fever, headache, vomiting, neck stiffness and others), laboratory testing (CSF culture and cytochemical analysis) and neurodiagnostic testing. The remaining 36 PCR negative samples were shown later to have other diseases, such as febrile seizure, sepsis, fungal or viral meningitis or encephalitis. Accordingly, there was a correlation between PCR results for the presence of bacteria in CSF specimens and the final clinical and laboratory diagnosis. When these cases were identified using specific primers, 7 were found to be *Haemophilus* spp., 1 *S. pneumoniae*, 1 *S. aureus*, and 1 *M. tuberculosis.*

Conclusion The test proved to be sensitive detecting bacteria down to 5 CFU/ml, specific since primers amplified exclusively specific target sequences and rapid since it was achievable in 9 to 10 hours.

Key words PCR, bacteria and meningitis

Introduction

Meningitis, the inflammation of the covering meninges may be caused by multiple organisms, yet bacteria remain the common causative agents (1). Bacterial meningitis being an infection of the CNS, where the normal host defense system is absent, is a clinical problem if prompt therapy is delayed. Early diagnosis, however, greatly improves the outcome. Accordingly, it is often essential to identify the bacterial pathogen in order to choose the preferred antimicrobial agents and to manage the infection control aspects of bacterial meningitis. Since the clinical features are often non specific especially in infants, different diagnostic methods are used (2). Neurodiagnostic tests such as computed tomography (CT), electroencephalography (EEG), and magnetic resonance imaging (MRI) of the brain are sometimes performed especially to suggest tuberculous bacterial meningitis (TBM). However, their abnormalities are non specific for a disease entity (3). Cytochemical analysis of the CSF is routinely done to detect certain characteristic inflammatory changes. Similarly, the CSF analysis is neither sensitive nor specific (2). Culture of bacteria remains the gold standard for the specific identification of the etiology, yet it is time consuming and it is often limited by its low sensitivity. Bacteria are not always easily recovered mainly due to prior antibiotic therapy, or it requires a long incubation time (24 hours to weeks) especially when a slow growing organism is involved (2). The other laboratory techniques include microscopy, serology, and antigen detection tests. These are more rapid but they may lack both sufficient sensitivity and specificity giving only circumstantial evidence of the bacteria. An optimal etiological concentration is often required to achieve a reliable positive result. In addition, these are limited by non-specificity and cross-reactivity (2).

Recently, the Polymerase Chain Reaction (PCR) was established for the diagnosis of bacterial meningitis (4,5). The PCR proved to increase the sensitivity, specificity, and speed of diagnosis of these conditions, and consequently it made a significant impact on management of meningitis. However, most of the PCR-based assays which detect bacterial DNA in CSF samples often include Southern Hybridization with genus/species-specific probes to identify the bacteria (4).

In this work, our objectives were to assess the utility of a two-step PCR-based assay for the detection and identification of the etiology of bacterial meningitis in CSF specimens, using universal primers for bacterial detection and genus / species-specific primers for the specific identification of the etiology.

Material and Method

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Case Definition: CSF samples were collected from patients suspected by the clinician to have bacterial meningitis, based on the clinical picture of the patients on admission. Typical symptoms of meningitis were sometimes available and these included: meningeal irritation, fever, vomiting, and neck stiffness or bulging fontanel with or without confusion. Whenever, these signs with or without neck stiffness were accompanied by the presence of hydrocephalous, tuberculous meningitis (TBM) was highly suggested rather than pyogenic bacterial meningitis. However, PCR diagnosis for both types of bacterial meningitis was performed whenever the picture was not clear and the state of the patient was deteriorating. The remaining cases were PCR diagnosed for bacterial meningitis as suggested by the clinician based on a variety of non-specific symptoms.

Source of CSF Samples: A total of 46 CSF samples were collected from 46 patients (one year period) for the diagnosis of bacterial meningitis, during the standard clinicalworkup of the patient. The ages of patients ranged from few days old (neonates) to 32 years old. The samples were provided from the American University of Beirut Medical Center (AUB-MC), Beirut, Lebanon. Lumbar puncture was performed on all patients except for one where CSF was collected from a Ventricular Peritoneal (VP) shunt. CSF specimens were PCR assayed immediately, otherwise they were stored at 4^0C up to 1 week, or at -20^0C for long term storage (more than one week).

***Medical Records of Patients*:** Clinical and laboratory information on patients with pyogenic bacterial meningitis, tuberculous meningitis, or any other neurological disease was collected from the medical records or clinicians. The information included 1) the clinical picture of the patient on admission, 2) culture and microscopy of CSF and blood, 3) CSF cytochemical analysis, 4) detection of bacterial antigen in CSF, 5) neurodiagnostic tests done (brain computed tomography; CT scan, electroencephalography; EEG, and Magnetic resonance imaging [MRI]), 6) response to therapy administered (antibiotics/and antituberculous drugs).

***Reference strains*:** Reference strains used in the study as positive controls included: *H. influenzae* ATCC 49766, *S. pneumoniae* ATCC 49619, *S. aureus* ATCC 29213 and *M. tuberculosis* DNA provided by Dr. R. Cooksey, Centers for Disease Control and Prevention (CDC).

***Culture*:** Culture of CSF specimens was done according to standard procedures (6).

***DNA extraction*:** A ten microliters portion of cultured reference strains or untreated CSF was overlaid with 2 drops of mineral oil in 0.5 ml microcentrifuge tube, and boiled for 15 minutes (7,8). Alternatively, DNA extraction was done according to Van Ketel et al. (9).

***PCR*:** PCR was initially done on DNA extracts using the universal primers RW01, 5'-AAC TGG AGG AAG GTG GGG AT-3', and DG74, 5'-AGG AGG TGA TCC AAC CGC A-3', which amplify a 370-bp region of the 16S rRNA gene, highly conserved among all bacteria (4). CSF specimens exhibiting the 370-bp amplicon were further identified in separate amplification reactions using the genus or species-specific primers of *Haemophilus, S. pneumoniae, S. aureus* and *M. tuberculosis.* The primers used for *Haemophilus, S. pneumoniae* and *S. aureus* were initially designed and used as probes by Greisen et al. (4). They anneal to genus or species-specific internal sequences within the 370-bp region of the detected bacteria obtained using universal primers RW01 and DG74 and were used along with the appropriate universal primer to amplify a DNA fragment within the 370-bp segment specific to each bacterium. For *Haemophilus*, the primer pair employed was RDR125: 5'GGA GTG GGT TGT ACC AGA AGT AGA T3' and DG74, which flank a 124-bp region within the 16S rRNA gene of *Haemophilus.* For *S. pneumoniae*, the primer pair, RDR462 5' AAC TGA GAC TGG CTT TAA GAG ATT A 3' and RW01 was used. These primers flank a 132- bp region within the 16SrRNA gene of *S. pneumoniae.* For *Neisseria meningitidis*, COR28 5' AAG CCG CGA GGC GGA GCC AAT CT 3' was used along with DG74 and these flank a 279-bp fragment also located within the 370-bp region. The pair of primer detecting *S. aureus* consisted of RDR327 5' GCC GGT GGA GTA ACC TTT TAG GAG C 3' and DG74. The length of the resulting amplicon was 105-bp. Identification of *M. tuberculosis*, was done using a specific pair of primers designed by Eisenach et al. (10) to amplify an Insertion Sequence IS6110 in the *M. tuberculosis* complex and the expected band size is about 123-bp. The sequences of these primers, T4 and T5, are: 5'-CCT GCG AGC GTA GGC GTC GG 3' and 5' CTC GTC CAG CGC CGC TTC GG 3' respectively.

PCR-amplification was carried in a 100 ul reaction mixture containing 61.5 ul sterile distilled water, 16 ul of deoxyribonucleoside triphosphate (dNTPs) (0.2 mM), 10 ul of 10 x PCR buffer (100 mM Tris-HCl[pH 8.3], 500 mM Kcl, and 15 mM $MgCl_2$), 1 ul of each primer-pair (0.3 ug/ul), and 0.5 ul of Taq DNA polymerase (5U/ul) and 10 ul of the DNA extract from reference strains and CSF specimens. The PCR reaction was performed in a thermal minicycler (MJ Research, Watertown, Mass, USA) using appropriate programs optimized for each primer set.

The PCR program for all primer pairs used except those specific for *M. tuberculosis* (T4 and T5) involved 34 cycles: each cycle consisted of: denaturation at 95C for 1 minute, annealing at 55C for 1 minute, and extension at 72C for 1 minute. Additional extensionat 72C for 10 minutes followed. The PCR program used for *M. tuberculosis* specific primers involved 25 amplification cycles. Each cycle consisted of: denaturation at 94^0C for 2 minutes, annealing of primers at 68^0C for 2 minutes, and primer extension at 72^0C for 2 minutes. The amplified DNA was detected after electrophoresis in 1.5% Seakem agarose (FMC BioProducts, Rockland, ME, USA) gel stained with 5 mg/ml ethidium bromide. The gel was visualized on a UV-light transilluminator (HaakeBuchler, Saddle Brook, NJ, USA) and photographed using polaroid films type 667, black and white (Polaroid Ltd., St. Albans, Hertfordshire, England).

***Sensitivity and specificity testing*:** The sensitivity of amplification using universal bacterial primers was estimated using purified bacterial cells added to normal CSF. Starting from $150x10^6$ CFU/ml as initial bacterial concentration, further dilutions 7were done to reach a concentration of 2.5 CFU/ml. Ten microliters aliquots from the same dilutions were used for PCR amplifications as described previously and for culture on chocolate or blood agar plates. Culture plates were incubated overnight at 37^0C with or without 5%CO_2 and colonies counted. Specificity of primers was determined by PCR amplification of DNA from reference strains with all primer pairs sets.

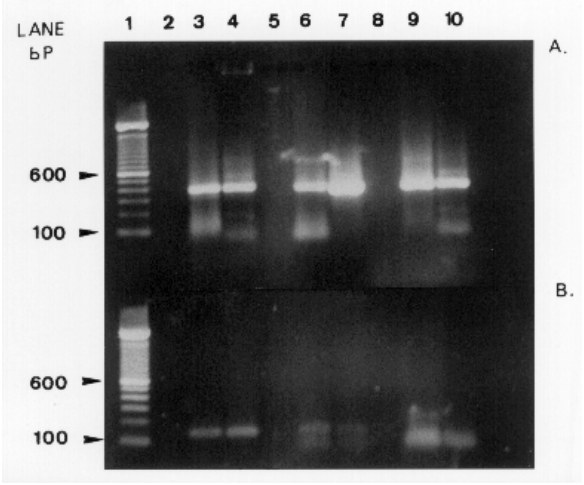

Figure 1. Agarose gels of representative amplicons using:
A. Universal primers RW01 and DG74: lane 1, 100-bp ladder; lane 2, empty; lanes 3,6,&9;positive control for PCR (*H. influenzae* ATCC 49766 DNA); lanes 4,7 & 10; PCR-positive sample (370-bp band); lanes 5 & 6 empty.
B. Genus/species-specific primers and a Universal primer: lane 1, 100 bp-ladder; lane, empty; lane 3, positive control for PCR (*H. influenzae* ATCC 49766 DNA); lane 4, PCR-positive *H. influenzae* CSF sample (124-bp band); lane 5, empty; lane 6, positive control for PCR (*S. pneumoniae* ATCC 49619); lane 7, PCR-positive *S. pneumoniae* CSF sample (132-bp band); lane 8, empty; lane 9, positive control for PCR (*S. aureus* ATCC 29213); lane 10, PCR-positive *S. aureus* CSF sample (105-bp band).

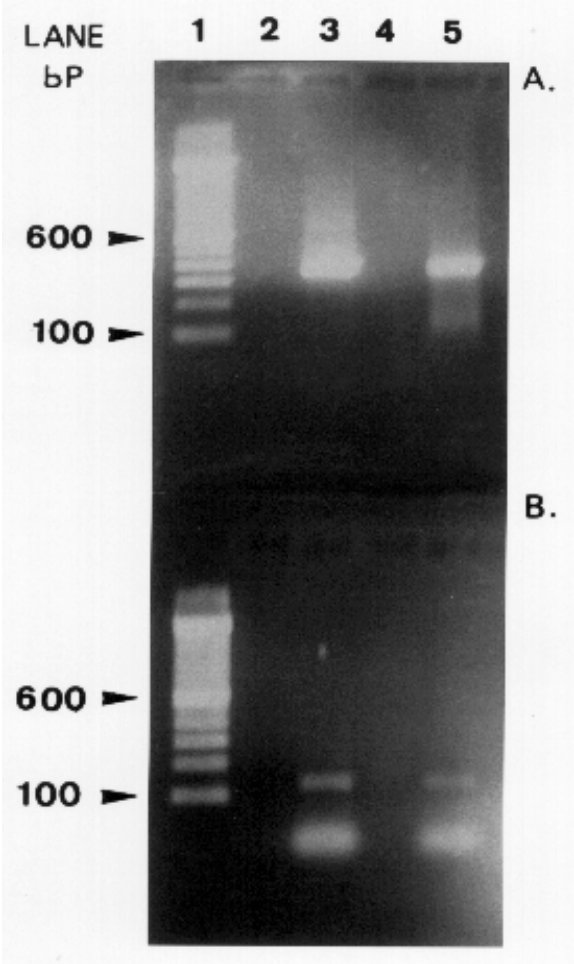

Figure 2. Agarose gels of representative amplicons using:
A. Universal primers RWO1 and DG74: lane 1, 100-bp ladder, lane 2, negative control for PCR; lane 3, positive control for PCR (*H. influenzae* ATCC 49619); lane 4, negative control for extraction; lane 5, PCR-positive CSF sample (370 bp).
B. *Mycobacterium tuberculosis* specific primer pair T4 and T5: lane 1, 100-bp ladder, lane 2, negative control for PCR; lane 3, positive control for PCR (*M. tuberculosis* clinical isolate CDC number H37 RV DNA); lane 4, negative control for extraction; lane 5, PCR-positive CSF sample (123-bp band).

Results

Two of 46 CSF samples were culture-positive for *H. influenzae*. Culture was negative on the remaining samples. Ten CSF samples including the 2 culture positive, were PCR-positive using universal primers and yielded the 370-bp band, indicating the presence of bacterial DNA in the samples. All the 10 PCR-

positive specimens belonged to cases of bacterial meningitis, based on clinical and laboratory information provided by the patients' medical records. Further PCR identification of the same samples at the genus or species level using specific primers, revealed that: 7 (70%) were positive for *Haemophilus* spp., 1 (10%) was positive for *S. pneumoniae*, 1 (10%) was positive for *S. aureus* and 1 (10%) was positive for *M. tuberculosis*. The age of patients PCR positive for *Haemophilus* spp.ranged from 5 months to 8 years. The age of patients PCR positive for *S. pneumoniae, S. aureus* and *M. tuberculosis*, was respectively, 2, 3 and 32 years. The remaining 36 PCR-negative samples were shown to have other diseases as revealed by the patients' medical records such as febrile seizures, sepsis, fungal or viral meningitis or encephalitis. Sensitivity testing has shown that PCR product of bacteria tested was detected down to a concentration of 5 CFU/ml while the detection limit of culture was 150 CFU/ml (Table I). Specificity testing has shown that all primers amplified exclusively specific target sequences.

Table I. PCR and Culture Sensitivity Results

Bacterial Concentration (CFU/ml)	PCR Results	Culture Results
1. $150x10^6$	+	+
2. $150x10^4$	+	+
3. 15000	+	+
4. 150	+	+
5. 50	+	-
6. 25	+	-
7. 5	+	-
8. 2.5	-	-

Discussion

In the present study, we evaluated a two-step PCR-based assay for the detection and identification of the etiology of bacterial meningitis in CSF samples collected from infected Lebanese patients. Ten of 46 CSF samples tested were PCR-positive using the universal primers. A high correlation was observed when PCR data was compared to clinical information. Similar observations were stated by other investigators. Radstrom et al (8) found that about 94% of the CSF samples from patients suspected to have bacterial meningitis were PCR-positive for the presence of bacteria. Those reported to be non-bacterial meningitis cases were PCR-negative. The test was sensitive, specific and rapid since it was achievable in 9 to10 hours.

Of 10 PCR-positive patients, 7 were PCR-positive for *Haemophilus* using genus-specific primers. All these were pediatric cases and had the typical meningitis signs: headache, vomiting, fever, neck stiffness or a bulging fontanel. Their clinical records revealed that only two of these were positive for *H. influenzae* none of these were vaccinated against *H. influenzae*. All 7 cases had improved clinical state after antibiotic therapy except for one patient who continued to suffer from irreversible neurological problems due to hydrocephalus complication.

The remaining 3 cases were due respectively to *S. pneumoniae, S. aureus,* and *M. tuberculosis*. *S. pneumoniae* was detected by PCR in the CSF of a partially treated pediatric bacterial meningitis case which was preceded by an upper respiratory tract infection. The CSF was only PCR-positive for *S. pneumoniae*, and it was PCR-negative for both *Haemophilus* and *N. meningitidis*. Clinical data confirmed PCR results. PCR was previously reported to detect *S. pneumoniae* in CSF with a similar high sensitivity regardless of culture results (7).

The CSF exhibiting an amplicon specific for *S. aureus* was collected from a pediatric case with bacterial meningitis suspected to be due to post VP shunt infection. PCR was done on this CSF sample using both *S. aureus* and *S. epidermidis* specific primers since *Staphylococci* are known to be the main causative agents of VP shunt induced bacterial meningitis (11). The clinical diagnosis confirmed PCR data. Meningitis has resolved when the shunt was removed and antibiotic therapy administered. The PCR-positive CSF for *M. tuberculosis* was collected from a patient with typical tuberculous meningitis (TBM) clinical symptoms which developed in a slowly progressive manner. CSF cytochemical analysis, the presence of hydrocephalus revealed by CT scan of the brain and EEG results were highly suggestive of TBM. Finally, the patient showed clinical improvement with anti-tuberculous drugs therapy. All this information clearly confirmed PCR data for TBM.

In conclusion, our data showed that the two-step PCR-based assay is efficient for identification of the etiology of bacterial meningitis. In addition, it is sensitive, specific, time efficient (9-10 hours duration) and less cumbersome than other available PCR-probe combination techniques used in the diagnosis of bacterial meningitis. This may allow the assay to serve as an early diagnostic method for this disease.

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