Application of 16S rDNA based seminested PCR for diagnosis of acute bacterial meningitis

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Background & objectives: The diagnosis of bacterial meningitis remains a challenge to the clinician because of its rapid lethal course lacking the consistency to particular clinical signs and symptoms. Moreover, in many clinical settings use of rampant and short course antibiotic therapy prior to lumbar puncture reduces the chance of isolation of bacteria in CSF culture making the diagnosis difficult. The present study was done to evaluate a multiplex seminested PCR based method for rapid diagnosis of bacterial meningitis even after initiation of antibiotics.

Methods: A 16S rDNA based PCR technique was evaluated using universal bacterial primers to detect any bacterial pathogen in CSF samples. The simultaneous use of three species-specific primers in a multiplex and seminested PCR format was done to identify Streptococcus pneumoniae, Haemophilus influenzae and Neisseria meningitidis within 4 h.

Results: Analysis of 267 CSF samples obtained from suspected cases of acute bacterial meningitis revealed 94 per cent concordance in results for conventional (Gram stain and culture) and molecular methods. Conventional techniques failed to detect five PCR positive samples where clinical diagnosis, cell count and biochemical findings of CSF supported the evidence of infection. The overall sensitivity, specificity, positive predictive value and negative predictive value of 16S rDNA PCR were 79.24, 97.6, 89.36 and 94.88 per cent respectively when culture was considered as gold standard. The detection limit of 16S rDNA PCR was determined to be 1000 cfu/ml of E. coli and 4000 cfu/ml of S. pneumoniae.

Interpretation & conclusion: The results suggest that 16S rDNA PCR can be used as a valuable supplementary test in routine clinical practice for diagnosis of acute bacterial meningitis in hospital setting.

Key words Bacterial meningitis - CSF - PCR - 16S rDNA

In spite of many improvements in health care system, acute bacterial meningitis remains a life threatening infectious emergency accounting a mortality rate not less than 10 per cent. An early diagnosis can reduce the mortality as well as post-meningitic neurological sequelae, which are more common in children and immunocompromised patients. Currently, the diagnosis of meningitis is mostly done by Gram stain and culture though the outcome is affected in many clinical situations by
the use of antibiotics before lumbar puncture. CSF-serology for detection of bacterial antigens is not widely practiced because of its lower sensitivity and specificity compared to Gram stain and culture.6

The use of PCR for rapid diagnosis of bacterial meningitis has the potential to overcome the poor sensitivity of culture when antibiotic had been already introduced.7-8. Strategic application of universal primers has been found to be superior to conventional techniques for detection of bacteria in sterile body fluids. However, the relative risk of contamination remains the major problem of using universal PCR alone as a specific test to confirm a diagnosis.7 The specificity of broad range universal PCR has been satisfactorily improved by further analysis of the PCR products by different methods like restriction fragment length polymorphism (RFLP), probing or sequencing.7-8 In the present study, we evaluated a 16S rDNA based PCR to amplify and detect bacterial DNA in the CSF and its applicability in routine diagnostic laboratories. A seminested strategy has been used for simultaneous detection of the three most common pathogens of bacterial meningitis, Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitidis as previously described.6

Material & Methods

Specimen collection: A total of 267 cerebrospinal fluid (CSF) samples were received from patients under 14 yr suspected of suffering from acute bacterial meningitis admitted to the paediatric and neurosurgery wards of All India Institute of Medical Sciences (AIIMS) Hospital, New Delhi, between July 2002 to December 2003. These clinical specimens were transported to the Microbiology Laboratory as a part of the routine diagnostic work-up of the admitted patients.

Medical records were reviewed for CSF biochemistry and white blood cell (WBC) count. On the basis of findings of culture, Gram staining and WBC counts in CSF, patients were divided into 3 groups. The “infected group” consisted of patients with microbiological evidence of infection as determined by positive result of either culture or Gram staining of the CSF specimens; the “probable infection group” included patients who did not have microbiological evidences of infection, as determined by negative results of gram staining and culture, but had positive cellular responses in CSF; and “no evidence of infection group” consisted of patients who had no microbiological or cellular evidences of infection.

Processing of samples: In the bacteriology laboratory, all CSF samples were subjected to direct smear and Gram staining followed by culture on 5 per cent sheep blood agar and chocolate agar (Himedia, Mumbai, India). One-hundred microlitre (100 µl) of CSF sample was also inoculated into brain heart infusion broth (BHI). The culture plates and the BHI broth were incubated in 5-10 per cent CO2 at 37°C for 24 h. Isolates obtained were identified by Gram stain and standard biochemical tests like Indole production (Hi-media), triple sugar iron agar test (Hi-media), citrate utilization (Hi-media), motility and urease production. About 500-600 µl of each CSF sample was stored at -70°C until further processing for DNA extraction and PCR assay. In case of repeat samples, only the first sample was considered for PCR.

PCR strategy: A seminested multiplex PCR was designed using two sets of primers, universal and specific primers as previously described by Radstrom et al. The universal primers (u3, ru8) amplify a specific 1000 bp region of the 16S rDNA of almost all bacteria. Specific primers were used for S. pneumoniae (STREP), H. influenzae (HI), and N. meningitidis (NM), the three commonest aetiological agents of childhood bacterial meningitis, which amplify a specific region within the already amplified 1000 bp 16S rDNA by universal primers.6 However, instead of carrying out two separate PCR reactions as described by Radstrom et al, we performed serial optimization experiments to standardize a PCR protocol using universal and the specific primers simultaneously in a single reaction.

Bacterial strain used: S. pneumoniae ATCC 49619, H. influenzae ATCC 33391, N. meningitidis ATCC 13077 group B strains were used for standard DNA preparation. Approximately 106 cfu/ml of each bacterial strain was taken and DNA extraction and purification were done by conventional phenol-chloroform method as described by Sambrook et al.

<table>
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<tr>
<th>Primer code</th>
<th>Primer sequence</th>
<th>Product size</th>
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<tr>
<td>u3</td>
<td>5’TGT CCT GCA GCC GCG GTA AT 3’</td>
<td>1000 bp</td>
</tr>
<tr>
<td>ru8</td>
<td>5’AAG GAG GGG TGT GTA C 3’</td>
<td></td>
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<tr>
<td>STREP (S. pneumoniae)</td>
<td>5’ GTA CAA CGA GTC GCA AGC</td>
<td>293 bp</td>
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<tr>
<td>HI (H. influenzae)</td>
<td>5’ CCT AAG AAG AGC TCG AG 3’</td>
<td>543 bp</td>
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<tr>
<td>NM (N. meningitidis)</td>
<td>5’TGT TGG GCA ACC TGA TTG 3’</td>
<td>710 bp</td>
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Table I. Oligonucleotide primers used in this study
DNA extraction from CSF samples: About 500 µl of CSF sample was boiled for 10 min followed by centrifugation at 12,000 g × 10 min and 2.5 µl of supernatant was used as template DNA.

PCR reaction: A master mix solution consisting of 2.5 µl of DNA template, 2.5 µl of 10× buffer, 1 µl of each primer, 1.25U Taq DNA polymerase (Takara Bio Inc., Japan), in a total volume of 25 µl was prepared. After 5 min initial denaturation at 94°C, the reaction mixture was run through 35 cycles (Techne Progene Thermal Cycler, USA) of denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C, and extension for 30 sec at 72°C, followed by an incubation for 15 min at 72°C. Finally, 5 µl of PCR product was electrophoresed on a 1.5 per cent agarose gel containing 10 µl of 0.1 per cent ethidium bromide in 0.5×Tris-borate-EDTA buffer. After electrophoresis, amplicons were made visible by UV light irradiation (BIORAD, USA) (Figs 1 & 2).

Sensitivity and specificity of PCR: In order to determine the sensitivity (in terms of cfu) of the PCR assay, CSF specimens from non meningitis (culture and PCR negative) patients were spiked with serial 10-fold dilution of E. coli ATCC 25922 and S. pneumoniae ATCC 49619. The analytical sensitivity of the 16S rDNA PCR was found 10^3 cfu/ml and 4 × 10^3 cfu/ml for E. coli and S. pneumoniae respectively (Fig. 3).

To verify the universal nature of primer u3 and ru8 for any bacterial DNA, several common bacterial species (E. coli, Staphylococcus aureus, Pseudomonas aeruginosa, N. meningitidis, S. pneumoniae, Enterococcus sp, Klebsiella sp, Acinetobacter sp, Mycobacterium tuberculosis) were subjected to 16S rDNA PCR amplification without using species-specific primers NM, STREP, and HI. Broad-range PCR assay was able to amplify all the bacterial strains tested. Fungal DNA like Cryptococcus neoformans and Candida albicans was not amplified. Use of specific primers NM, STREP, and HI had correctly amplified only the respective organisms; no false positive result was found.

To evaluate the effect of systemic antibiotics on sensitivity of culture and PCR assay, a culture and PCR negative CSF specimen obtained from a patient who had been administered systemic antibiotic (ceftriaxone) was spiked with E. coli ATCC 25922 to have the final concentration of 10^6 cfu/ml and incubated at 37°C. Repeated subculture of the CSF specimen (10 µl each) was carried out at regular interval of 0, 2, 4, 6, 8, 10, 12, 14, 16 and 18 h to examine the recovery of viable bacteria. At 0-12 h incubation interval, no bacteria was isolated (sensitivity < 10^3 cfu/ml), at 14 h

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**Fig. 1.** Amplification of standard DNA of S. pneumoniae, H. influenzae, N. meningitidis and E. coli by standardized PCR protocol. Lane 1: S. pneumoniae; Lane 2: H. influenzae; Lane 3: N. meningitidis; Lane 4: E. coli and Lane 5: negative control. Molecular marker size (lane M) in base pairs is given on the left side of the picture.

**Fig. 2.** Application of 16S rDNA PCR on clinical samples. Lane 1-3: Positive controls. Lane 1: S. pneumoniae; Lane 2: H. influenzae; Lane 3: N. meningitidis; Lane 4: negative control. Lane 5: sample 1 positive (1000bp). Lane 6-12: sample 2-8 negative. Molecular marker size (lane M) in base pairs is given on the left side of the picture. Note: Sample 1 was also positive by culture showing growth of Klebsiella pneumoniae.
interval 10³ cfu/ml and at 16 h interval 10⁷-10⁸ cfu/ml were recovered in culture. Application of 16S rDNA PCR assay detected bacteria in all the samples taken at 2, 4, 8 and 12 h respectively.

Statistical analysis: Statistical analysis was done for comparison of three groups of patients by standard descriptive statistics like mean and standard deviation. Comparative analysis was performed by Kruskal-Wallis Chi-square test and ANOVA.

Results

A total of 267 CSF samples were subjected to Gram stain, bacterial culture and 16S rDNA based PCR assay. Microbial growth in bacterial culture was detected in 53 (19.8%) of CSF samples (Table II). Total number of samples examined from neurosurgery ward was 117 (43.8%). Remaining 150 (56.2%) samples were received from general paediatric ward. Bacterial growth was found in 32 of 117 (27.3%) samples collected from neurosurgical patients. *S. aureus* (9 isolates) and *Pseudomonas* (9 isolates) remained the most common bacteria isolated from neurosurgery followed by *Acinetobacter* sp (7 isolates), *E. coli* (2 isolates), *K. pneumoniae* (2 isolates), *Citrobacter* sp (2 isolates), and *Proteus vulgaris* (1 isolate). The growth was detected among 21 of 150 (14%) samples from paediatric ward. The bacteria isolated were *Acinetobacter* sp (5 isolates), *E. coli* (4 isolates), *Pseudomonas* (3 isolates), *K. pneumoniae* (3 isolates), *S. aureus* (3 isolates), *Enterobacter* sp (2 isolates), *S. pneumoniae* (1 isolate).

Broad-range 16S rDNA based PCR could detect organisms among 52 (19.4%) specimens. Two cases of *S. pneumoniae* have been identified by PCR, one was culture positive and the other was culture negative but Gram staining showed presence of diplococci. No case of *H. influenzae* or *N. meningitidis* was detected.

Bacterial pathogen was not detected in majority of samples, *i.e.*, 80.2 per cent (214 of 267 specimens) were negative by culture, 93.3 per cent (249 specimens) by Gram staining and 80.5 per cent (215 specimens) by PCR (Table II). For 251 (94%) samples, conventional and molecular methods were in complete concordance. Gram staining report was also negative in all those 11 culture-positive but PCR-negative cases. Organisms identified by culture among 11 PCR negative samples were *S. aureus* (4 isolates), *Acinetobacter* sp (3 isolates), *P. aeruginosa* (2 isolates), *E. coli* (1 isolate) and *K. pneumoniae* (1 isolate). In all of the five CSF specimens, where Gram staining was positive but culture did not show growth, PCR successfully detected the presence of organisms. In those samples, the high cell count (average: 340/mm³) and CSF biochemistry values (average protein: 317.2 mg/dl and glucose: 56 mg/dl) also went in favor of infection. For five samples, which were negative both by culture and Gram stain, the broad range 16S rDNA based PCR yielded positive result (Table III). All these five samples were negative for *S. pneumoniae*, *H. influenzae* or *N. meningitidis* by

| Table II. Results of culture, Gram stain and PCR for CSF samples (n=267) |
|-----------------|-----------------|-----------------|
|                 | Culture No. (%) | Gram stain No. (%) | PCR No. (%) |
| Positive        | 53 (19.8)       | 18 (6.7)         | 52 (19.5)   |
| Negative        | 214 (80.2)      | 249 (93.3)       | 215 (80.5)  |

Fig. 3. Determination of the sensitivity of 16S rDNA PCR with seminested strategy. (A) PCR results with *E. coli* DNA from 10⁷ to <10 organisms (lanes 1 to 8) and no bacteria (lane 9). (B) PCR results with *S. pneumoniae* from 4 × 10⁷ to <10 organisms (lanes 1 to 8) and no bacteria (lane 9). Molecular marker size (lane M) in base pairs is given on the left side of the picture.
specific PCR primers. These five samples were also negative for *M. tuberculosis* by Zeihl-Neelsen staining and culture of CSF.

The ‘infected group’ consisted of 58 patients who were diagnosed to have meningitis by either Gram stain or culture and 47 of them were PCR positive. The proportion of infection was higher among neurosurgical patients (33/117 or 28.2%) than patients from general pediatric ward (25/150 or 16.7%). The mean protein level was comparatively higher than the patients of other two groups (Table IV).

Broad-range 16S rDNA PCR detected organisms in five samples among “probable infection group”. All these patients had elevated WBC count (mean 318 cells/mm$^3$) in comparison to “infected group” (*P*>0.05) with predominance of polymorphs and simultaneous peripheral blood leukocytosis. Of 5 positives mean CSF protein level was 209.4 mg/dl and mean CSF-glucose was 39.4 mg/l (*P*>0.05).

**Discussion**

Analysis of CSF samples collected from children under 14 years with a clinical suspicion of acute bacterial meningitis revealed 19.9 per cent cases to be culture positive for various organisms. *Pseudomonas* sp. and *S. aureus* were the commonest organisms identified from post-neurosurgical meningitis cases, 40 per cent of those cases were on ventricular shunt. *Acinetobacter* sp. was the commonest organism in the pediatric ward. Incidence of *S. pneumoniae* was low (5%). Among the Gram-negative bacteria, 82 per cent were third generation cephalosporin resistant and 55.5 per cent of *S. aureus* were MRSA. The only strain of *S. pneumoniae* isolated was sensitive to penicillin. *H. influenzae, N. meningitidis, group B streptococci* or *Listeria monocytogenes* were not isolated. The spectrum of organisms and their nature of sensitivity pattern in our study also suggest that most of these meningitis cases were of nosocomial origin. Besides, as AIIMS is a referral tertiary care centre,
almost all the patients have received broad-spectrum intravenous antibiotic before lumbar puncture. This reduces the chance of isolation of the three common capsulated organisms, which are sensitive to most of the commonly used antibiotics\textsuperscript{12}.

Comparison of conventional methods and 16S rDNA PCR showed a good agreement for 94 per cent of the samples. Broad-range 16S rDNA PCR correctly identified 79.2 per cent of all culture positive cases. In one sample where culture yielded a strain of \textit{S. pneumoniae}, 16S rDNA PCR with seminested strategy was also able to identify the organism within 4 h. In another sample, which was negative by culture but Gram staining showed Gram-positive diplococci, final identification of \textit{S. pneumoniae} was possible by 16S rDNA PCR. In this sample the broad-range seminested PCR showed supplementary value to culture.

Broad-range 16S rDNA PCR was able to detect organism in five cases where conventional techniques were negative but other laboratory data like peripheral leukocytosis, elevated CSF cell count and CSF protein and low CSF glucose inclined towards bacterial meningitis. The cell counts and CSF biochemistry from the available data suggested probable bacterial meningitis that was confirmed by 16S rDNA PCR. Moreover, all these five samples were re-tested with appropriate control to rule out contamination during PCR process. As all these patients were on antibiotics, the conventional techniques like Gram stain and culture failed to detect organism showing the potential value of PCR in diagnosing culture-negative bacterial meningitis. All these patients were discharged after appropriate treatment.

In a simple experiment, we failed to isolate \textit{E. coli} ATCC 25922 in culture up to 12 h from a spiked ($>10^6$ cfu/ml) CSF specimen that had been obtained from a patient on intravenous ceftriaxone. This indicates that the antibiotic pressure pushes the bacteria into viable but non-cultivable state for a certain period that depends on the nature of antibiotic, organism and site of infection\textsuperscript{13}. 16S rDNA based PCR successfully detected organisms even in non-cultivable state showing its practicability to diagnose meningitis in antibiotic treated patients.

When culture is considered as gold standard, the sensitivity and specificity of broad-range seminested PCR were 79.24 and 97.6 per cent with the positive and negative predictive values of 89.41 and 94.9 per cent respectively. Compared to culture, sensitivity of Gram stain was only 24.5 per cent. The relative low positive predictive value of the PCR can be explained by the fact that PCR remained positive in five culture-negative samples where cell count and CSF biochemistry went strongly in favour of infection. Broad-range 16S rDNA PCR indeed was negative for all samples in the ‘no evidence of infection’ group indicating false positivity is not common.

Sensitivity of our universal PCR was comparatively lower than the study by Radstrom \textit{et al}\textsuperscript{6}. They followed a two-step PCR protocol, where the second step PCR increased the lower limit of detection of their assay from $3 \times 10^5$ cfu/ml (after 1\textsuperscript{st} reaction) to $3 \times 10^2$ cfu/ml (after 2\textsuperscript{nd} reaction). Recently following the similar protocol Luciane \textit{et al}\textsuperscript{14} reported 92.3 per cent sensitivity of broad range seminested PCR applied to CSF samples of 182 children with suspected bacterial meningitis. They reported one case of false positive result contaminated with \textit{N. meningitis} DNA. We tried amplification in a single step PCR putting all the primers together to eliminate the chance of contamination during transfer of PCR products. Moreover, we used boiling method of DNA extraction that might have affected the overall sensitivity of PCR\textsuperscript{7}.

Detection of bacteria in the CSF sample depends on number of cfu, different concentrations of PCR inhibitors and different efficacies of extraction methods of DNA from bacteria. Reports from various studies indicate that in a significant proportion of CSF samples, amplification process is problematic due to presence of PCR inhibitors. Ratnamohan \textit{et al}\textsuperscript{15} described that the inhibitory effects may be due to increased levels of proteins and increased cell numbers, but not due to cellular DNA. Our findings also supported this observation as CSF protein and cell count were high in 11 culture-positive but PCR-negative samples. We also observed presence of inhibitors in some clear CSF where cell count and protein concentration were normal, when we tried to associate the presence of inhibitors with the turbidity of CSF (data not shown). We did not find any such association because amplification was possible in many CSF samples irrespective of turbidity, whereas some clear samples have not been amplified. The finding was also supported by Luciane \textit{et al}\textsuperscript{14}. The role of PCR inhibitors interfering the performance of CSF-PCR has also been recently observed by Welinder \textit{et al}\textsuperscript{16} who reported the sensitivity of broad range PCR as 64.1 per cent in comparison to culture of CSF samples from patients with community acquired bacterial meningitis.
As characterization of various PCR inhibitors is still not known in different population, it is difficult to predict the exact success rate of amplification from different CSF samples.

In conclusion, overall performance of broad-range 16S rDNA PCR was found to be promising as a screening test for diagnosis of acute bacterial meningitis, particularly in a hospital setting where most of the patients are on antibiotic. Using the seminested strategy identification up to species level can be done within 4 h provided the appropriate species-specific primers are applied according to the epidemiological pattern in aetiology of meningitis. In fact, single step seminested PCR is simpler and more rapid than RFLP, DNA probing or sequencing which are not always feasible in routine laboratory set up.

References


