Original Article

Access this article online
Quick Response Code:

Website:
www.jacmjournal.org

DOI:
10.4103/jacm.jacm_11_18

Nested polymerase chain reaction targeting 16S rRNA gene in diagnosis of acute bacterial meningitis

Harshita Lal, Abhinav Agarwal[1], Ashish Kumar Singh, Rajniti Prasad[1], Anil Kumar Gulati, Pradyot Prakash

Abstract:

CONTEXT: Diagnosing acute bacterial meningitis (ABM) among children presenting to tertiary health care settings is often difficult because of prior administration of antimicrobials.

AIMS: The present study was an attempt to diagnose cases of ABM in children with the help of nested polymerase chain reaction (PCR).

SETTINGS AND DESIGN: It is a prospective observational study, in which a total of 84 clinically suspected cases and cerbrospinal fluid (CSF) biochemical parameters suggestive of ABM were included in the study.

METHODS AND MATERIAL: CSF samples were subjected to Gram staining, bacterial culture and biochemical identification tests as well as panbacterial nested PCR targeting 16S rRNA gene sequence. Subsequently, a nested multiplex PCR for detection of the three fastidious organisms, viz. *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae,* was performed.

STATISTICAL ANALYSIS USED: The sensitivity, specificity, PPV, NPV, LR+, and LR− of the tests were calculated.

RESULTS: The sensitivity of Gram stain, bacterial culture, and nested PCR targeting 16SrRNA were observed to be 16.21%, 9.45%, and 97.29% respectively. Further, the NPV and LR− the PCR were found to be 83.33 and 0.02 respectively. Species specific nested multiplex PCR was able to detect *S. pneumoniae* (*n* = 7), *N. meningitidis* (*n* = 2) and *H. influenzae* (*n* = 1).

CONCLUSIONS: The results indicates that nested PCR targeting 16S rRNA gene may be used in diagnosis of ABM. Further, nested multiplex PCR targeting the three important fastidious bacterial pathogens in ABM cases has showed their presence in our region.

Keywords:

Haemophilus influenzae, Neisseria meningitidis, *Streptococcus pneumoniae*

Introduction

Acute bacterial meningitis (ABM) is the most common form of suppurative central nervous system (CNS) infection. It is a rapidly progressive, life-threatening condition that requires prompt recognition and treatment. Worldwide, 1.2 million cases of bacterial meningitis are estimated to occur every year, and without treatment, the case fatality rate has been observed to be as high as 70%.[1] Overall, about 50% of bacterial meningitis cases occur in children under the age of five years. The second peak occurs in adolescents aged between 15 and 19 years of age.[2]

The organisms most often responsible for community-acquired bacterial meningitis are *Streptococcus pneumoniae* (~50%), *Neisseria meningitidis* (~25%), Group B streptococci spp. (~15%) and *Listeria monocytogenes* (~10%), and these pathogens

Departments of Microbiology and [1]Paediatrics, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Address for correspondence:
Dr. Pradyot Prakash, Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi - 221 005, Uttar Pradesh, India.
E-mail: pradyot_micro@bhu.ac.in

How to cite this article: Lal H, Agarwal A, Singh AK, Prasad R, Gulati AK, Prakash P. Nested polymerase chain reaction targeting 16S rRNA gene in diagnosis of acute bacterial meningitis. J Acad Clin Microbiol 2018;20:77-83.

constitute more than 90% of ABM pathogens. *Haemophilus influenzae* type b (Hib) accounts for <10% of cases of bacterial meningitis in most series. *N. meningitidis* is the causative organism of epidemics of meningitis every 8–12 years.[3]

Various studies from India and neighbouring countries report that *S. pneumoniae* is the major cause of ABM. Das *et al*.[4] and Mani *et al*.[5] from India reported that around 61%–61.8% of cases of ABM among children are caused by *S. pneumoniae*, whereas Abro *et al*.[6] and Alamgir *et al*.[7] reported that *S. pneumoniae* is associated with 25.58% and 37.5% cases of ABM in Pakistan and Bangladesh, respectively.

In the recent years, there has been a change in the spectrum of bacteria causing community-acquired bacterial meningitis, after the introduction of Hib vaccine and pneumococcal vaccine. The incidence of *H. influenzae* meningitis has dropped down significantly, and today, the most common cause of bacterial meningitis in North America is *S. pneumoniae*.

In children, the clinical manifestations of bacterial meningitis are fever, headache, vomiting, photophobia, nuchal rigidity, lethargy, confusion and coma. The condition deteriorates very rapidly, and therefore, prompt diagnosis and early initiation of antibiotics is very important. The conventional methods for the diagnosis of ABM include cerbrospinal fluid (CSF) examination by direct microscopy (Gram stain) which is quick but nonspecific and has a low sensitivity. Gram stain of CSF culture-positive specimens are usually positive in 75%–90%; but in patients who have received antimicrobial therapy before lumbar puncture positivity decreases to 40%–60%.[8] CSF culture is considered as the 'gold standard'; however, it may yield false-negative results due to antibiotic treatment before sampling.

Various methods have been developed for rapid diagnosis of ABM, based on the detection of bacterial antigens by immunological methods. None of these tests either alone or in combination is entirely satisfactory because of limited sensitivity and specificity. Rapid antigen detection tests may yield false-positive results, particularly when Gram stain results are negative among meningitis patients.[9]

In recent years, molecular diagnostic techniques, such as polymerase chain reaction (PCR)-based methods, have evolved to be the most substantial technical advancement in the field of rapid detection of infection in clinical samples. In various studies, it has been observed that PCR is extremely useful in the early laboratory diagnosis of meningitis, particularly in samples obtained after administration of antibiotics.[10]

In the present study, we attempted to determine the bacterial aetiology among children clinically diagnosed as cases of ABM using conventional tests as well as nested PCR targeting the 16S rRNA gene. Further, nested multiplex PCR (NMPCR) was attempted for the detection of *N. meningitidis, H. influenzae* and *S. pneumoniae*.

Subjects and Methods

This study was carried out on 84 patients between five days and 15 years of age suspected of having ABM, at a tertiary care hospital in North India. Meningitis was suspected based on the following criteria: acute onset of fever (usually >38.5°C rectal or 38°C axillary), headache and one of the following signs: neck stiffness, altered consciousness or other meningeal signs. Among the suspected cases of ABM only those cases, were included in this study in which cytological and biochemical findings of CSF are suggestive of bacterial meningitis, i.e. elevated WBC counts, usually in the range of 1000–5000 cells/mm^3 with neutrophil predominance, typically between 80% and 95%, CSF glucose concentration <40 mg/dL in approximately 50%–60% of patients; a ratio of CSF to serum glucose of 0.4 in children 12 months of age and a ratio of 0.6 is considered to be abnormal in neonates, elevated CSF protein concentration (>45 mg/dl).[3,11] Only those cases were considered as true cases of ABM which were having clinical symptoms and signs along with positive CSF culture and/or positive Gram stain and/or positive for nested PCR targeting the 16S rRNA gene.

Venous blood specimens and CSF samples of all the above cases were obtained and were subjected to routine microbiology investigation, CSF biochemistry, cell count and PCR before administration of antimicrobials. Most of the cases included in this study had already received antibiotics before being referred to the paediatric outpatient department.

The samples were processed employing standard microbiological procedures. CSF sample for PCR was stored at −20°C till DNA isolation was done.

Gram stain

CSF was centrifuged at 1000 rpm for 15 min. After centrifuging, the supernatant was discarded and the deposit was used to make smear for Gram staining.

Bacterial culture

Centrifuged deposit of CSF was inoculated onto blood agar plate enriched with haemophilus spp. growth factor (HiMedia), chocolate agar plate and MacConkey agar. Blood agar and chocolate agar plates were incubated overnight in a candle jar at 37°C. In case no growth observed after 24 h, they were re-incubated for another 24 h.

The blood samples were collected in a blood culture bottle and were incubated at 37°C for 24 h. First sub-culture was done on blood agar and MacConkey agar plates after 24-h incubation. Isolated colonies of bacteria were further identified by biochemical tests.

Polymerase chain reaction

DNA isolation

DNA from CSF samples was extracted by the CetylTrimethylammonium Bromide (CTAB)–Phenol–Chloroform–Isoamyl alcohol method. Briefly, centrifuged deposit of 1 ml CSF was resuspended in 500 µl 10 mM Tris, 1 mM EDTA (TE; pH 8.0) buffer. This was followed by addition of 100 µl of 10% Sodium dodecyl sulphate (SDS) and 3 µl proteinase K (20 mg/ml) and incubated overnight at 37°C. Subsequently, 100 µl of 5 M NaCl and 80 µl of 10% CTAB added, mixed well and then incubated at 60°C for 10 min. This was followed by extraction with Phenol–Chloroform–Isoamyl alcohol. The DNA was precipitated using isopropanol, washed with 70% ethanol and resuspended in 30 µl sterile TE buffer. Five microlitres of this was used as template for PCR. Quantity and quality of extracted DNA was checked by spectrophotometer (NanoDrop®). After validating the DNA extraction results, DNA was stored at – 20°C until PCR was performed.

Polymerase chain reaction-based detection of bacterial pathogens

The PCR-based diagnosis was attempted using two different protocols of nested PCR.

- Detection of bacterial DNA in CSF by nested PCR targeting the pan bacterial 16S rRNA gene.
  - The first PCR protocol, which is a nested PCR, was attempted for bacterial DNA, targeting the 16S rRNA gene sequence employing primers as described earlier by Okhravi *et al*[12]
  - Master mix for the first round of PCR: Master mix (25 µl) for the first cycle of PCR was prepared using ×10 buffer (Merck, GeNei, Bengaluru, India), 10 mM deoxyribonuceotide triphosphate mix (Merck, GeNei, Bengaluru, India), *Taq* polymerase (Merck, GeNei, Bengaluru, India), 10 pmol of consensus forward primer and reverse primers namely F1F5′-TTGGAGAGTTTGATCCTGGCTC-3′ and R1R5′-ACGTCATCCCCACCTTCCTC-3′ (Eurofins, Bengaluru, India), 5 µl DNA template and deionised water (*qs*)
  - The master mix for the first cycle of PCR was subjected to 40 cycles of amplification in the thermal cycler (Bio-Rad, USA); 10 s denaturation at 94°C, 10 s annealing at 57°C and 15 s elongation at 72°C, with a final elongation step extended to 7 min
  - Master mix for the second round of PCR: Master mix (25 µl) for the second round of PCR contained the same constituents as described for the first cycle, except the primers namely F2 F5′-GGCGGCAKGCCTAAYACATGCAAGT-3′ and R2 R5′-GACGACAGCCATGCASCACCTGT-3′ (Eurofins, Bengaluru, India) were used and only 1 µl product of first cycle was used as template
  - The reaction mixture was again subjected to 25 cycles of amplification in the thermal cycler (Bio-Rad, USA) at annealing temperature of 57°C.
- Detection of *N. meningitidis*, *H. influenzae* and *S. pneumoniae* by NMPCR
  - The second PCR protocol is NMPCR for the detection of *N. meningitidis, H. influenzae and S. pneumoniae* targeting species-specific gene employing primers as described in Table 1, of which primers F6, R6, F7, R7, F8 and R8 used in the study are designed and used for the first time.

Designing of the primers F6, R6, F7, R7, F8 and R8

The primers used for the detection of nspA gene of *N. meningitidis* (GenBank Accession No. GU968247.1) namely F3, R3, F4 and R4 were previously described by de Filippis *et al*.[13] For PCR amplification of *S. pneumoniae*, the gene encoding pneumolysin was chosen (GenBank Accession No. GU968247.1). The primers targeting pneumolysin gene used in the first cycle of the PCR

Table 1: Primers employed in *Neisseria meningitidis* polymerase chain reaction (for the detection of *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*

Pathogen	Target gene	Cycle	Primer sequence	Expected size of amplicon
Neisseria meningitidis	*nspA*	First	F3 5'-AGCACTTGCCACACTGATTG-3'	481 bp
			R3 5'-GGAACGGACGTTTTTGACAG-3'	
		Second	F4 5'-TAGGTTCTGCCAAAGGCTTC-3'	354 bp
			R4 5'-CAGTGTTGACTTTGCCGATG-3'	
Streptococcus pneumoniae	Pneumolysin	First	F5 5'-ATTTCTGTAACAGCTACCAACGA -3'	347 bp
			R5 5'-GAATTCCCTGTCTTTTCAAAGTC -3'	
		Second	F6 5'-CCCACTCTTCTTGCGGTCGA - 3 '	208 bp
			R6 5'-GAGCCGTGATTTTTTCATACTG- 3'	
Haemophilus influenzae	*BexDCBA* gene	First	F7 5'-CAGATAACCCACAGGTGCCAG 3'	360 bp
			R7 5'-GCTCTCTTATGCTCGCGAT-3'	
		Second	F8 5'-GCGACACAAACTGCGGATTA-3'	152 bp
			R8 5'-GGCTTGCTTGGTTGGCTAAT-3'	

Primers F6, R6, F7, R7, F8 and R8 used in the present study are designed and used for the first time in the present study. Source: F3, R3, F4, R4: de Filippis *et al*., 2005, F5, R5: Toikka *et al*., 1999

were taken from Toikka *et al*.[14] Primers for the second round (F6 and R6) were designed using online software Primer3 (Primer3.ut.ee). Primers for both the rounds of PCR detection of *H. influenzae* (F7, R7, F8 and R8) was designed using online software Primer3 targeting bexDCBA gene (GenBank Accession No. X54987.1).

Master mix for the first round of PCR: Master mix (25 µl) for the first cycle of PCR was prepared by using ×10 buffer (Merck, GeNei, Bengaluru, India), 10 mM dNTP mix (Merck, GeNei, Bengaluru, India), *Taq* polymerase (Merck, GeNei, Bengaluru, India), 10 pmol of primer F3, R3, F5, R5, F7 and R7 (Eurofins, Bengaluru, India), 5 µl DNA template and deionised water (qs).

The master mix for the first cycle of PCR was subjected to 30 cycles of amplification in the thermal cycler (Bio-Rad, USA) at annealing temperature of 52°C.

The master mix for the second round of PCR: The master mix (25 µl) for the second round of PCR contained the same constituents as described for the first cycle, except the primers F4, R4, F6, R6, F8 and R8 (Eurofins, Bengaluru, India) were used and only 1 µl product of first cycle was used as template.

The reaction mixture was again subjected to 40 cycles of amplification at annealing temperature of 57°C. DNA isolated from biochemically confirmed strain of *S. pneumoniae* has been used as the positive control.

Amplicons obtained after amplification from both PCR protocols were subjected to 1.5% agarose gel electrophoresis along with positive control and 100 bp DNA ladder.

Statistical analyses

The statistical analysis was done considering cumulative positivity of Gram stain, culture isolation and 16S rRNA gene nested PCR as gold standard as utilised previously by Richardson *et al*.[15] The statistical calculations were done following the methods described by Greenberg *et al*.[16]

Ethical issues

The approval for conducting this study was obtained from the Institution Ethics Committee, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India.

Results

Of 84 children enrolled for this study, majority, i.e. 36.9%, were in the age group of >1–5 years followed by 28 days to one year age group which constituted 27.38%.

The result of cytological and biochemical laboratory tests is shown in Table 2. CSF leucocyte count was raised with neutrophilia in 98.8% of suspected cases and CSF C-reactive protein level was raised in 91.6% of the cases. CSF glucose was low in 77.3% of the cases and protein level was elevated in 94.0% of the suspected cases. Eighty-four such cases with suggestive features of bacterial meningitis were included in the study.

Of 84 CSF samples, Gram stain of 12 centrifuged CSF samples showed the presence of organisms whereas only seven samples of CSF yielded bacterial growth in culture. In one of the cases, the same bacteria were isolated from both the blood and CSF samples [Table 2]. On culture isolation, seven CSF samples yielded pseudomonas species (n = 3), *Klebsiella pneumoniae* (n = 2), *Escherichia coli* (n = 1) and *Acinetobacter lwoffii* (n = 1) [Table 3].

A total of 72 samples were positive by 16S rRNA PCR amplification [Table 4a and Figure 1]. The Gram stain had shown the presence of organisms in all the culture-positive cases. In addition, three samples which showed the presence of organisms in Gram stain also yielded 1026 bp amplicon of 16S rRNA sequence by nested PCR. There were two CSF samples which were

Table 2: Results of cytological and biochemical tests used in the diagnosis of acute bacterial meningitis in suspected cases (n=84)

Cytological and biochemical tests	Tests positive	Percentage
CSF leucocytes with neutrophilia (10-10,000/µl)#	83	98.8
CSF glucose (<40 mg/dl)#	65	77.3
CSF protein (protein>45 mg/dl)#	79	94.0

#CSF: Cerebrospinal fluid

Table 3: Bacteria isolated by culture from cerebrospinal fluid and blood of suspected cases of acute bacterial meningitis (n=84)

Bacterial isolates	n (%)
Pseudomonas species	3 (3.57)
Klebsiella pneumoniae	2 (2.3)
Escherichia coli	2* (2.38)
Acinetobacter lwoffii	1 (1.19)

**Escherichia coli* was isolated from both the blood and CSF samples of same patient. CSF: Cerebrospinal fluid

Table 4a: Positivity of polymerase chain reaction, Gram stain and culture among clinically diagnosed patients with acute bacterial meningitis (n=84)

Method	Test positive (%)	Test negative (%)
Nested PCR (16SrRNA)	72 (85.7)	12 (14.2)
Gram stain (centrifuged CSF deposit)	12 (14.2)	72 (85.7)
Culture	7 (8.3)	77 (91.7)

CSF: Cerebrospinal fluid; PCR: Polymerase chain reaction

Gram stain positive but conspicuously negative by both culture and PCR [Table 4b].

Table 4b demonstrates the statistical parameters of the three tests namely Gram stain, culture isolation and nested PCR targeting the 16S rRNA gene in the diagnosis of ABM. Cumulative positivity of all the three tests, i.e. Gram stain, culture isolation and nested PCR, was taken as the gold standard. The sensitivity and likelihood ratio for a negative test result of nested PCR was turned out to be 97.29% and 0.02, respectively.

When the 72 PCR-positive samples were subjected to multiplex nested PCR targeted for *S. pneumoniae, N. meningitidis and H. influenzae*, the PCR protocol detected 7 (9.7%) cases of *S. pneumoniae*, 2 (2.7%) cases of *N. meningitidis* and 1 (1.3%) case of *H. influenzae* [Figure 2].

Discussion

ABM remains a major cause of mortality and long-term neurological sequel in India and worldwide. In the United States from 1978 through 1981, studies showed that more than 80% cases of ABM were caused by *S. pneumoniae*, *H. influenzae, N. meningitidis* and *H. influenzae*.[17] However, currently, *S. pneumoniae* is a major cause of childhood bacterial meningitis whereas the incidence of *H. influenzae* meningitis has declined dramatically following the introduction of Hib vaccine in the United States.[18]

In India, the largest epidemic of ABM on record began in New Delhi in the winter of 1984.[19] Meningococcal disease in India is endemic in Delhi and sporadic cases occurred in Haryana, Uttar Pradesh, Rajasthan, Sikkim, Gujarat, West Bengal and Odisha. In the last decade, the incidence of meningitis due to *H. influenzae* in India has changed, and *S. pneumoniae* has emerged as the major cause of community-acquired bacterial meningitis followed by *H. influenzae* and *N. meningitidis*.[6] As compared to data from the Western countries, the incidence of meningitis caused by *H. influenzae*, *N. meningitidis* and *L. monocytogenes* is lesser in Southeast Asia including India. On the contrary, Gram-negative bacilli such as *K. pneumoniae* and *Pseudomonas aeruginosa* are increasingly being recognised as important pathogens of community-acquired as well as nosocomial meningitis.

In our study, we attempted to know the aetiological profile of ABM in children (five days to 15 years) in our region using conventional as well as molecular diagnostic methods.

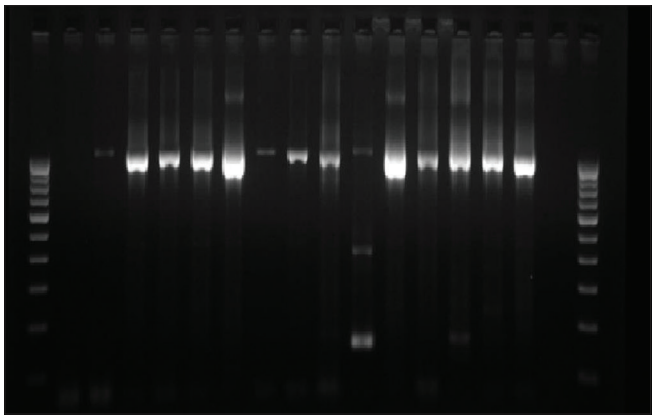

Figure 1: Gel picture of 16SrRNA amplicons obtained after nested polymerase chain reaction from CSF samples. Lane 1 and 18: 100 bp DNA ladder; Lane 2: Negative control; Lane 3: Positive control Pneumococcal DNA (1024 bp); Lane 4 to 16: Samples positive for 16S rRNA gene; Lane 17: Samples negative for 16S rRNA gene

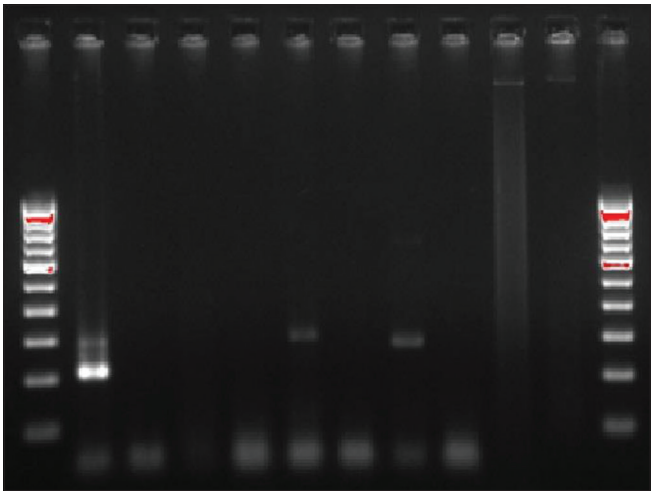

Figure 2: Detection of *Streptococcus pneumoniae* and *Neisseria meningitidis* specific amplicons employing multiplex nested polymerase chain reaction. Lane 1 and 12: 100 bp DNA ladder; Lane 2: Positive control (Pneumococcal DNA) (208 bp); Lane 3: Negative control; Lane 6 and 8: Samples positive for *Neisseria meningitidis* (345 bp); Lane 4, 5, 7, 9, 10, 11: Samples negative for *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae*

Table 4b: Gram stain, culture and polymerase chain reaction in the aetiological diagnosis of acute bacterial meningitis (*n*=74)

Test	Number of samples		Sensitivity	Specificity	NPV	PPV	LR^+	LR^-
	Positive	Negative						
Gram staining	12	62	16.21	100	13.88	100	∞	0.84
Culture	7	67	9.45	100	12.98	100	∞	0.90
nPCR (16S rRNA gene)	72	2	97.29	100	83.33	100	∞	0.02

Two CSF samples which were Gram stain positive were conspicuously negative by both culture and PCR. PCR: Polymerase chain reaction; CSF: Cerebrospinal fluid; NPV: Negative predictive values; PPV: Positive predictive values; LR: Likelihood ratio

Number of attempts have been made to detect the true cases of ABM based on the detection of universal 16S rRNA gene sequence, but most of these protocols are based on the single-round PCR along with restriction fragment length polymorphism, probing or sequencing.[20] Few workers have also attempted to detect some of the important pathogens associated with ABM targeting the species-specific region of 16S rRNA gene sequence employing semi-nested PCR protocol, in which a target sequence is amplified using two consecutive PCR runs. For the second PCR run, one of the primers used in the first run is used again and the other primer is within the target sequence;[21] however, none has observed benefit of the pan bacterial nested PCR targeting the universal sequence in cases of bacterial meningitis. Elsewhere, there are attempts for simultaneous detection of these three organisms in single tube or real-time PCR assay, which monitors the amplification of a targeted DNA molecule during the PCR, i.e. in real-time, and not at its end, as in conventional PCR.[22,23] Although probe-based real-time PCR using specific primers can be very useful when using a multiplex PCR for simultaneous detection of different specific target sequences, it is not of much advantage in performing a broad-range PCR; instead, there may be chances of possible cross-reactions with human DNA and it is also very expensive for routine testing in laboratory.

Nested PCR involves the sequential use of two PCR primer sets: the first primer set is used to amplify a target sequence and the amplicon generated serves as the template for a second amplification using primers internal to those of the first amplicon, thus increasing both sensitivity and specificity of reaction.

Nested PCR has the sensitivity of detecting even one bacterium in a given sample within a few hours, even in the presence of non-target human chromosomal DNA which acts as a relative PCR inhibitor.[24,25] CNS infections are usually paucibacillary and the sample obtained is often very less in volume which makes most of the laboratory tests difficult to perform, whereas nested PCR can be performed even using few microliters of CSF.[26]

In this study,pan bacterial nested PCR was performed targeting the 16S rRNA gene sequrence ,which turned out to be a promising tool with a sensitivity of 97.29% ,far better than that of culture and gram staining. The LR+ and LR− values of the nested PCR of >10 and <0.1, respectively, indicate that the test may be considered as a test of clinical utility in the diagnosis of ABM.

In the present study, NMPCR protocol was designed and used for the detection of three fastidious pathogens, namely *S. pneumoniae*, *H. influenzae* and *N. meningitidis*, in these 72 PCR-positive cases, for which primers were designed for the second round of PCR for *S. pneumoniae*, targeting pneumolysin gene. In addition, primers for both the rounds of PCR detection of *H. influenzae* were designed targeting the *bexDCBA* gene. The NMPCR could detect seven samples positive for *S. pneumoniae*, two samples for *N. meningitidis* and one positive *for H. influenzae*. Similar observation has been made by other authors also,[5,21] which can be attributed to the climate conditions in India.The aetiology of acute bacterial meningitis in children varies in different regions .The ususal pathogens causing meningitis – *H. influenzae*, *N. meningitidis*, *S. pneumoniae* are more commonly found in temperate west, however, in South east Asian countries like India ,owing to the semitropical climate gram negative bacilli such as *E.coli*, *K. pneumoniae* and pseudomonas species, flourish whereas relatively more fragile bacteria such as *H. influenzae*, *N. meningitides*, *S. pneumoniae*, *S. agalactiae* and *L. monocytogenes* do not have a survival advantage.[27] This was also indicated by isolation of common Gram-negative bacteria in the present study.

The poor culture isolation in the present study might be due to prior administration of antimicrobials, as almost all the cases coming to tertiary care set up are on empirical antibiotics before referral. The consequences of prior antibiotic therapy have been most thoroughly studied in children with bacterial meningitis, in whom antibiotics have been shown to decrease the yield of CSF culture and alter the cellular count significantly.[28]

In this study, latex agglutination (LA) antigen detection tests were not performed, taking into account the cost factors and variable sensitivities of different LA antigen detection kits as well as variable sensitivities for different organisms.[29] The aetiological agent in cases which were PCR positive for universal 16S rRNA gene and culture negative could be identified by performing sequencing of the amplicons but was beyond the scope of the present study.

Conclusions

The results indicate that nested PCR targeting the 16S rRNA gene is a useful tool in the diagnosis of ABM. NM PCR targeting the three important fastidious bacterial pathogens in ABM cases has shown the presence of these pathogens in our region, but isolation of Gram-negative bacteria such as pseudomonas species, *K. pneumoniae*, *E. coli* and *A. lwoffii* indicated the diverse aetiology of the disease.

Financial support and sponsorship

The study was supported by the Laboratory Grant, Departments of Microbiology, Institute of Medical

Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India.

Conflicts of interest

There are no conflicts of interest.

References

1. World Health Organization, Centers for Disease Control and Prevention. Laboratory methods for the diagnosis of meningitis caused by neisseria meningitidis, streptococcus pneumoniae, and haemophilus influenzae: WHO manual. Available from: https://www.cdc.gov/meningitis/lab-manual/full-manual.pdf. [Last accessed on 2018 Oct 05].
2. Brouwer MC, Tunkel AR, van de Beek D. Epidemiology, diagnosis, and antimicrobial treatment of acute bacterial meningitis. Clin Microbiol Rev 2010;23:467-92.
3. Roos KL, Tyler KL. Meningitis, encephalitis, brain abscess and empyema. In: Harrison's Textbook of Internal Medicine. 19th ed. Kasper SL, Fauci AS, Hauser SL, Longo DL, Jameson JL, Loscalzo J, editors. New Delhi: McGraw Hills Education; 2015. p. 883-906.
4. Das BK, Gurubacharya RL, Mohapatra TM, Mishra OP. Bacterial antigen detection test in meningitis. Indian J Pediatr 2003;70:799-801.
5. Mani R, Pradhan S, Nagarathna S, Wasiulla R, Chandramuki A. Bacteriological profile of community acquired acute bacterial meningitis: A ten-year retrospective study in a tertiary neurocare centre in South India. Indian J Med Microbiol 2007;25:108-14.
6. Abro AH, Abdou AH, Ali H, Ustadi AM, Hasab AAH. Cerebrospinal fluid analysis acute bacterial versus viral meningitis. Pak J Med Sci 2008;24:645-50.
7. Alamgir F, Miah RA, Saleh AA. Bacterial antigen detection and CRP estimation. Bangladesh Med J 2008;13:17-20.
8. Dalton HP, Allison MJ. Modification of laboratory results by partial treatment of bacterial meningitis. Am J Clin Pathol 1968;49:410-3.
9. Perkins MD, Mirrett S, Reller LB. Rapid bacterial antigen detection is not clinically useful. J Clin Microbiol 1995;33:1486-91.
10. Welinder-Olsson C, Dotevall L, Hogevik H, Jungnelius R, Trollfors B, Wahl M, *et al.* Comparison of broad-range bacterial PCR and culture of cerebrospinal fluid for diagnosis of community-acquired bacterial meningitis. Clin Microbiol Infect 2007;13:879-86.
11. Tunkel AR, Hartman BJ, Kaplan SL, Kaufman BA, Roos KL, Scheld WM, *et al.* Practice guidelines for the management of bacterial meningitis. Clin Infect Dis 2004;39:1267-84.
12. Okhravi N, Adamson P, Matheson MM, Towler HM, Lightman S. PCR-RFLP-mediated detection and speciation of bacterial species causing endophthalmitis. Invest Ophthalmol Vis Sci 2000;41:1438-47.
13. de Filippis I, do Nascimento CR, Clementino MB, Sereno AB, Rebelo C, Souza NN, *et al.* Rapid detection of *Neisseria meningitidis* in cerebrospinal fluid by one-step polymerase chain reaction of the nspA gene. Diagn Microbiol Infect Dis 2005;51:85-90.
14. Toikka P, Nikkari S, Ruuskanen O, Leinonen M, Mertsola J. Pneumolysin PCR-based diagnosis of invasive pneumococcal infection in children. J Clin Microbiol 1999;37:633-7.
15. Richardson DC, Louie L, Louie M, Simor AE. Evaluation of a rapid PCR assay for diagnosis of meningococcal meningitis. J Clin Microbiol 2003;41:3851-3.
16. Greenberg RS, Daniels SR, Flanders WD, Eley JW, Boring JR, editors. Diagnostic testing. In: Medical Epidemiology. 3rd ed. New York: McGraw-Hill; 1996. p. 77-88.
17. Schlech WF 3rd, Ward JI, Band JD, Hightower A, Fraser DW, Broome CV, *et al.* Bacterial meningitis in the united states, 1978 through 1981. The national bacterial meningitis surveillance study. JAMA 1985;253:1749-54.
18. Dery MA, Hasbun R. Changing epidemiology of bacterial meningitis. Curr Infect Dis Rep 2007;9:301-7.
19. Basu RN. Meningococcal meningitis in Delhi and other areas. Community Dis Bull 1985;2:1-10.
20. Kalghatgi AT, Praharaj AK, Sahni AK, Pradhan D, Kumaravelu S, Prasad PL, *et al.* Detection of bacterial pathogens in cerebrospinal fluid using restriction fragment length polymorphism. Med J Armed Forces India 2008;64:29-32.
21. Chakrabarti P, Das BK, Kapil A. Application of 16S rDNA based seminested PCR for diagnosis of acute bacterial meningitis. Indian J Med Res 2009;129:182-8.
22. Rådström P, Bäckman A, Qian N, Kragsbjerg P, Påhlson C, Olcén P, *et al.* Detection of bacterial DNA in cerebrospinal fluid by an assay for simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and streptococci using a seminested PCR strategy. J Clin Microbiol 1994;32:2738-44.
23. Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, Kaczmarski EB, *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;39:1553-8.
24. Tzanakaki G, Tsopanomichalou M, Kesanopoulos K, Matzourani R, Sioumala M, Tabaki A, *et al.* Simultaneous single-tube PCR assay for the detection of *Neisseria meningitidis*, *Haemophilus influenzae* type b and *Streptococcus pneumoniae*. Clin Microbiol Infect 2005;11:386-90.
25. Diggle MA, Clarke SC. Detection and genotyping of meningococci using a nested PCR approach. J Med Microbiol 2003;52:51-7.
26. du Plessis M, Smith AM, Klugman KP. Rapid detection of penicillin-resistant *Streptococcus pneumoniae* in cerebrospinal fluid by a seminested-PCR strategy. J Clin Microbiol 1998;36:453-7.
27. Khan F, Rizvi M, Fatima N, Shukla I, Malik A, Khatoon R. Bacterial meningitis in North India: Trends over a period of eight years. Neurol Asia 2011;16:47-56.
28. Nigrovic LE, Malley R, Macias CG, Kanegaye JT, Moro-Sutherland DM, Schremmer RD, *et al.* Effect of antibiotic pretreatment on cerebrospinal fluid profiles of children with bacterial meningitis. Pediatrics 2008;122:726-30.
29. Talan DA, Hoffman JR, Yoshikawa TT, Overturf GD. Role of empiric parenteral antibiotics prior to lumbar puncture in suspected bacterial meningitis: State of the art. Rev Infect Dis 1988;10:365-76.