Clinical use of multiplex-PCR for the diagnosis of acute bacterial meningitis

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ABSTRACT

Background and Objectives: Prompt and accurate diagnosis of acute bacterial meningitis (ABM) is critical for patient management. We designed and evaluated two sets of multiplex-PCR assays for the simultaneous detection of six major etiologies of ABM i.e., *Streptococcus pneumoniae, Haemophilus influenzae* type b, and *Neisseria meningitidis* in one set and *Listeria monocytogenes, Streptococcus agalactiae, and Escherichia coli* in another set of multiplex-PCR in CSF of patients with suspected ABM. **Methods:** A total of 113 CSF specimens from patients of all ages having clinical features suggestive of meningitis were tested for bacteriological evidence by Gram's smear, culture, and our designed multiplex-PCR. **Results:** Multiplex-PCR assay performed excellently by increasing the overall detection rate by 6% when compared to culture as of total 113 samples tested, 17 (15%) were positive by multiplex-PCR whereas only 9% (10/113) were positive by culture. It detected the DNA in eight culture negative samples revealing the presence of *S. pneumoniae* in three and other possible bacterial pathogens in five of them. Our assay showed a DNA detection limit of 1 pg/µL. Compared to CSF culture, the sensitivity and specificity of the multiplex-PCR were 90% and 92.2%, respectively. **Conclusion:** This study accentuates the importance of multiplex-PCR assay that is efficiently fast and reliable for the diagnosis of acute bacterial meningitis that can substantially improve the diagnosis in culture negative cases, especially in patients who were previously started on antimicrobial therapy.

Keywords: Acute bacterial meningitis, multiplex-PCR, *Streptococcus pneumoniae*

Introduction

Acute bacterial meningitis (ABM) is a serious health emergency warranting early diagnosis and treatment. CSF culture, the gold standard for its diagnostic confirmation, is time-consuming and due to prior antibiotic therapy, produces false negative results.[1] In the current study, we evaluated two sets of multiplex-PCR for detection of six bacterial pathogens from CSF causing meningitis to meet the need for its rapid and accurate diagnosis. In the first set, a seminested PCR strategy was used to simultaneously identify *Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitidis* whereas in the second set, three sets of paired primers were used for simultaneous detection of *Listeria monocytogenes, Streptococcus agalactiae,* and *Escherichia coli.*

Methods

CSF samples

CSF samples from 113 patients of all ages suspected of having ABM (defined below in clinical case definitions for ABM) referred to different OPDs and wards were prospectively received in the Bacteriology laboratory in the Department of Microbiology of our institute between February and May 2019. The study was approved by the Institutional Ethics

Access this article online

Quick Response Code:

Website:
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DOI:
10.4103/jfmpc.jfmpc_1162_21

How to cite this article: Sharma N, Gautam H, Tyagi S, Raza S, Mohapatra S, Sood S, *et al*. Clinical use of multiplex-PCR for the diagnosis of acute bacterial meningitis. J Family Med Prim Care 2022;11:593-8.

Committee (IECPG-379/2019). The samples were subjected to routine bacteriological workup (Gram's staining and culture) and the remaining sample from it was kept at -80°C and thawed immediately before testing by multiplex-PCR. Clinical case definitions for acute bacterial meningitis[2]: (i) Suspected meningitis: Any person with sudden onset of fever (>38.5°C rectal or 38.0°C axillary) and one of the following signs: neck stiffness, altered consciousness, or other meningeal sign; (ii) Probable meningitis: A suspected case with CSF examination showing at least one of the following: turbid appearance; leukocytosis (>100 cells/mm³); leukocytosis (10–100 cells/mm³) AND either an elevated protein (>100 mg/dl) or decreased glucose (<40 mg/dl); (iii) Confirmed meningitis: A case that is laboratory-confirmed by growing (i.e., culturing) or identifying (i.e., by Gram stain or antigen detection methods) a bacterial pathogen (Hib, pneumococcus or meningococcus) in the CSF or from the blood in a child with a clinical syndrome consistent with bacterial meningitis.

Biochemical and cytological CSF parameters

Other CSF biological parameters (cytology, protein, and sugar levels) analyzed for samples were also recorded. CSF total leukocyte count >10 mm³, protein >100 mg/dL, and glucose <40 mg/dL were taken as altered.

Direct smear and bacterial isolation by culture

The total volume of CSF was centrifuged at 3000 g for 5 min. The sediment was cultured onto 5% sheep blood agar, chocolate agar (BD BBL) and, MacConkey agar. The plates were incubated in a BOD incubator at 37°C for 24 hours except for chocolate agar plate, which was incubated in a 5-10% CO_2 incubator at 37°C for 24 hours. A smear was also prepared for gram staining. If bacterial growth was seen on the plates, its identification was done by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF).

Multiplex-PCR

Multiplex-PCR design

Two sets of multiplex-PCR assays were utilized for the diagnosis of ABM: **(1)** In the first set of multiplex-PCR, a seminested PCR strategy in a single reaction was used, as described previously,[3,4] where paired universal primers (U3, U8) and unpaired specific primers [Table 1] for *Streptococcus pneumoniae, Haemophilus influenzae,* and *Neisseria meningitidis* were used. U3 and U8 amplified 1000 bp universal *16S rRNA* gene common for almost all bacteria; specific primers then amplified regions specific to these three species within the 1000 bp amplified product. Detection of other possible bacterial pathogens was an added advantage with the use of universal primers in this multiplex-PCR. (2) In the second set of multiplex-PCR, three sets of paired primers [Table 1] targeting *cspB, hylA* and *16S rRNA* genes specific for *S.agalactiae, L.monocytogenes,* and *E. coli,* respectively, were used for their simultaneous detection. This set of the assay was designed to perform if the CSF sample is positive for only universal gene

in first set of multiplex-PCR to diagnose ABM especially in neonates and in patients with immunocompromised status.

Bacterial strains used for multiplex PCR standardization

ATCC culture strains of *S.pneumoniae (*ATCC 49619)*, N.meningitidis* (ATCC 13090), *H.influenzae* (ATCC 33391)*, E. coli* (ATCC 25922), *L.monocytogenes* (ATCC 19112)*,* & *S.agalactiae* (ATCC 13813) purchased from HiMedia Laboratories were used. They were stored at -80°C in Brain Heart Infusion Broth (BHI) with 15% of glycerol and were cultured on chocolate and blood agar and incubated at 37°C for 18 to 48 hours with 5% CO2, before DNA extraction.

Limit of detection

To determine the lower detection limit of the proposed assay for any bacterial DNA, the extracted DNA of *S.pneumoniae* was quantified by NanoDrop ND, diluted to decreasing concentrations in a range from 1000 pg/μL to 0.1 pg/μL and analyzed by PCR using universal primers.

DNA extraction from CSF samples

DNA from 200 μL of CSF sample was extracted using QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions.

Detection of bacterial meningitis by multiplex-PCR

First set of multiplex-PCR was performed in a 25-μL reaction volume, containing 2.5 μL 10 × PCR buffer (containing $MgCl_2$), 150 μM each deoxyribonucleoside triphosphates, 1.25U of Taq Polymerase (Thermo Scientific), 0.4 μL of 10 pmol universal primers each (U3, U8), 0.2 μL of species specific primers each (STREP, HI, NM), and 3 μL of DNA template. PCR cylcling conditions included initial denaturation for 5 min at 94°C then 30 cycles of denaturation for 30s at 94°C, annealing for 30s at 55°C and extension for 30s at 72°C followed by final extension for 10 minutes at 72°C. In second set of multiplex-PCR, reaction volume of 25 μL was prepared with 1 μL of each EC primers, 1 μL of each LM primers, and 0.2 μL of each SA primers were used with other PCR reagents and DNA template as used in first set. Cycling conditions were set as initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s and extension at 72°C for 30s followed by final extension at 72°C for 6 min. PCR products were separated on a 1.5% (wt/vol) agarose gel stained with 0.1% ethidium bromide for 1 hour at 9.5 V/cm and visualized under UV illuminator. A 100-bp DNA ladder (ThermoFischer) was used as an external molecular size standard.

Results

Demographic characteristics of patients

A total of 113 patients suspected of acute bacterial meningitis were included in the study. The mean age ± SD of patients was

Table 1: Primers used in multiplex-PCR

Primer code	Bacterial species	Sequence 5'-3'	Amplicon size (bp)
Universal	Universal for all bacteria	U3 (Forward) - gtg cct gca gcc gcg gta at RU8 (Reverse) - aag gag ggg atc caa ccg ca	1000 bp
STREP	*S. pneumoniae*	gta caa cga gtc gca agc	293 bp
HI	*H. influenzae*	cct aag aag agc tcg ag	543 bp
NM	*N. meningitidis*	tgt tgg gca acc tga ttg	710 bp
SA	*S. agalactiae*	Forward - aca acg gaa ggc gct act gtt Reverse - acc tgg tgt ttg acc tga act a	255 bp
LM	*L. monocytogenes*	Forward - cat tag tgg aaa gat gga atg Reverse - gta tcc tcc aga gtg atc ga	730 bp
EC	*E. coli*	Forward - tgc ctg atg gag ggg gat aa Reverse - ttt aac ctt gcg gcc gta ct	776 bp

26.12 ± 21.29 years (age of patients <1 year old was taken as 1 year).

Direct smear and bacterial isolation by culture [Table 2]

Out of 113 CSF samples tested, eight samples were positive by smear, whereas ten were positive by culture (*Klebsiella pneumoniae n* = 2, *Acinetobacter species n* = 2, *Staphylococcus epidermidis n* = 1, *Pseudomonas aeruginosa n* = 1, *Enterococcus faecium n* = 1, *Stenotrophomonas maltophilia n* = 1 and two were reported as contaminants but were not excluded from the study to report the correct sensitivity of our PCR assay).

Multiplex-PCR standardization [Figure 1]

The designed multiplex-PCR efficiently amplified standard DNA of *S.pneumoniae, H.influenzae, N.meningitidis*, and *16S rRNA* (universal for bacteria)*, cspB (S.agalactiae), hylA (L.monocytogenes),* and *16S rRNA (E. coli)* genes and showed their respective bands on agarose gel.

Limit of detection

The lowest detection limit of the multiplex-PCR for bacterial DNA in this study was 1 pg/μL

Multiplex-PCR [Table 2]

Of 113 samples tested by multiplex PCR, 17 (15%) were positive. Out of these, three were positive for *Streptococcus pneumoniae* where rest 14 showed bands for only *16S rRNA* gene (universal for bacteria) and were reported positive for any bacterial species other than six bacteria included in our two sets of multiplex PCR panel. No sample was positive for other five bacteria (other than *Streptococcus pneumoniae*) included in the multiplex-PCR panels.

Multiplex-PCR detected DNA in culture-positive and culture-negative CSF samples [Figure 2, Table 3]

Nine out of ten culture positive samples were also positive by multiplex-PCR for *16S rRNA* gene. Additionally, in 7.8% (8/103) culture-negative CSF specimens, *S. pneumoniae* (*n* = 3) and other possible bacterial DNA (n = 5) were detected by multiplex-PCR. Compared to CSF culture, the sensitivity and specificity of the
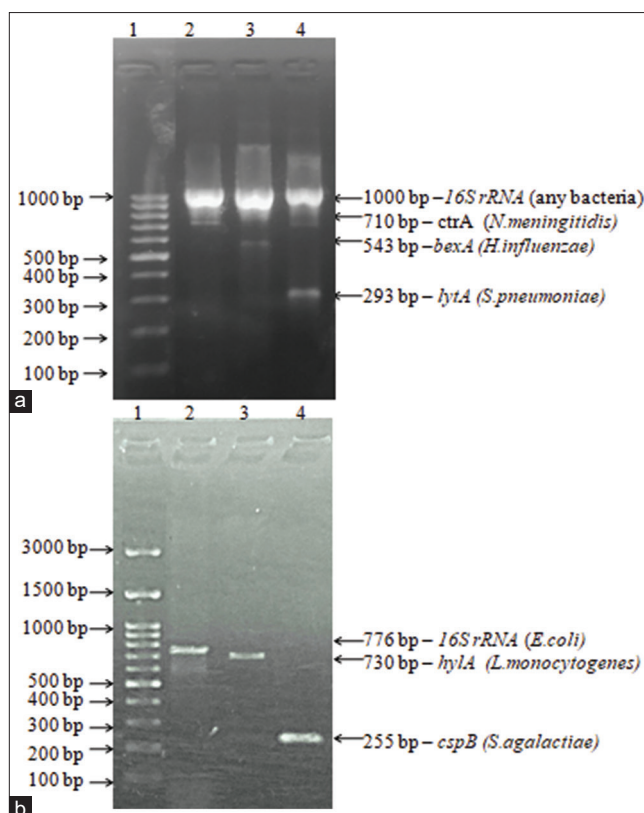
Figure 1: Multiplex-PCR standardization: (a) Amplification of *N.meningitidis, H.influenzae,* and *S.pneumoniae* DNAs in first set of multiplex-PCR. Lane 1-100bp DNA ladder, *2-N.meningitidis, 3- H.influenzae,* and *4-S.pneumoniae*. (b) Amplification of *E. coli, L.monocytogenes* and *S.agalactiae* in second set of multiplex-PCR. Lane 1-100bp ladder, 2-*E. coli,* 3-*L. monocytogenes* and 4-*S.agalactiae*

multiplex-PCR was 90% and 92.2%, respectively, with complete agreement in the identification of organism. Multiplex-PCR yielded positive result in six samples that were negative in both culture and smear. There was one sample that was positive only by culture and three samples that were positive only in smear.

Biochemistry and cytology of CSF samples supported the multiplex-PCR results [Table 4]

Of 17 patients with positive multiplex-PCR, 12 presented clinical evidences of meningitis, with significant biochemical

Table 2: Culture, Gram stain, and multiplex-PCR results for CSF samples (*n*=113)

	Gram stain	Culture	Multiplex-PCR
Positive	8 (7%)	10 (8.8%)	17 (15%)
Negative	105 (93%)	103 (91.2%)	96 (85%)

Table 3: Comparison of multiplex-PCR with culture and gram stain results

	Culture positive	Culture negative, smear negative	Culture negative, smear positive	Total
PCR positive	9 (91%)	6 (6%)	2 (50%)	17 (15%)
PCR negative	1 (9%)	92 (94%)	3 (50%)	96 (85%)
Total	10	98	5	113

Percentages are column percentages

### Table 4: CSF biochemistry and cytology

	Mean (multiplex-PCR positive, *n*=12)	Mean (probable meningitis, no bacteriological evidence, *n*=26)
CSF TLC (cells/mm³)	3360.45	649.23
CSF glucose (mg/dl)	52.75	49.84
CSF protein (mg/dl)	237.41	263.5

and cellular alterations in their CSFs whereas for five patients, these parameters were found to be normal. Twenty-six patients, despite fulfilling the criteria for probable meningitis with altered levels of CSF cellular counts and biochemistry, showed no evidence of bacterial detection in their smears or PCR or culture examinations.

Discussion

Even with advances in vaccine development and the availability of newer drugs, the mortality rate due to meningitis, remains considerably high in India. More than 90% of the world's cases of meningitis are caused by *S.pneumoniae, H. influenzae* & *N. meningitidis*.[5] Patients' susceptibility to its bacterial etiology seems age-related. Usually, in neonates, *Listeria monocytogenes, Streptococcus agalactiae, Escherichia coli* and, other Enterobacteriaceae members are the etiological agents[6]; from 1 month to 15 years, *Streptococcus pneumoniae, Haemophilus influenzae, Neisseria meningitidis* are the most frequent etiologies[7,8] whereas in adults, these are *Streptococcus pneumoniae* and *Neisseria meningitidis*.[9,10] The sensitivity of CSF culture, the gold standard method for its microbiological confirmation is around 85% in patients without antibiotic therapy prior to sample collection[1,11,12] with 30% more reduction under antibiotic therapy,[13] which limits its utility. A common and rapid method of diagnosis, Gram staining, alone or in combination with latex agglutination test also has limited sensitivity.[8,9] Polymerase chain reaction (PCR) based methods though have shown to provide the additional aid in the diagnosis of meningitis with better sensitivity, specificity, and rapidity that detects pathogenic DNA even in samples
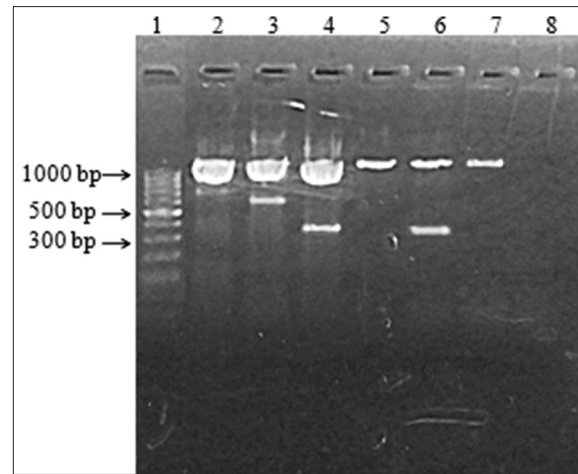
Figure 2: Multiplex-PCR results on CSF samples: Lane 1- 100bp DNA ladder; 2,3,4-Positive controls of first set multiplaex-PCR (; 5,7- Samples positive for *16S rRNA* gene, 6- Sample positive for *16S rRNA* & *Streptococcus pneumoniae*; 8- Negative control

with low bacterial load without being dependent on its growth in culture.[3,13-16]

In previous studies, PCR techniques have shown high sensitivity and specificity for the detection of ABM etiologies and have focused mainly on the simultaneous detection of only three important etiologies of ABM i.e., *S. pneumoniae, H. influenzae,* and *N. meningitidis* using multiplex-PCR and multiplex real-time PCR.[3,17-19] Through this study, we aimed to develop and validate two sets of multiplex-PCR for rapid and accurate diagnosis of these important etiologies of acute bacterial meningitis in addition to the identification for three more bacterial causes i.e., *E. coli, L.monocytogenes,* and *S.agalactiae* that are important causes of ABM in neonates and in patients with compromised immune status.

Of 113 specimens, only 10 (9%) were culture positive [Table 2]. This low rate of positivity in CSF culture is similar to the results of previous studies[15,20] and is attributed to either the use of antibiotics prior to sample collection or small bacterial load in CSF or even the poor quality of specimen. Table 3 shows that 9/10 culture-positive samples were also positive for multiplex-PCR, showing that the sensitivity of this method i.e., 90% was comparable to the gold standard. This finding is similar to those reported by Upadhyay *et al.* (85.7%) and Wang *et al.* (80%).[21,22] The specificity of our assay was however much higher (92.2%) than these studies (76.7% by Upadhyay *et al*,; 76.4% by Wang *et al.*) Eight culture-negative samples were positive by multiplex-PCR. These patients were on antibiotic treatment before CSF sample collection but as DNA based detection methods do not require viable cells, its diagnostic ability is therefore less affected by prior antibiotic treatment. For six samples, these results are also supported by significant cellular and biochemical alterations in their CSF samples, whereas for two samples presence of organism was indicated in Gram stained smear.

A total of 17 (15%) out of 113 samples tested were positive by multiplex-PCR, whereas only 9% samples were positive by culture [Table 2]. These percentages are higher than those reported by Albuquerque *et al.*[15] who found 9% positivity by multiplex-PCR and 6% by culture; the ratio of percentages of multiplex-PCR positives to culture was however similar. Other studies have also reported higher positivity by multiplex-PCR,[23-25] however, a recent study by Sharma *et al.*[26] did not report a significant difference in detection rate by multiplex-PCR (8.59%) and culture (7.55%). Furthermore, a study conducted by Seth *et al.*[18] reported higher positivity in culture than by PCR. This could be because of not including universal primers for the identification of other possible organisms. 9/17 samples were culture positive and multiplex-PCR positive only for 16S rRNA gene. These were identified in culture as *Klebsiella pneumoniae n = 2, Acinetobacter species n = 2, Staphylococcus epidermidis n = 1, Pseudomonas aeruginosa n = 1, Stenotrophomonas maltophilia n = 1* and two were reported as contaminants. Eight multiplex-PCR positive samples were culture negative, out of which three were detected as *S.pneumoniae.* On the whole, the multiplex-PCR results increased the overall detection rate by 6% when compared to culture. No other bacterial species included in the multiplex PCR panel was detected by multiplex PCR. This is because *S.pneumoniae* among all six etiologies is the most common cause of infection in ABM patients. Also, after the introduction of *H. influenzae* type b vaccine for children between 2 months and 5 years of age in Universal Immunization Programme, it was possible to observe a great decline in the incidence of *H. influenzae* among children.[27]

In our study, 26 patients who had the clinical manifestations of meningitis [Table 4], an increased number of leukocytes and/or a high protein level, and/or low glucose level in their CSF samples, were found with no evidence of the bacterial detection in their smears, culture or multiplex-PCR. Also, there was one sample that was positive only by culture that showed the growth of *E. faecium.* Three other samples showed the presence of bacteria only in smear with evidence of cellular derangements. The failure of detection by multiplex-PCR in these specimens could be due to the small amount of DNA in the sample, beyond the detection limit of the assay and/or the presence of a PCR inhibitory substance (which may be high amount of protein or leukocytes). The lowest concentration of DNA for bacterial detection was found to be 1 pg/μL indicating high sensitivity of the assay.

The limitations of the current study are that it was designed as a laboratory-based, single-center, prospective approach evaluation study and the total number of samples testing positive for specific organisms in the multiplex-PCR panel was low, being the initial phase study. This impeded us to calculate the positive and negative predictive values of the multiplex-PCR assays.

Our study has several significant strengths. The multiplex-PCR proposed in the study has a differential ability to simultaneously identify six important etiological agents of bacterial meningitis within 5 hours with low per sample cost. The automated multiplex-PCR systems such as FilmArray® System, are also emerging as useful modalities in the diagnosis of ABM, but their relatively higher cost makes their utility less feasible.[28]

In the context of its utility in clinical practice, this multiplex-PCR can provide the clinicians a prompt, comprehensive and accurate diagnosis for the initiation of early treatment of acute bacterial meningitis to reduce the mortality and long-term neurological sequelae associated with ABM. Moreover, the assay provides a valuable addition in cases with high rates of culture-negative results as it includes the testing for not only six bacterial pathogens included in the panel, but also for any other possible bacterial pathogen present in the sample responsible for causing acute bacterial meningitis. With traditional (singleplex) PCR however, a clinician has a limitation to order each test independently, which puts him under pressure of selecting a correct test or ordering multiple individual tests but multiplex-PCR assay allows comprehensive testing in a shorter time frame. The implementation of such tests for ABM has been reported to in fact reduce the duration of empiric antimicrobial therapy and possibly the length of hospital stay when compared with conventional diagnostic methods.[29]

In summary, there were 15% positive pathogen detections by our multiplex-PCR assay while only 9% pathogen detections were recorded by gold standard method, culture. Multiplex-PCR detected DNA in eight culture negative samples with *S. pneumoniae* in three and other possible bacterial pathogens in five of them with the use of universal primers. The detection limit of the assay was found to be 1 pg/μL with sensitivity and specificity of 90% and 92.2%, respectively.

Conclusions

In conclusion, the proposed multiplex-PCR technique is fast and reliable for the diagnosis of acute bacterial meningitis that can substantially improve the diagnosis in culture negative cases, especially in patients who were previously started on antimicrobial therapy. Thus, the use of this assay into routine diagnostic testing is suggested for the diagnosis of acute bacterial meningitis that would supplement other diagnostic tests, and would potentially limit the unnecessary exposure of antibiotic therapy and hospitalization.

Financial support and sponsorship

Conflicts of interest

There are no conflicts of interest.

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