

IMPACT OF MOLECULAR METHOD FOR THE DIAGNOSIS OF ACUTE BACTERIAL MENINGITIS IN A TERTIARY HEALTH CARE CENTRE IN NORTH INDIA

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Background. Acute bacterial meningitis is one of the significant infectious diseases that add an immense burden to the health system. Proper management of meningitis is an invincible need to overcome the severe consequences.

Objectives. The aim of the study is detection of the etiological agents of acute bacterial meningitis by PCR.

Methods. Total 267 CSF samples collected from suspected bacterial meningitis cases were processed for the detection of *S. pneumoniae*, *H. influenzae*, *N. meningitidis*, *E. coli* and Group B streptococci by conventional and molecular diagnosis method. CSF was inoculated on Blood, chocolate and MacConkey agar plates and incubated at 37 °C for 24-48 hrs. Bacteria grown were identified by Matrix assisted laser desorption/ionization time of flight (MALDI-TOF). Multiplex PCR of the enlisted bacteria was performed using DNA extracted from CSF by DNA extraction kit (Qiagen, USA).

Results. 5 (1.87%) out of the total 267 CSF samples were culture positive (3 *S. pneumoniae* and 2 *E. coli*) and 28 (10.49%) had detectable DNA by conventional PCR. Out of these 28 samples, 20 (71.43%) were *S. pneumoniae* and 8 (28.57%) were *E. coli*. 15 (53.57%) out of total *S. pneumoniae* were present in children below 5 years of age. Similarly, *E. coli* was predominant in neonates. Sensitivity and specificity of the PCR was 100% and 95-98% respectively.

Conclusions. *Streptococcus pneumoniae* is the commonest cause of community acquired bacterial meningitis in children below five years of age. Hence, for the promising outcome, PCR should be implemented for the diagnosis of acute bacterial meningitis.

KEY WORDS: cerebrospinal fluid (CSF); acute bacterial meningitis; PCR; MALDI-TOF.

Introduction

Bacterial meningitis is one of the dangerous infectious diseases of central nervous system (CNS). Meningitis is an inflammation of delicate membrane known as meninges covering brain and spinal cord and marked by intense headache, fever and nuchal rigidity. Despite advances in treatment, acute bacterial meningitis (ABM) is the major cause of mortality and morbidity in developed countries as well as in developing ones [1]. In India and other developing countries mortality rate varies 16-32% [2-5]. Individuals with suspected meningitis require prompt diagnosis and treatment. Delay in management can result in poor outcome of the disease [6].

The most common bacteria causing ABM are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Listeria*

monocytogenes, Group B *Streptococci* and *E. coli* [7]. Etiological agents of ABM vary globally depending on age, geographical area and immune status [8, 9]. Hence, there is need for regularly reviewing of bacterial meningitis.

Culture is considered as the 'Gold standard' for the diagnosis of acute bacterial meningitis as well as equally important for antibiotic susceptibility testing of the causative agents that account for treatment [10]. However, it takes longer for identification of the pathogens and its outcome is influenced by intake of antibiotics prior to lumbar puncture and number of bacteria (CFU/ml) present in the CSF. To overcome this diagnostic glitch, polymerase chain reaction (PCR) is gaining momentum for a rapid diagnosis of ABM [11-17]. Hence, this experiment have been designed and performed to detect the etiological agents of community acquired bacterial meningitis by molecular method as PCR does not rely on viability of the organism.

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Methods

A perspective study was designed and carried out at the Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India. Total 267 cerebrospinal fluids (CSF) samples from suspected acute bacterial meningitis cases were collected in March, 2015 – April, 2018. All samples were processed for cytological, biochemical, Microbiological (Gram stain, Latex agglutination and culture) and molecular examination. CSF was centrifuged at 10,000 rpm for 10 minutes. Sediment was used for Gram stain and culture. Blood, chocolate and Mac-conkey agar plates were used for growth of the bacteria. After inoculation, the plates were incubated at 37 °C for 24-48 hrs with 5% CO₂. Isolates grown on the culture plates were identified by MALDI-TOF (Vitek MS Biomerieux, France). The isolates were further processed for antibiotic susceptibility tests. Nosocomial infection was defined as a positive bacterial infection that was not present at the time of hospital admission or clinical evidence of an infection no sooner than 48 hours after admission. Remaining patients were considered to have community acquired infection.

Identification of isolates by MALDI-TOF.

For identification of the bacteria, a single bacterial colony was taken, and the smear was made on the MALDI-TOF slide with the help of wooden stick and 0.5 µl matrix (α-cyano-4-hydroxycinnamic acid) was added. The slide was kept in air at a room temperature for 5 minutes to dry and finally slide was kept in the MALDI-TOF machine (VITEK MS Biomerieux, France) for acquisition and identification. The isolates identified were further processed for antibiotic susceptibility test.

Antibiotics susceptibility test. Mueller Hinton Agar was used to perform an antibiotic

susceptibility test. The isolate was inoculated in 0.5% peptone water and incubated at 37 °C for 2 hrs. Turbidity was compared with 0.5 McFarland solutions so that bacterial count to be >10⁵ CFU/ml. Antibiotics used were amikacin (10 µg), co-trimoxazole (25 µg), cephataxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), ciprofloxacin (30 µg), erythromycin (30 µg), penicillin G (10units), piperacillin + tazobactam (100/10 µg), imipenem (10 µg), Netilmicin (30 µg), chloramphenicol (30 µg) and vancomycin (30 µg). The antibiotic discs were obtained from Hi Media Laboratories, Mumbai, India. Antibiotic susceptibility results were interpreted as per CLSI guideline. The *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC25923 strains were used for quality control.

Latex agglutination test (LAT). A supernatant of the centrifuged CSF was used for detection of soluble bacterial antigen of *Streptococcus pneumoniae*, *Neisseria meningitidis* A, B, C, and W135, *Streptococcus agalactiae*, *Escherichia coli*, and *Haemophilus influenzae* type B by latex agglutination test (LAT) following manufacturer's instructions. The Directigen™ Meningitis combo Kit (BD, USA) was used for the LAT.

Extraction of DNA. Extraction of DNA from CSF samples was carried out using the Mini DNA extraction Kit (Qiagen, USA) following manufacturer's instructions. DNA was eluted in 100 µl elution buffer and stored at -20 °C for future use in the experiment.

Primer design and PCR conditions. For detection of *S. pneumoniae*, *H. influenzae* and *N. meningitidis* semi-nested multiplex PCR was designed. However, for *E. coli* & Group B streptococcus the Multiplex PCR was done. Primers were designed accordingly that is presented in Table 1.

Table1. Sequence of primers used in this study

Organisms	Target Gene	Primer	Amplicon length (bp)	Reference
Seminested Multiplex PCR				
Universal Primer	U3 (16S rRNA)	5'-GTG CCT GCA GCC GCG GTA AT-3'	1000	Chakrabarti P et al, 2009 [18]
	U8 (16S rRNA)	5'-AAG GAG GGG TGT GTAC-3'		
<i>S. pneumoniae</i>	ply	5'-GTA CAA CGA GTC GCA AGC-3'	293	
<i>H. influenzae</i>	bex	5'-CCT AAG AAG AGA TCG AG-3'	543	
<i>N. meningitidis</i>	ctr	5'-TGT TGC GCA ACC TGA TTG-3'	710	
Multiplex PCR				
<i>E. coli</i>	16S rRNA	5'-TGC CTG ATG GAG GGG GAT AA-3'	776	This study
		5'-TTT AAC CTT GCG GCC GTA CT-3'		
<i>S. agalactiae</i>	sip	5'-ACA ACG GAA GGC GCT ACT GTT C-3'	255	Bergh K et al, 2004 [19]
		5'-ACC TGG TGT TTG ACC TGA ACT A-3'		

Amplification of DNA extracted from the CSF. The PCR mixture (25 µl) contained 1×PCR buffer 2.5 µl with 2.5 mM MgCl₂, 0.5 µl dNTPs (10mM each), 0.25 µl Taq polymerase (Promega, 500U) & Primer 0.2 µl (10 µM each) for semi-nested multiplex PCR. However, for multiplex PCR, primer concentration used was 1 µl (10 µM each) and 2.5 µl of the target DNA. Finally, the volume of the PCR mixture was adjusted by adding milli Q water. PCR was performed in the Thermocycler (Agilent Technologies, Surecycler 8800, USA) with the following cycling condition for multiplex PCR, i.e. initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 1 min, Annealing 55 °C for 30 sec, extension 72 °C for 30 sec and final extension at 72 °C for 10 min with 4 °C holding temperature, the number of cycles was 35. After amplification of the bacterial DNA, gel electrophoresis of the PCR product was run for 30 minutes in 1.5% agarose with ethidium bromide and visualize under UV fluorescence to check the product size. Representative Image of the Gel electrophoresis is presented in Fig. 1.

Statistical analysis. of the tests was performed by the Wilcoxon Singed-rank test. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were evaluated.

Ethical Approval. The study was approved by the Institute Ethics Committee of All India Institute of Medical Sciences, New Delhi (No: IESC/T-20)

Results

The finding of the study was interpreted and correlated with the etiological agents of ABM in all age group (0 days-84 years) with conventional and molecular techniques. Out of total 267 cases of suspected meningitis patients, 194 (72.60%) were male and 73 (27.34%) were female. The main clinical feature shown by the patients were fever 258 (96.63%), headache 210 (78.65%) and altered sensorium 181 (67.79%) details are given in Table 2.

Table 2. Demographic details of the meningitis patients

Clinical Features	Number of patients
Fever	258 (96.63%)
Headache	210 (78.65%)
Nuchal rigidity	57 (21.34%)
Altered sensorium	181 (67.79%)
Nausea/Vomiting	54 (20.24%)
Kernig's and Brudzinski's sign	51 (19.10%)
Bulging anterior fontanelle	32 (11.98%)

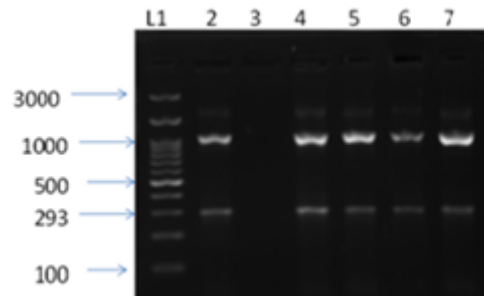


Fig.1. A gel image of PCR product of *S. pneumoniae* in 1.5% agar.

L₁ = Ladder, L₂ = Positive control, L₃ = Negative control & L_{4,5,6,7} = CSF sample.

The CSF glucose and protein level of the majority of the patients were <45 mg/dl and >55 mg/dl respectively. Out of 267 CSF samples, 5 (1.87%) were proved to be positive by culture. Among these 5 isolates, 3 (60%) were *S. pneumoniae* and 2 (40%) were *E. coli*. Similarly, total 28 (10.49%) bacteria were detected by conventional PCR (CPCR) as the culture positive bacteria were also positive by PCR; out of 23 bacteria detected by PCR only, 6 (26.09%) were *E. coli* and 17 (73.91%) were *S. pneumoniae*. Main cases of the organisms revealed were of the age group of 0-5 yearsold. Likewise, 4 (50%) cases of *E. coli* were revealed in neonates. In case of *S. pneumoniae*, 15 (53.57%) isolates were revealed in children below 5 years of age. The details of distribution of bacteria revealed are presented in Table 3.

Latex agglutination test (LAT) was used to detect the bacterial soluble antigen of the common etiological agents of acute bacterial meningitis: *Streptococcus pneumoniae*, *Neisseria meningitidis* A, B, C & W135, *Streptococcus agalactiae*, *Escherichia coli*, and *Haemophilus influenzae* type B. However, only *S. pneumoniae* and *E. coli* was detected by the LAT in this study. The LAT has detected more organisms than the culture. Total 15 isolates were detected by the LAT. Out of 15 LAT positive isolates 10 (5 *S. pneumoniae* and 5 *E. coli*) were positive by the LAT only. However,

Table 3. Age wise distribution of the isolates detected from CSF

Isolates	Methods of detection	Age group						Total
		Neonates	1-3 months	3-24 months	2-5 yrs	5-50 yrs	>50 yrs	
<i>E. coli</i>	Culture	01	-	-	-	-	01	02
	CPCR	03	02	-	-	-	01	06
<i>S. pneumoniae</i>	Culture	-	-	01	01	01	-	03
	CPCR	-	02	07	04	02	02	17
Total		04	04	08	05	03	04	28

3 *S. pneumoniae* and 2 *E. coli* that were culture positive were also positive by the LAT.

Taking culture as gold standard, the sensitivity, specificity, positive predictive value and negative predictive value were evaluated. Sensitivity and specificity of CPCR for *S. pneumoniae* and *E. coli* were found in 100% and 95-98% cases, respectively, with 95% CI that is presented in Table 4.

The antibiotic susceptibility test was performed for *E. coli* and *S. pneumoniae* detected from CSF by culture. Most of the antibiotics tested were susceptible to *S. pneumoniae* (100%). One (33.36%) *S. pneumoniae* showed resistance to co-trimoxazole. For *E. coli*, the most susceptible antibiotic was amikacin (100%) followed by colistin (100%) and imipenem (100%).

Table 4. Sensitivity, specificity, PPV, NPV of CPCR comparing with culture

Statistics	Conventional PCR			
	<i>E. coli</i> (N=6)		<i>S. pneumoniae</i> (N=17)	
	Value	with 95% CI	Value	with 95% CI
Sensitivity	100%	15.81% - 100%	100%	28.24% - 100%
Specificity	98.28%	96.29% - 99.36%	95.26%	95.53% - 97.22%
PPV	25%	13.10% - 42.42%	15%	9.99% - 21.92%
NPV	100%	-	-	95.30%
Accuracy	98.29%	96.31% - 99.37%	95.30%	92.59% - 97.24%

Discussion

Community acquired acute bacterial meningitis is an urgent health issue as most of the infection occurs in children below 5 years of age. In this study, total 267 CSF samples were included irrespective of the age limit. Of the total number of CSF, only 5 (1.87%) were found positive by culture for different etiological agents of community acquired bacterial meningitis. A study conducted by Zahra B. H et al. has shown culture positivity 2.5% [20]. Positivity of culture decreases 60-70% in case of CSF collected after administration of antibiotics [21]. In our study, low culture positivity may be due to intake of antibiotics prior to lumbar puncture as our hospital is a referral center where majority of the patients admitted had already been administered with various antibiotics before getting admitted.

We detected 28 (10.14%) positive CSF by CPCR which were almost 10% more than the culture positives. Our finding is concurrent with the finding (11.76%) shown by Nour M et al. [22]

but less than the finding by Zahra B. H. et al. and Chokrabarty P. et al. who have reported 15% and 19.8% positivity by mPCR respectively [18, 20]. Low positivity may be due to difference in sample size and organisms detected. Sensitivity and specificity of convention PCR was found 100% and 95-98% respectively for *E. coli* and *S. pneumoniae* with 95% CI. Finding was concurrent with the findings of Wu et al. [23].

In children below 1-24 months of age *S. pneumoniae* 10 (50%) was the main organism. However, *E. coli* 4 (50%) were found in neonates that was also a significant finding as *E. coli* and *S. agalactiae* are the major pathogens of the neonates [24, 25]. We have detected total 20 *S. pneumoniae*, 15 (75%) of which were in those less than 5 years of age. Likewise, out of these 15 cases, 8 (53.3%) were detected in those 3-24 months of age. More number of *S. pneumoniae* in children below 5 years of age was reported from India [26, 27]. However, 1 previous study conducted in 1999 by ISIB has reported *H. influenzae* as the main cause of bacterial menin-

gitis prior to the introduction of pentavalent vaccines [28].

In our study, only *E. coli* and *S. pneumoniae* were detected among the etiological agents of community acquired acute bacterial meningitis that may be a result of implementation of the Hib vaccination in national immunization program in India. *H. influenzae* detection rate has decreased drastically in recent times. Similarly, in India, incidence of *N. meningitidis* increased greatly during the epidemics and the epidemic cycle of *N. meningitidis* is at an interval of 15-20 years. Last episode of *N. meningitidis* epidemics was in 2005 [29].

Antibiotic susceptibility test result showed that most of the antibiotics used were susceptible to *S. pneumoniae*. One (33.3%) *S. pneumoniae* out of 3 was resistance to co-trimoxazole. However, no penicillin resistance *S. pneumoniae* was found. Similarly, amikacin, colistin and imipenem were the most susceptible (100%) antibiotics for *E. coli* though ciprofloxacin has shown (50%) resistance followed by ceftiofur + salbutamol (50%).

Conclusions

Acute bacterial meningitis is a severe medical emergency and *S. pneumoniae* is still the a major cause of community acquired bacterial

meningitis in children. Molecular diagnosis is gaining momentum for a rapid detection of etiological agents with high sensitivity and specificity since it is unaffected by viability of the organisms. Hence, PCR should be implemented in the diagnosis to overcome the dependency on conventional method.

Conflict of Interest

The authors declare no conflict of interest.

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Authors Contributions

M.S. Raza – conceptualization, investigation, writing (original draft), B.K. Das – conceptualization, project administration, resources, supervision (review and editing), V. Goyal, R. Lodha, R. Chaudhry, S. Sood, D. Nair, S. Mohapatra, A. Kapil – conceptualization, supervision, writing (review and editing), H. Gautam – conceptualization, writing (review and editing), V. Sreenivas – formal analysis, visualization writing, data curation.

МОЛЕКУЛЯРНІ МЕТОДИ ДІАГНОСТИКИ ГОСТРОГО БАКТЕРІАЛЬНОГО МЕНІНГІТУ НА ТРЕТИННОМУ РІВНІ НАДАННЯ МЕДИЧНОЇ ДОПОМОГИ У ЛІКАРНЯХ ПІВНІЧНОЇ ІНДІЇ

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Вступ. Гострий бактеріальний менінгіт – одне з найбільш небезпечних інфекційних захворювань, що слугує додатковим тягарем для системи охорони здоров'я. Відповідне лікування – необхідна умова для попередження та лікування тяжких наслідків хвороби.

Мета дослідження – встановлення етіологічних чинників гострого бактеріального менінгіту за допомогою ПЛР.

Методи. Загалом було відібрано 276 зразків спинномозкової рідини (СМР) від пацієнтів з підозрою на бактеріальний менінгіт для визначення його основних збудників *S. pneumoniae*, *H. influenzae*, *N. meningitidis*, *E. coli* та стрептококів групи В типовим способом та за допомогою методів молекулярної діагностики. Для проведення культурального дослідження зразків СМР використовували живильні середовища: кров'яний агар, шоколадний агар, середовище МакКонкі. Зразки інкубували при 37°C протягом 24-48 годин. Ріст бактерій досліджували методом мас-спектрометрії з лазерною десорбцією/іонізацією MALDI-TOF. Мультиплексна ПЛР зразків СМР проводилася з використанням наборів для екстракції ДНК Qiagen (USA).

Результати. Із 276 досліджених зразків СМР, п'ять (1.87%) були культуро-позитивними (три – *S. pneumoniae* та два зразки – *E. coli*). За допомогою типової ПЛР ДНК збудника було знайдено у 28 зразках (10.49%). З цих 28 зразків у 20 (71.43%) – були ідентифіковані *S. pneumoniae*, а у 8 зразках (28.57%) – *E. coli*.

Серед зразків, де було виявлено *S. pneumoniae*, 15 (53.57%) належали дітям до 5 років. *E. coli* переважала у новонароджених. Чутливість та специфічність ПЛР була 100% та 95-98% відповідно.

Висновки. *Streptococcus pneumoniae* – найбільш поширений збудник бактеріального менінгіту у дітей віком до 5 років. Враховуючи результати наших досліджень, ПЛР-діагностика повинна впроваджуватися у комплекс методів діагностики гострого бактеріального менінгіту.

КЛЮЧОВІ СЛОВА: **спинномозкова рідина (СМР); гострий бактеріальний менінгіт; ПЛР; MALDI-TOF.**

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