Detection of bacterial meningitis via Ply, Lyt and Bex gene in Pakistani Population

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***Abstract*-** Bacterial meningitis diagnosis mostly involves non-specific and painful procedures. An alternative strategy has been proposed in this study utilizing Ply and Bex genes for *Streptococcus pneumoniae* and *Haemophilus influenzae* identification. Blood samples were collected from meningitis patients at Services hospital Children's hospital, Lahore, with a subsequent sub-culturing and enrichment. DNA was isolated from bacteria and blood using a kit, and Polymerase Chain Reaction was performed with five primer sets. Six strains were isolated from Tryptic Soy Broth (TSB) and oxidase test. PCR confirmed Ply and Lyt gene amplification for *Streptococcus pneumoniae*, while amplified Bex gene amplification suggested a potential combinatorial approach as early diagnostic biomarkers for bacterial meningitis. The study, conducted at KAM School of Life Sciences, Forman Christian College University, Lahore, from January to May 2019, enhances understanding of the reliability of diagnostic methods. The consistent presence of amplified Bex and Ply genes in bacterial meningitis patients underscores their utility for early disease diagnosis.

Index Terms- Bacterial meningitis, S*treptococcus pneumoniae*, *Hemophilus influenzae*, Blood culture, Polymerase Chain Reaction

I. INTRODUCTION

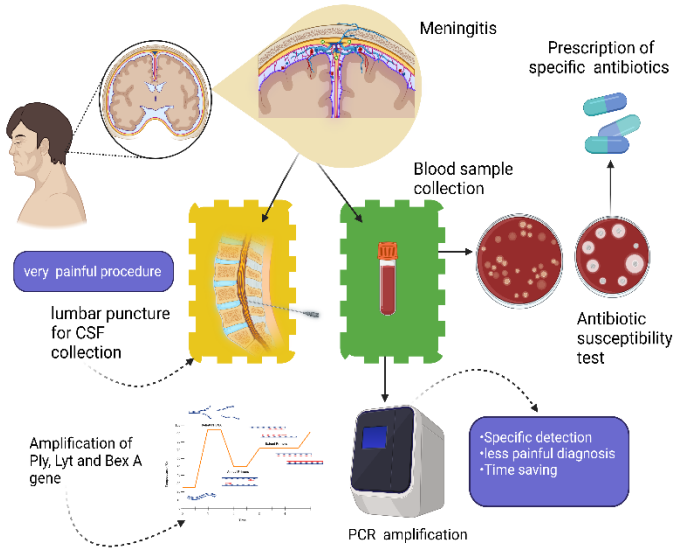
The yearly prevalence of Bacterial Meningitis (BM) is 4-6 incidences per 100,000 adults worldwide. 1,2 The frequency was estimated to range from 0.5 to 1.5 per 100,000 people in settings with adequate resources, but in sub-Saharan Africa also referred to meningitis belt; can reach up to 1000 per 100,000 cases.3 According to the WHO, there were approximately 20,000 meningitis deaths in Africa, 18,000 in America, 73,000 in South East Asia, 15,000 in Europe, 25,000 in the Eastern Mediterranean, and 20,000 in the Western Pacific. Infant fatalities in the USA from meningitis were 0.9%. 4 In Pakistan, meningitis is a major cause of newborn mortality. Two-thirds of meningitis cases worldwide occur in children under the age of 15. Older adults and newborns who have meningitis exhibit irritability and restlessness. The three main types of meningitis include viral meningitis, tuberculosis meningitis and bacterial meningitis. However, viruses involved in meningitis include Enteroviruses, Human immunodeficiency virus (HIV), Herpes simplex virus (HSV-1 and HSV-2), Cytomegalovirus, Varicella-zoster virus, Mumps and West Nile virus.5 Meningitis caused by bacteria, which affects the meninges that surround the brain and spinal cord, is the leading cause of illness and mortality worldwide. It is connected to pia metre, Cerebrospinal Fluid (CSF), and arachnoid. Thus, in 10 to 20% of the population, it progresses to more severe neurological disorders, which highlights the need for quick treatment, diagnosis, and preventative measures.

There are several diagnostic markers that can be used for detection of disease including opc and rmpM genes for bacterial meningitis through PCR. The available methods for detection of disease include gram staining, culture method, 16s rRNA, PCR and Lateral Flow Assay (LFA).6 For the purpose of identifying acute bacterial meningitis, CSF culture method is initially used with only 0.3% chances of accuracy in obtained results.7 Therefore, it becomes hard to detect pathogen in specimen if patient have already taken excessive amount of antibiotics. Even samples that are not detected through CSF culture approach can be used with PCR to identify the pathogen that is causing the problem. Sometimes the lack of sufficient DNA prevents PCR from producing accurate results. Moreover, the bacterial type causing the infection also affects how well the diagnostic assay works.8

The administration of antibiotics must be initiated right after the diagnosis because early antibiotic use lowers the death rate. Patients with delayed meningitis require longer healing times. Meningococcal meningitis was formerly treated with ampicillin and penicillin G. But, Neisseria meningitis later developed resistance to it, necessitating modifications to this antibiotic. When treating BM effectively, dexamethasone is the most suitable treatment. Its dose should be given with the combination of antibiotics.9 80% of cases are caused by Streptococcus pneumoniae and Neisseria meningitidis, the most prevalent causal pathogens. Disease burden is greater in nations with insufficient resources. However, the prevalence of the HIV, widespread use of vaccines, the increased accessibility of antimicrobial therapy, and

the notable advancements in development and poverty-reduction strategies, including better maternal and neonatal care, all have an impact on the global epidemiology of meningitis in recent years.10 In contrast to viral meningitis, which is the most common type; is typically less severe and self-limiting. BM is a more dangerous and potentially fatal condition.11 Despite having well-established diagnostic and treatment options, BM has a high morbidity and fatality rate. Although cerebrospinal fluid (CSF) analysis is crucial for the detection of bacterial meningitis, it is unknown how predictively it can identify sepsis.12

In this study an alternative strategy was suggested to combat painful procedures of Lumbar Puncture for CSF collection before diagnosis of BM. Primers were designed against the Ply and Lyt genes of Streptococcus pneumoniae and Bex gene of the Haemophilus influenzae. Conventional PCR was used to determine the accuracy of bacterial detection. Antibiotic resistance profiles of bacterial strains were also determined using ampicillin and ceftriaxone. The scheme adopted in this study has been shown in Figure 1. "created with Biorandeer.com"



II. MATERIALS AND METHODS

Sample collection

A total 40 samples of meningitis patients were collected from services hospital and children hospital Lahore, Pakistan after the ERC approval (ERC-45—2019). Informed consent was obtained from all the participants of study before sample collection. Blood samples were collected in blood culture bottles containing Tryptic Soy Broth (TSB). Patients lying in the age range of 4 months to 85 years, diagnosed with BM on the basis CT scan and Lumber Puncture and who never used the drug for the meningitis were included in the study. And Patients of age less than 4 months, taking drugs for meningitis and having complaints of seizure were excluded.

Sub-culturing and enrichment of blood culture

The blood samples were streaked on Chocolate Agar (CAP) media after an incubation period of 7 days following TSA media for enrichment purposes. After overnight culturing of TSB, isolated colony was inoculated in media and culture tubes were placed into shaking incubator at 37 ˚C for 24 hours. Turbidity of culture determined the presence of bacteria.

Biochemical test

The oxidase and catalase tests were performed for the identification of bacteria. 1% Kovac's oxidase reagent was prepared and the colony was selected using the swab method. Soaked the swab with oxidase reagent and touched it to the plate to select a single colony. Waited for 10 seconds to observe color change.

PCR amplification

DNA was extracted from bacteria with the help of a kit (Thermo Fisher Scientific K0721) according to the manufacturer's protocol and also directly extracted from blood. Gene specific primers were used to amplify the targeted region of the DNA. The 5 different primer sets for Ply, Lyt gene of *Streptococcus pneumoniae* and BexA gene of *Haemophilus influenzae* were designed using Primer 3 software. Details of primers are given in Table 1.

Table 1. Sequences of primers for Ply, Lyt and Bex gene

Sr. No	Primers Name	Sequence 5' to 3'
1	Ply (Sp1F)	TGCAGAGCGTCCTTTGGTCTAT
	Ply (Sp1R)	CTCTTACTCGTGGTTTCCAACTTGA
2	Lyt (Sp2F)	ACGCAATCTAGCAGATGAAGCA
	Lyt (Sp2R)	TCGTGCGTTTTAATTCCAGCT
3	Bex (Hi1F)	GGCGAAATGGTGCTGGTAA
	Bex (Hi1R)	GGCCAAGAGATACTCATAGAACGTT
4	Bex (Hi2F)	TGCGGTAGTGTTAGAAAATGGTATTATG
	Bex (Hi2R)	GGACAAACATCACAAGCGGTTA
5	Bex (Hi3F)	TATCACACAAATAGCGGTTGG
	Bex (Hi3R)	GGCCAAGAGATACTCATAGAACGTT

For the Bex, Ply and Lyt gene amplification, PCR conditions were set the same except the annealing temperature. Initial Denaturation was set at 95 ˚C for 5 min. Denaturation at 95 ˚C for 1min, extension at 72 ˚C for 1 min and Final Extension at 72 ˚C for 10

min. Annealing temperature was 58 ℃, 60 ℃ and 64 ℃ for 1 min for Bex, Ply and Lyt genes respectively. Lastly, antibiotic sensitivity of the isolated pathogens was detected against ampicillin and ceftriaxone to determine status of antibiotic resistance.

III. RESULTS

Blood samples were collected from 40 patients who were suspected for bacterial meningitis. It was also recorded that antibiotics are given to patients for their early recovery. Samples collected from patients were of different age groups and gender. Samples were streaked on the selective media for the isolation of all bacterial pathogens. After streaking plates were placed in the incubator at 37°C for 24 hours. Colony morphology of grown pathogens on CAP were observed as shown in Figure 2 and 3.

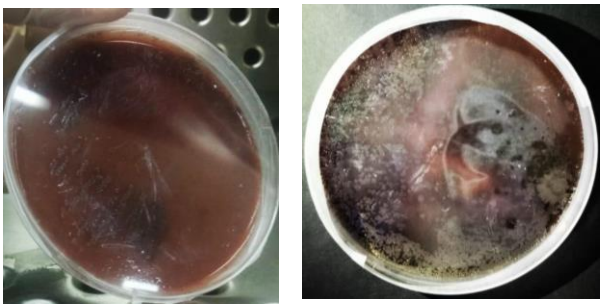


Figure 2. (A,B) Suspected growth of *Haemophilus influenzae* and *Streptococcus pneumoniae* on CAP

Haemophilus influenzae and *Streptococcus pneumoniae* were confirmed through observation of colonies.

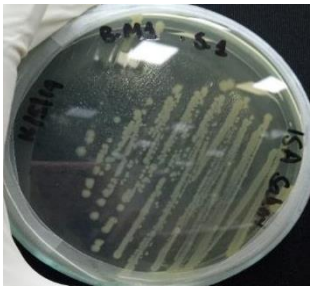


Figure 3. Suspected colonies of *Haemophilus influenzae* on TSA plates

Colonies on TSA media confirmed the presence of *Haemophilus influenzae* as shown in Figure 3,4 and 5.

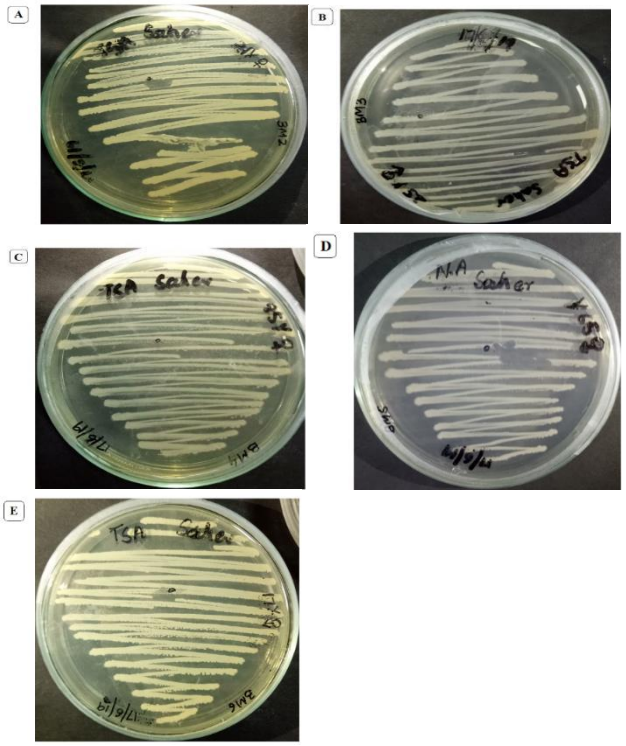


Figure 4. (A-E) Colonies growth of *Streptococcus pneumoniae* on TSA plate

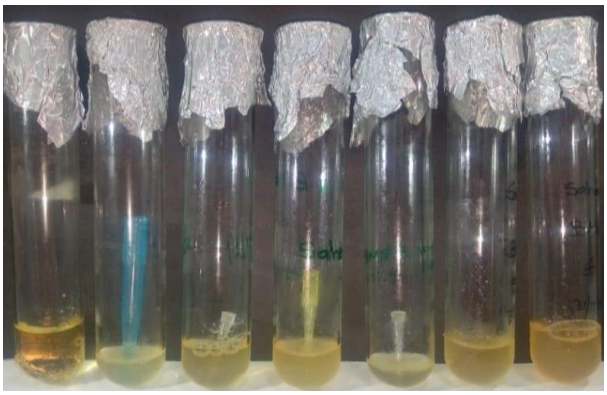


Figure 5. Culture tubes showing growth of bacteria in TSB media

Culture tube shown on the left hand side had no growth of bacteria and turbidity. Whereas, remaining tubes represented bacterial growth after 24-48 hours. The purple color obtained after the swab dipped in 1% Kovac's oxidase reagent confirmed the presence of *H. influenza* bacteria after the time period of 10 seconds.

Amplification of Ply, Lyt and Bex gene

Six strains were isolated and DNA was extracted following the methodology given by the manufacturer in kit; Thermo Fisher Scientific K0721. Concentration of DNA was checked by nanodrop. 81 bp fragment of Ply gene in four samples and 75bp Lyt gene in two samples confirmed the detection of *Streptococcus*

pneumoniae in 6 samples. And a bright band of Bex gene of 182 bp confirmed the detection of *Haemophilus influenzae* in multiple samples with the primer set Hi3. Amplification of the Bex gene was also confirmed through other two sets of primers as shown in Figure 6.

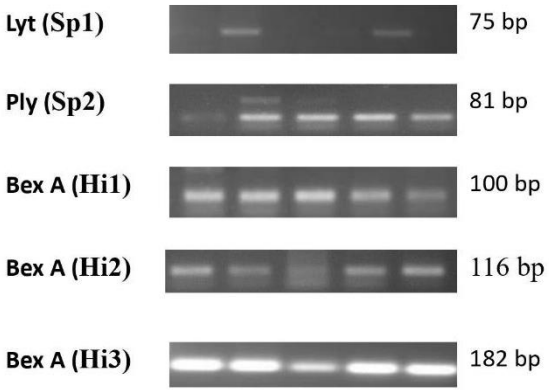


Figure 6. Agarose gel electrophoresis for PCR products showing expression pattern of Lyt, Ply and Bex genes in clinical samples of bacterial meningitis

The zone of inhibition was observed around disks and their diameters were measured. These measurements help in choosing the most suitable antibiotic for future use. It also shows the range of distribution of medication via media as shown in Figure 7.

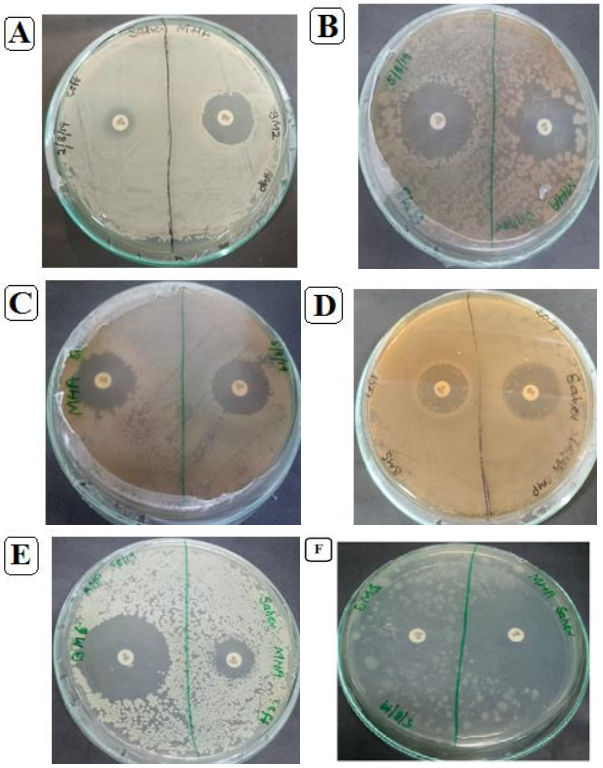


Figure 7. (A-F) Antibiotic susceptibility test for all isolated bacteria using ceftriaxone and ampicillin disks

IV. DISCUSSION

Meningitis is a severe condition that can be caused by both viruses and bacteria. Effective antimicrobial treatment should be started to cure the disease at early stages. Infection causing pathogens like bacteria are isolated by CSF culture mainly. Molecular methods, such as gram staining, CSF culture and latex agglutination test, are used as benchmark for detecting any type of meningitis.13 CSF is a sensitive method that is dependent on the type of microbe present, patient attributes, and laboratory resources.14 However, it becomes challenging to detect bacteria using the CSF culture approach in case of small number of bacteria present in sample. The most practical technology for identifying pathogens in a disease is PCR. Occasionally, PCR fails to provide results if there is not enough bacterial DNA present. Different *S. pneumoniae* serogroups are involved in the disease-causing process. Serogroup 19A infection can be avoided by using pneumococcal conjugate vaccination 13 (PCV13), but serogroup 23A infection can be avoided by using PCV10, PCV13, and PCV7. Meningitis patients in Pakistan received ciprofloxacin and ceftriaxone treatment just after they got admitted in hospital. Despite this, 34% of patients passed away due to antibiotic resistance evolved in pneumococci, which raised the high mortality and morbidity rates especially in developing countries. A crucial part in preventing such a deadly illness is the use of vaccines against resistant microorganisms. 15 CSF culture method is considered to be more useful for identification of *Haemophilus influenzae* and *Streptococcus pneumoniae* as compared to blood culture method. Limitation of this research was that we did not find *Neisseria meningitidis* and other many bacteria causing meningitis. Diagnosis of bacterial meningitis using a combination of Ply and Bex genetic markers can be devised as a reliable strategy that can overcome the disadvantages of lumbar puncture. It was concluded that meningitis should be controlled by improving the immunization in the Ministry of National Health services, Pakistan.

V. CONCLUSION

Our study recommends a different approach to the painful lumbar puncture for CSF collection prior to BM diagnosis by using Ply genes from *Streptococcus pneumoniae* and the Bex gene from *Haemophilus influenzae.* The precision of bacterial detection was evaluated using conventional PCR. Using ampicillin and ceftriaxone, the antibiotic resistance profiles of various bacterial strains were also identified. The failure to identify *Neisseria meningitidis* and other bacteria known to cause meningitis was one of the research's weaknesses. It is imperative to prioritize the improvement of immunization programs within the Ministry of National Health services in Pakistan, also focusing on the development of advanced technologies for the enhanced diagnosis of bacterial meningitis.

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