



Diagnosis of Bacterial Meningitis and AMR Profile Using Molecular and Immunological Techniques

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Study Protocol

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ABSTRACT

Background: Bacterial meningitis (BM) is severe complication of central nervous system (CNS) and is often associated with high mortality and morbidity rates if not timely diagnosed and treated. Current diagnostic tools for BM and drug resistance suffer from lack of sensitivity due to paucibacillary nature of Cerebrospinal fluid (CSF).

Objectives: The objective of the study is to develop rapid and efficacious immunological tools (Enzyme linked immunosorbent assay and Lateral flow) for diagnosis of BM in hospital settings. In addition, in-house molecular assays will be developed for diagnosis of extended spectrum beta lactamases and Carbapenems drug resistance in community and hospital acquired BM infection

Methodology: A prospective observational study will be carried out in patients admitted in IPD awards of CIIMS, Nagpur. Diagnosis of BM will be done using conventional gold standards and by in-house designed nested polymerase chain reaction (PCR) for 8 etiological pathogens. An in-house ELISA assay will be standardized and optimized based on culture filtrates antigen isolated from clinical isolates of etiological pathogens associated with BM for immunological diagnosis. For diagnosis of drug resistance, in-house conventional PCR assay targeting ESBL (TEM, SHV and CTX-M) and Carbapenems (NDM, OXA-48, VIM) resistance genes will be standardized and

optimized. The sensitivity and specificity of developed test will be determined by standard ROC curve using medical software. The diagnostic accuracy will be determined based on concordance with gold standards.

Expected results: A simple, rapid ELISA assay will be developed for etiological diagnosis BM in hospital settings. In addition, in house molecular assay will be developed for rapid identification of ESBL and Carbapenems drug resistance in BM cases.

Conclusion: A simple, rapid, efficacious immunological and molecular tools for diagnosis of BM and drug resistance will be developed for improved management of cases in hospital settings as governance of bacterial meningitis in India is poor.

Keywords: Bacterial meningitis; ELISA; PCR; antibiotic resistance; antibiotic stewardship.

1. INTRODUCTION

Bacterial meningitis (BM) remains a significant cause of concern worldwide due to associated mortality and lasting neurological sequelae. In spite of the recent development of potent new antibiotics, the mortality rate due to BM is found to be significantly high ranging from 16-32% in India owing to the lack of awareness and diagnosis [1]. Meningococcal disease, in India, is endemic in New Delhi and sporadic cases have occurred in Uttar Pradesh, West Bengal, Haryana, Gujarat, and Orissa [2,3]. Various demographic studies have so far revealed that several pathogens are responsible for causing meningitis depending on the patient's age group [4,5,6].

The diagnosis of bacterial meningitis is however still very low in resource poor settings and developing countries established on basis gram staining and culture [7,8]. The diagnostic accuracy of these investigations is, more often than not, affected by the use of antibiotics administered prior to the lumbar puncture and due to paucibacillary nature of etiological agents in CSF [9,10]. The use of molecular techniques like polymerase chain reaction (PCR) for the expeditious diagnosis of BM has the potential to overcome the poor sensitivity of culture when antibiotics have already been introduced but even so PCR is not available in many parts of developing countries [11,12]. The rapid detection and estimation of the severity of the disease are important factors, both in diagnosis and initiating the treatment [13].

Moreover in recent years, increasing trend in cases of Antimicrobial resistance in etiological agents of BM has added a fatal blow to the already limited treatment leading to increase in morbidity of infected cases [14]. In India, resistance to Carbapenems and β -Lactams has increased due to Extended-spectrum β -

Lactamases (ESBL) production among members of gram negative organisms. Moreover, the commercially available Drug susceptibility testing techniques for detection of drug resistance are not only dependent on culture positivity but are also exorbitant for common public making the treatment of this disease even more strenuous and challenging [15,16].

Simpler yet accurate diagnostic techniques should be introduced that can help overcome this drawback as they do not require a sophisticated lab setup and over the odds instruments so that diagnosis and treatment can reach the low resource setting areas of our country where appropriate facilities are not available.

Rapid and efficacious techniques like ELISA and lateral flow can be developed and introduced in such remote locations in order to help overcome the limitation of diagnosis. This culture secretome of an organism contains cocktail of several bacterial proteins, with broad yet specific immune response. The antibodies against such secretome may be detected using simple ELISA based test which are rapid yet cost effective and even have scale up capacity to be converted into rapid point of care in form of lateral flows. Development of specific yet cost effective in-house molecular technique can help reduce the burden of AMR and subsequently the challenges faced in treatment of BM infection.

Such assays can aid clinicians in bedside screening for BM and initiate specific treatment in suspected cases to avoid complications associated with empirical regimens.

2. METHODS

1. Study Design

Present prospective study will be carried out at IPD wards of CIIMS, Nagpur. Patients suspected

of suffering form BM will be routinely screened using pre designed in house semi-nested PCR by Bhagchandani et al for detection of bacterial meningitis [17].

2.2 Test Methods

2.2.1 Nested PCR

DNA Extraction Protocol: The reagents and instruments used in laboratory for DNA extraction are mentioned in Table 1 and Fig. 1 respectively.

1. Collect CSF by lumbar puncture from suspected admitted cases of BM in IPD wards. Store at -20⁰C.
2. Before processing, thaw and centrifuge in a 1.5 mL micro centrifuge tube at 22536g for 10 minutes.
3. Supernatant will be removed and stored. To the pellet, 400 µL of 1 X PBS, 15 µL of Sodium dodecyl sulphate (SDS) and 3 µL of Proteinase K will be added and followed by incubation at 55⁰C for 1 hour in a water bath.
4. After incubation, 3 µL of Proteinase K will be added and again incubate 55⁰C for 30 mins.
5. After incubation, 100 µL of (5M) NaCl and 80µL of 10% cetyl trimethylammonium bromide (CTAB) will be added. Again incubate at 65⁰C for 10 minutes on a heating block.
6. Allow the tubes to cool at room temperature (RT) for 10mins. Add 350µL of

Phenol and 350µL of Chloroform: Isoamyl alcohol solution (24:1) to the vial. Centrifuge as above.

7. Two distinct layers are obtained, transfer the upper aqueous layer to a fresh tube and add equal volume of the Chloroform: Isoamyl alcohol solution. Centrifuge as above.
8. Transfer the upper aqueous layer again to a fresh vial and add 30µL sodium acetate and 0.6 of the total volume of Isopropanol and incubate at -20⁰C for 30minutes. After incubation, centrifuge as above.
9. Discard the supernatant, wash the pellet with 1mL ice cold 70% Ethanol. Centrifuge as above.
10. Discard the supernatant and allow the pellet to air dry at RT.
11. Add 25µL of TE buffer and store at -20⁰C for further use [17].

The schematic flowchart of DNA extraction can be seen in Fig. 2.

2.2.2 16s rDNA PCR

In developing countries, the advanced technique for detection of broad range 16s rDNA gene in bacteria is being introduced in recent years, though not very extensively. [17] Reagents and figures required for 16s rDNA protocol can be seen in Table 2 and Fig. 3 respectively. Table 3 depicts the sequence of primers used while the PCR protocol can be studied in Table 4 and amplification program consecutively in Table 5.

Table 1. Reagents required for DNA extraction

Sr. No.	Reagents
1	10X PBS
2	Sodium dodecyl sulphate (SDS)
3	Proteinase K
4	5M Sodium chloride (NaCl)
5	10% cetyl trimethylammonium bromide (CTAB)
6	Molecular grade Phenol [Caution: Phenol is potentially degrading in nature and may cause severe burns on skin if brought in contact]
7	Chloroform
8	Isoamyl alcohol
9	Isopropanol
10	Sodium acetate
11	Ethanol



Sr. No	Instrument Name	Reference Images
1	Water Bath (Make : REMI, India)	
2	Heating Block (Make : REMI, India)	
3	Cooling Centrifuge (Make : REMI, India)	
4	Vortex Mixture (Make: REMI, India)	

Fig. 1. Lab instruments for DNA extraction

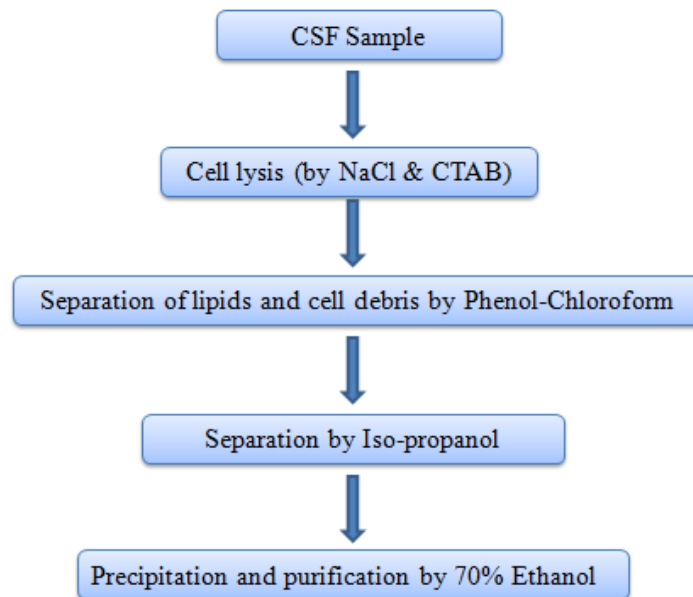


Fig. 2. Flowchart of DNA extraction method

Table 2. Reagents required for 16s rDNA PCR protocol

Sr. No.	Reagents
1	10 X PCR Buffer (Takara bio. Inc.)
2	2.5 mM Concentration of each deoxynucleotide triphosphate (Takara bio. Inc.)
3	Primers (Sigma-Genosys, USA)
4	5U/uL Taq DNA Polymerase (Takara bio. Inc.)
5	25 mM MgCl ₂ (Thermo Fisher)
6	Nuclease free water (MP Biomedicals)
7	Extracted DNA

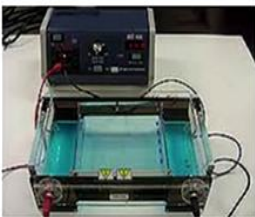


Sr No	Instrument Name	Reference Image
1	Electrophoresis Assembly (HiMedia, India)	
2	Thermal cycler-Proflex (Applied biosystems, USA)	
3	Gel documentation system (BIORAD, California)	

Fig. 3. Lab instruments required for PCR

Table 3. Universal primers sequence, annealing temp and product size for 16s rDNA protocol [17]

Gene	Primer	Sequence (5'-3')	Ta°C	Product size
16s rDNA	U1 Forward	CCAGCAGCCGCGGTAATACG	55	1.1kbps
	U2 Forward	ATCGGCTACCTTGTTACGACTTC	55	1.1kbps

Table 4. Protocol including concentration and final reaction mixture for 16s rDNA amplification [17]

Sr. No	Reagents	Initial Conc.	Final Conc.	Final volume in 50 µL for 1 reaction (µL)
1	Buffer	10 X	1 X	4µL
2	MgCl ₂	25 Mm	2.5 mM	5 µL
3	dNTPs	2.5 mM	0.4 mM	2.4 µL
4	Forward Primer	10 µM	0.2 µM	0.3 of primer mix
5	Reverse Primer	10 µM	0.2 µM	
6	Taq Polymerase	5U/µL	2.5 U/µL	0.2 µL
7	Nuclease free Water			35.6 µL
8	DNA template			2.5 µL
	Total			50 µL

2.2.3 Nested protocol for detection of 8 bacterial species

Using the purified 16s PCR product as DNA template, prepare a PCR reaction mix (20 µL) to perform PCR amplification in thermal cycler (Applied Biosystems). Run the obtained product on 2% Agarose gel containing Ethidium bromide

for visualization of bands on a gel documentation system (Bio-Rad Laboratories). [17] Primers used for the Nested PCR have been mentioned in Table 6 while PCR protocol and Amplification protocols of 8 organisms can be studied in Table 6, Table 7, Table 8, Table 9, Table 10, Table 11 and Table 12 respectively.

Table 5. Thermal cycler temperature range with time and number of cycles per stage for 16s rDNA PCR protocol [17]

Stages	Temperature	Time(minutes)	Cycles
1	94°C	5 min	1
2	94°C	1 min	15
	71°C	1 min	
	72°C	1 min	
3	94°C	1 min	20
	70°C	1 min	
	72°C	1 min	
4	72°C	7min	1
5	4°C	∞	-

Table 6. Primers used for detection of 8 bacterial genus using a nested PCR tool [17]

Sr. No.	Genus	Primer name	Primer Sequence	Product size
1	Pseudomonas	U 1 Pseud R	CCAGCAGCCGCGGTAATACG ACAGGAAATTCCACCACCCTC	190
2	Streptococcus	Strep F Strep R	ACGAGTCGCAAGCCGGTGAC ATCCCACCTTAGGCGGCTG	320
3	Acinetobacter	Acinet F Acinet R	GTGCGAGCGTTAATCGGAT TCGTTTACGGCATGGACTA	278
4	Staphylococcus	Staph F Staph R	AATCAGAAAGCCACGGCTA TATCTCTGCGCATTTCACC	218
5	Micrococcus	Mic F Mic R	TAAAGAGCTCGTAGGCGGT CGTATCTCTACGGCGATCGAG	453
6	Hemophilus	Hemo F Hemo R	ATATGAGCTTGGTGCCCGTAGC TACTGAGATTCGCTCCACCT	444
7	Neisseria	NM Mod Ru8	TGTTGGGCAACCTGATTG TGATCCAAGCCGCACGCGTTC	703
8	Enterobacteriaceae	Uni515F Ent826R	GTGCCAGCAGCCGCGGTAA GCCTCAAGGGCACAACCTCCAAG	334

Table 7. Final concentration and volume of PCR reagents used for amplification for nested PCR [17]

Sr. No.	PCR Reagents	Concentration	Volume for 1 reaction (20µL)
1	Buffer	1X	2µL
2	MgCl ₂	2.5mM	1µL
3	dNTPs	0.8µM	1.6µL
4	Forward Primer	0.25µM	0.6µL
5	Reverse Primer	0.25µM	0.6µL
6	Taq Polymerase	1 U	0.08µL
7	Nuclease free Water		12.12µL
8	DNA template		2µL

Amplification programs for thermal cycler:**Table 8. Thermal cycler protocol of *Acinetobacter and Streptococcus* genus [17]**

Stage	Temperature	Time	Cycles
1	94°C	5mins	1
2	94°C	1min	15
3	71°C	1min	20
	72°C	1min	
	94°C	1min	
	70°C	1min	
4	72°C	1min	1
	72°C	7mins	

Table 9. Thermal cycler protocol of *Micrococcus* genus [17]

Stage	Temperature	Time	Cycles
1	95°C	7mins	1
2	95°C	1min	35
3	64.5°C	1min	1
	72°C	1min	
	72°C	5mins	

Table 10. Thermal cycler protocol of *Enterobacteriaceae* family [17]

Stage	Temperature	Time	Cycles
1	95°C	10 mins	1
2	95°C	1 min	35
	67°C	1 min	
	72°C	1 min	
3	72°C	7 mins	1

Table 11. Thermal cycler protocol of *Hemophilus and Neisseria* genus [17]

Stage	Temperature	Time	Cycles
1	95°C	7 mins	1
2	95°C	1 min	35
	55°C	1 min	
	72°C	1 min	
3	72°C	5 mins	1

Table 12. Thermal cycler protocol of *Pseudomonas* genus [17]

Stage	Temperature	Time	Cycles
1	94°C	10 mins	1
2	94°C	1 min	20
	72°C	1 min	
	72°C	1 min	
3	94°C	1 min	1
	69°C	40 sec	
	72°C	1 min	
4	72°C	7 min	1

2.2.4 PCR protocol for Antimicrobial Resistance (AMR) in gram negative bacteria

Antibiotic resistance can be termed as the reduction in the overall effectiveness of a drug in treating an infection or a disease. Most common type of resistance is seen in gram negative bacteria against Beta Lactams antibiotics and Carbapenems. Literature based primers for standardization of PCR for diagnosis of antimicrobial resistance genes are mentioned in Table 13.

Primers and Amplification Protocol for PCR:

The inhouse primers for these genes will be designed in later stage of study and optimization will be done by changing concentrations of primers and DNA templates in a standard PCR protocol.

2.2.5 Gel Electrophoresis

Materials and reagents required for visualization of PCR products on 2% agarose gel electrophoresis are mentioned in Table 14.

2 g Agarose will be dissolved in 100 mL of 1X TAE buffer. Boil until a clear solution is obtained. Add 12 µL of 1mg/mL of Ethidium Bromide to it. Pour the gel into casting tray and allow solidifying with casting combs. Mix the PCR products with 6X Gel Loading dye and load into the wells with 5 µL of 100 bp DNA ladder. Allow the samples to run at a constant voltage of 150V and then observe the gel under UV light or in a Gel documentation system.

2.3 Whole Cell Lysate

Fig. 4 depicts equipments and instruments necessary for performing whole cell lysate extraction while a schematic flowchart of the same can be studied in Fig. 5.

2.3.1 Procedure

1. Fresh bacterial culture first subjected to heat killing at 65°C for 10-15 mins should be cooled and dispensed in a 50 mL centrifuge tube, followed by centrifugation at 22536g for 10 mins.
2. Without disturbing the pellet, decant the supernatant.
3. Add 1X PBS buffer (ice cold) to the pellet and mix thoroughly. Centrifuge as above; discard the supernatant.
4. Repeat this thrice to wash the pellet. Add 4-5mL of 1X PBS, dispensing the pellet completely.
5. Add equal amount of lysis buffer and incubate on a shaker for 45mins. Maintain ice cold conditions.
6. Seven to eight cycles of sonication should be done following the incubation. Centrifuge the tube again as above.
7. Transfer the supernatant to a fresh tube and add 4 times the amount of ice cold acetone. Incubate at -20°C for 2 hours. Centrifuge as above.
8. The extracted proteins are found adhered to the wall of centrifuge tube. Discard the supernatant against this direction.
9. Dispense the extracted proteins in minimum amount (200 µL) of 1X PBS.

Table 13. Literature based primers for 3 genes each of ESBL and carbapenems resistance [18-21]

Sr. No.	Gene	Sequence	Reference
ESBL			
1	TEM	TTGGGTGCACGAGTGGGTTA TAATTGTTGCCGGAAGCTA	[18]
2	SHV	CGCCGGGTTATTCTTATTTGTCGC TCTTTCCGATGCCGCCGCCAGTCA	[18]
3	CTX-M	ATGATGACTCAGAGCATTCCGCCG TCAGAAACCGTGGGTTACGATTTT	[19]
Carbapenems			
1	NDM	GGCAGTCGCTTCCAACGGT GTAGTGCTCAGTGTCCGGCAT	[20]
2	VIM	TTTGGTTCGCATATCGCAACG CCATTAGCCAGATCGGCAT	[20]
3	OXA-48	TTGGTGGCATCGATTATCGG GAGCACTTCTTTGTGATGGC	[21]

Table 14. Reagents required for gel electrophoresis

Sr. No.	Reagents
1	TAE buffer 10X
2	Agarose powder 2%
3	6X Gel Loading dye
4	100 bp DNA ladder
5	Ethidium bromide (1 mg/mL) [Caution: EtBr is a known carcinogen. Precaution is advised while use]




Sr No	Instrument Name	Reference Image
1	BacT-Alert 3D system (Biomerieux, USA)	
2	Sonicator (Trans-o-Sonic, India)	
3	Nanodrop (Thermofisher, USA)	

Fig. 4. Lab equipments required for WCL protocol

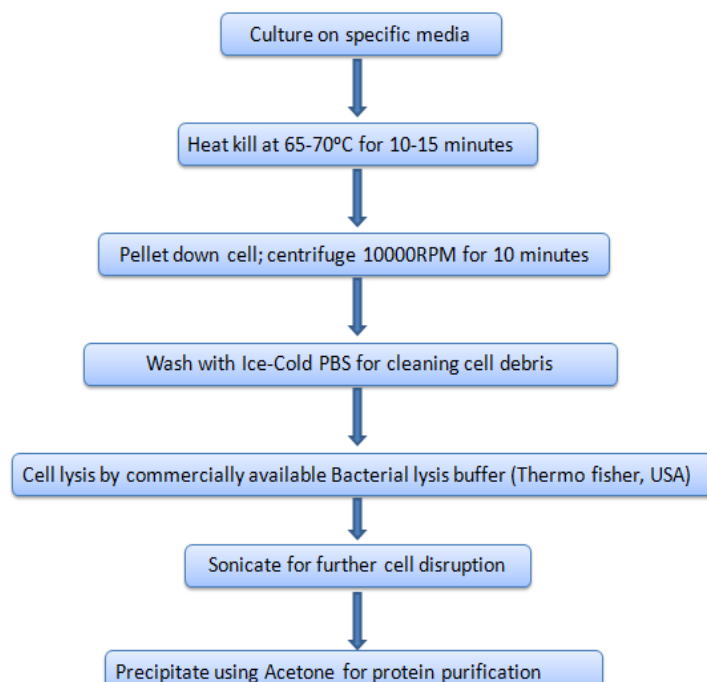


Fig. 5. Schematic representation for extraction of whole cell lysates using commercially available lysis buffer (Thermofisher scientific, USA)

Alternatively, different protocols for cell lysate extraction will be processed and optimized to develop a clinically suitable protocol for protein extraction. Some of the methods are listed in Table 15.

2.3.2 Protein quantification

Determine the exact concentrations of micro proteins in the whole cell lysates by Nanodrop technique (Thermofisher, USA).

2.3.2.1 Procedure

1. Start the program on Nanodrop software module
2. Set **Blank** using distilled water or TE buffer based on diluents used in sample.
3. Add 2µL of distilled water/TE buffer on the Nanodrop instrument pedestal and click **Blank**
4. A 2µL sample size is recommended for protein measurements. Add 2µL extracted whole cell lysate on the Nanodrop pedestal and select the tab labelled **Measure**
5. The software module measures concentration of protein (mg/mL) along with purity ratio
6. Dilute the lysates to the concentration of 1mg/mL before proceeding to ELISA.

2.4 Elisa

Enzyme-linked immunosorbent assay (ELISA) works based on an enzyme system to provide distinct combination of an antibody to its specific antigen. This method is used for quantifying an antigen immobilized on a solid surface like a micro-well tray. ELISA technique uses a very specific secondary antibody which has an enzyme coupled covalently to it. Fig. 6 shows the diagrammatic representation of ELISA technique and Fig. 7 shows the equipments required for ELISA technique in laboratory.

2.4.1 Standardization of ELISA technique

1. Standardization of the ELISA will be conducted by using Checker board method to know the exact concentration of antigen and secondary antibody in which the results can be optimal.
2. Several dilutions of antigens (WCL) and antibody (IgM) will be used. Each dilution of antibody should be used against each dilution of antigen prepared as can be studied in Fig. 8 and Table 16.
3. The initial concentration of antigen should be 1 mg/ml.
4. Use different dilutions of WCL for coating of ELISA plates.

Table 15. Various methods of bacterial cell lysis for extraction of WCL

Sr. No.	Methods	Cell lysis protocols
1	Method-1	Commercially available cell lysis buffer + Sonication
2	Method-2	SDS + Lysozyme + Urea + Sonication
3	Method-3	10% Glycerol + 0.1% Triton + 100µg/mL Lysozyme + 1mM Phenyl-methyl-sulphonyl fluoride (PMSF)
4	Method-4	10% SDS and 2x Laemmli buffer; heat for 10mins
5	Method-5	Homogenization at 6000-10000 psi

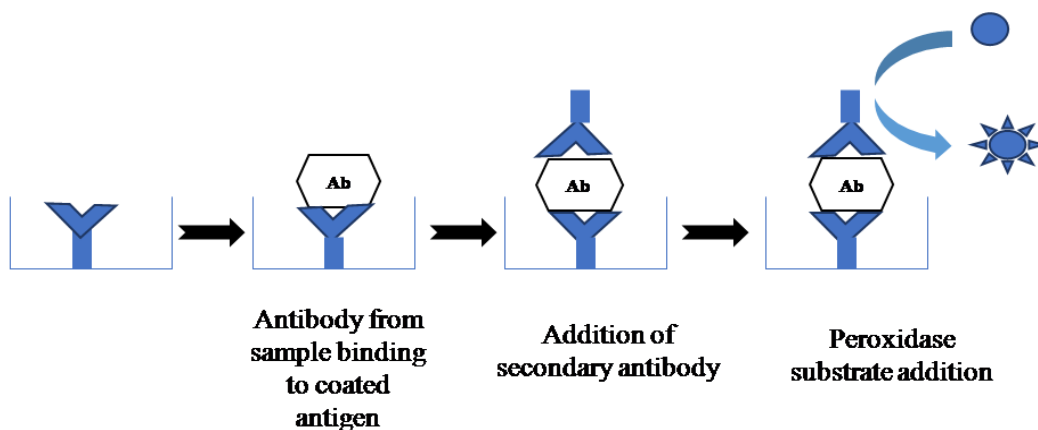


Fig. 6. Diagrammatic representation of ELISA technique






Sr No	Instrument Name	Reference Image
1	Incubator (I-Therm, India)	
2	Cyclo- Mixer for Vortexing (REMI, India)	
3	ELISA plate reader (Robonik, India)	
4	pH Meter (Labman, India)	
5	Laboratory Centrifuge (REMI, India)	

Fig. 7. Lab equipments required for ELISA technique

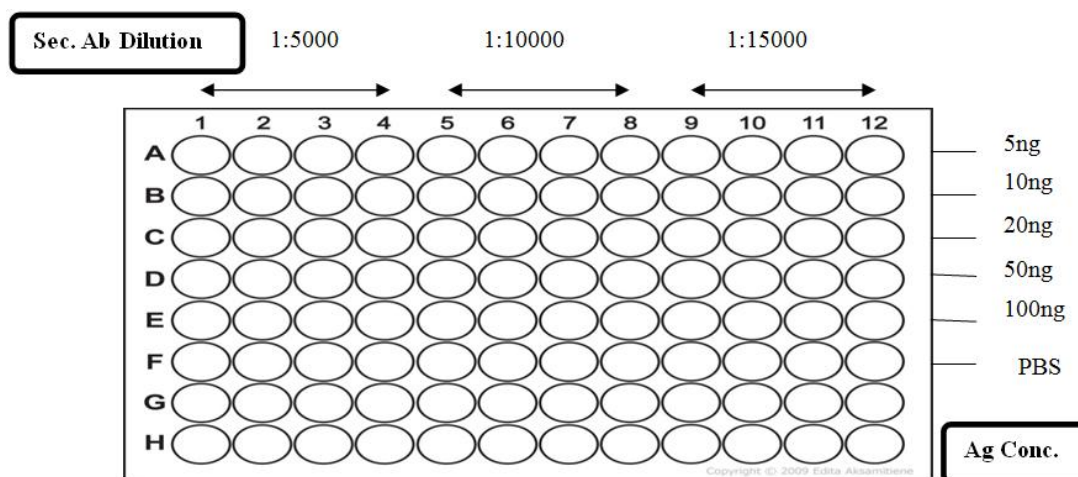


Fig. 8. ELISA plate coated with different dilutions of antigens (vertically) and secondary antibody (horizontally) for standardization

Table 16. Checkerboard method for standardization of ELISA technique

Sr. No.	Concentration	Antibody dilution		
		1:5000	1:10000	1:15000
1	5 ng/mL			
2	10 ng/mL			
3	20 ng/mL			
4	50 ng/mL			
5	100 ng/mL			

5. After coating, add 100 μ L of the CSF samples in each well. Each sample was added in duplicate with a negative control and PBS.
6. Antibody (IgM) of following concentrations should be used for all five dilutions of antigens.
 - A 1:5000 dilution: 1 μ l of IgM in 5ml of 1X PBS.
 - A 1:10000 dilutions: 1 μ l of IgM in 10ml of 1X PBS.
 - A 1:15000 dilutions: 1 μ l of IgM in 15ml of 1X PBS.
7. For first set, to the wells coated with 5ng/mL dilution of antigens, add the CSF samples and incubate at 37°C for 35 mins. Following this, add 100 μ L of secondary IgM antibody (Goat anti-rabbit HRP conjugated) diluted to 1:5000, 1:10000 and 1:150000 respectively to the wells and incubate for 30mins.
8. Wash the well 4 times with wash buffer. Add 100 μ L substrate to each well followed by incubation at RT for 1 minute.
9. Add stop solution after 1 minute and measure OD at 450 nm on an ELISA plate reader.
10. Repeat the same procedure for wells coated with the concentration of 10 ng/mL, 20 ng/mL, 50 ng/mL and 100 ng/mL.
11. Based on the results obtained, the appropriate dilution of antigen and secondary antibody should be decided for further processing of clinical samples.

2.4.2 Coating of ELISA plates

1. Calculate the number of ELISA wells for the samples in duplicate
2. For coating the wells, add 1.2 μ L of 1mg/mL of cell lysate to 12 mL of 1X PBS
3. Dispense 100 μ L of the above dilution in each well to be coated and then incubate for 3 hours at 37°C
4. Post incubation, wash these wells with the help of wash buffer for 1 min.
5. For blocking the antigens dispense 0.5% BSA in the sample wells and incubate for 2 hours at 37°C.
6. Wash the plates using wash buffer for 1 minute and then dry the wells by gently tapping the ELISA plates on tissue paper.
7. Cover the plate with tissue and ELISA plate cover. Store in inverted manner at 4°C.

2.4.3 ELISA protocol for Antibody detection

1. Before starting the procedure, wash the coated wells twice with wash buffer.
2. Dispense the optimized amount of CSF in duplicates for accurate results and incubate for 35 minutes at 37°C
3. Follow this by washing thrice with wash buffer
4. Add the optimized amount of secondary antibody to all the wells and incubate as above.
5. Wash the wells again 4 times with wash buffer before adding 100 μ L substrate to each well and incubate for 1 minute
6. Add Stop solution and measure OD at 450nm
7. This protocol will be followed through in order to develop the ELISA technique for the 8 bacteria included in the study individually.

3. RESULTS

3.1 Participants

The recruitments of participants with clinical as well as baseline criteria can be seen in Fig. 9 also depicting clinical inclusion and exclusion criteria designed for the present study.

The reference test in the present study will be Microbiological culture, the universal gold standard for diagnosis of Bacterial meningitis. If in case, the microbiological culturing yields undetermining results, the results of in house Molecular PCR technique developed by Sharda Bhagchandani et al will be considered as reference. For the molecular tests developed to study antibiotic resistance, the reference standard will be automated BD Phoenix system for drug susceptibility testing. The clinical information and results of reference standards and index tests will be available to the performer, reader, assessor alike.

The test positivity cut off for serology based technique can be defined as that value, where above if the result obtained, will be considered positive. There will be no cut off determination required for the molecular tests. For the test that will be developed based on ELISA, test cut-offs, sensitivity and specificity will be determined by ROC using the MedCalc statistical software (10.1.2.0).

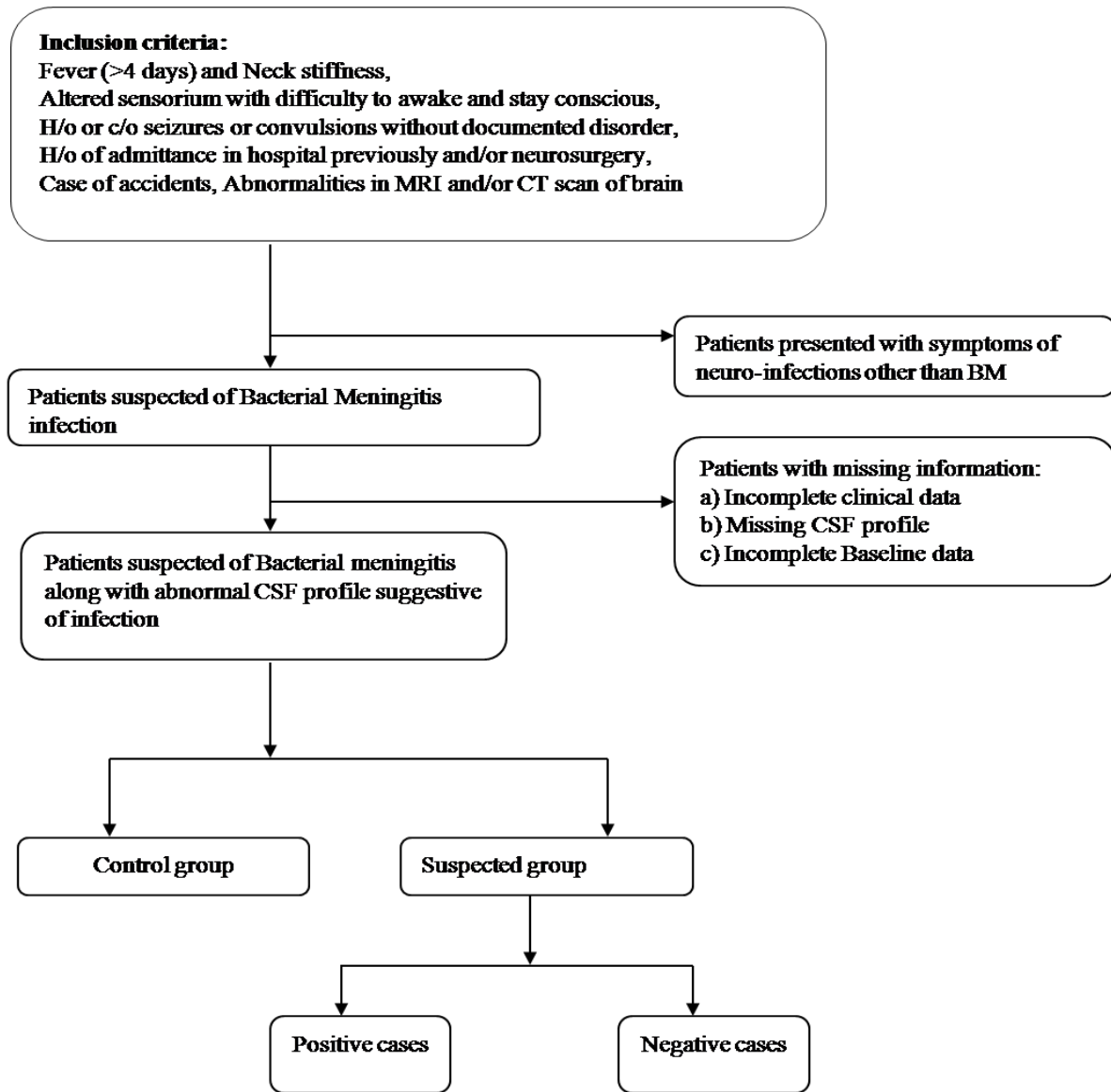


Fig. 9. Diagram for flow of participants inclusive of baseline and clinical characteristics

3.2 Analysis

Test concordance between molecular and serological tests developed will be assessed using the kappa (κ) statistic. Indeterminate test or reference standard will be handled by repeated evaluation of the particular test until valid results are obtained. Missing data for index test or reference standard will be dealt with retrospective follow up via telephonic conversations.

The sample size of the study has been determined by a two-stage sampling approach adopted using R 3.4.3-programming tool, taking into consideration the prevalence of 15% and the

confidence interval of 95%, a sample size of 307 was determined.

3.3 Test Results

The distribution of BM in patients with suggestive condition will be studied by the effects of the disease observed in the patient as well as by studying the reports of routine analysis performed for CSF after lumbar puncture. The higher the amount of total cells present in the CSF more will be the severity of infection. This can further be determined by testing the organisms causing infection for antimicrobial resistance as multidrug resistant bacteria generally tend to cause more severe form of

infections due to lack of appropriate treatment. The time interval from the point of patient's admittance to diagnosis will depend from case to case.

The patient after being suspected or diagnosed of a neurological complication will be proceeded for lumbar puncture and the CSF sample will be received for diagnosis of BM using predesigned in-house nested PCR technique at the same time, a portion of this will be sent for CSF routine analysis for studying aspects including Total cell count, Differential cell count, CSF sugar, CSF protein, CSF lactate and more. This can also be helpful in preliminary diagnosis of Bacterial Meningitis. The positivity will be determined based on results obtained on gel electrophoresis following 16s rDNA and Nested PCR amplifications of the extracted DNA.

ELISA technique will be developed using the whole cell lysate obtained by the optimized

protocol of protein extraction. The lysate containing antigens will be used for coating the ELISA wells which subsequently help in identifying the antibodies present in the sample. Standardization of the ELISA technique will be performed according to the above mentioned Checkerboard method and the evaluation of the standardized method will be further conducted using the positive and negative samples characterized by nested PCR technique.

The frequency of the demographic, baseline and clinical factors will be measured on a nominal scale. MedCalc statistical software (version 10.1.2.0) will be used to perform statistical analysis. Baseline data will be compared in the fore mentioned groups using Chi square analysis and a difference of $P < 0.05$ will be considered to be significant. Negative predictive and positive predictive value will be estimated using a 2×2 table for diagnostic test evaluation with healthy controls. (Software: MedCalc; Version 10.1.2.0).

Troubleshooting:

Table 17. Troubleshooting for PCR and ELISA technique

Sr. No.	Step	Problem	Possible reason	Possible solution
1	PCR	Carry over contamination	Contamination can be carried over from PCR due to aerosols, contaminating pipettes, surfaces, gloves and reagents	Carryover contamination can be reduced by unidirectional workflow, proper cleaning of pipettes, making frequent and small aliquots of reagents, aseptic cleaning techniques, proper separation of reagents stored together
2	PCR	Low purity	Purity of DNA affected	Use of purification kits, remove traces of contaminants like phenol, EDTA, Proteinase K with chemical or enzymatic DNA purification protocol, re-wash the DNA with 70% ethanol to remove impurities and ions
3	ELISA	Uniform low readings	Incorrect wavelength, insufficient development time, capture antibody does not bind to plate	Check filters and readers, increase development time, dilute PBS without additional proteins, re-qualify the reagents of choice.
4	ELISA	High signal	Substrate solution mixed too early and turning colour uniformly	Make fresh buffers, substrate solution to be freshly prepared and used, wash the plates properly, check dilutions, titrate if necessary

4. DISCUSSION

In the proposed study, the aim is to aid the diagnosis and treatment of BM in low resource setting areas and tertiary care hospitals by development of rapid, efficacious yet affordable techniques for detection. Initial screening will be conducted on the basis of clinically structured proforma and the patients suspected of BM will be further diagnosed on basis of Nested PCR tool developed by Bhagchandani et al. [17].

Moreover, data of antimicrobial resistance in BM samples is severely lacking in India and needs to be studied. Molecular based PCR techniques that can detect these antimicrobial resistant genes with specifically targeted primers will tremendously help in antibiotic stewardship programs [18-20].

5. CONCLUSION

ELISA for diagnosis of BM will be developed using the whole cell lysates of the organisms which contain an array of bacterial proteins. These proteins will be used for detection of the infection with specific antibodies present in the sample. This semi quantitative test can be performed in matter of few hours thus initiating appropriate treatment to prevent co-morbidities incurred. A few commercial ELISA tests available have a disadvantage of being expensive which cannot be sustained by a common person [21-23]. This can be overcome by development of affordable inhouse ELISA that can produce accurate results in a short period of time. The lysates can also be scaled up to development of point of care tests like lateral flow for diagnosis in remote areas where sophisticated laboratory cannot be established [24-27].

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

Admitted patients suggestive of BM will be recruited after taking written consents and verbal

explanation about the study using predefined clinical inclusion and exclusion criteria.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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