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Research Protocol

Biochemistry Section

Metagenomic Identification of Uncommon Meningoencephalitis Pathogens and Development of Peptide-based Immunoassays for Early Diagnosis: A Prospective Observational Study Research Protocol

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ABSTRACT

Introduction: Meningoencephalitis (ME) is an inflammatory condition of the meninges and brain parenchyma, remains a major diagnostic challenge. Metagenomic Next-Generation Sequencing (mNGS) has emerged as a promising tool for unbiased detection of viral, bacterial, fungal, and parasitic pathogens often missed by conventional methods. It also enables identification of regional pathogen profiles to guide development of locally tailored diagnostics. However, mNGS-based studies from India, particularly Central India, remain limited.

Need of the study: The lack of diagnostic tests for uncommon and regionally prevalent pathogens causing ME in Central India contributes to a high burden of undiagnosed cases. Even in well-equipped healthcare settings, a proportion of ME cases remain without confirmed aetiology due to diverse pathogens and overlapping clinical presentations. Addressing this gap is essential for improving aetiological diagnosis and guiding appropriate treatment. Therefore, the present study is designed to identify these uncommon pathogens using mNGS approach and to develop targeted, clinically applicable diagnostic tools to improve early detection and patient management.

Aim: To characterise the aetiology of pathogens in ME cases using mNGS and to develop peptide-based immunoassays for uncommon pathogens enabling early diagnosis.

Materials and Methods: The present prospective observational study will be conducted at the Neurological Intensive Care Unit (NICU) of Central India Institute of Medical Sciences (CIIMS), Nagpur, Maharashtra, India from April 2026 to April 2028, enrolling 146 clinically suspected ME patients (18-60 years). Cerebrospinal Fluid (CSF) samples will be analysed using conventional and metagenomic approaches. Patients will be categorised into undiagnosed, confirmed, and non infectious groups. Peptide-based Enzyme-Linked Immunosorbent Assay (ELISA) will be developed based on mNGS findings. Statistical analysis will include sensitivity, specificity, and Receiver Operating Characteristic (ROC) curve evaluation.

Keywords: Biomarkers, Cerebrospinal fluid, Enzyme-linked immunosorbent assay, Metagenomic next-generation sequencing

INTRODUCTION

Central Nervous System (CNS) diseases such as ME are associated with significant cause of mortality and morbidity in tertiary neuro care Intensive Care Unit (ICU) settings [1]. ME is an infectious neurological emergency characterised by inflammation of the meninges and brain, presenting with symptoms such as fever, seizures, and altered sensorium. Despite advances in diagnostics, ME remains a significant public health concern in India, with overlapping clinical features and diverse aetiologies complicating diagnosis and management [2]. In Western countries, bacterial meningitis occurs at approximately three per 100,000 population, while viral meningitis is more common, with an incidence of 10.9 per 100,000 [3,4].

At the CIIMS, Nagpur, Maharashtra, India, approximately 300-400 ME cases are reported annually (unpublished data). Despite the availability of in-house Polymerase Chain Reaction (PCR)-based diagnostic panels, 30-40% of cases remain undiagnosed highlighting the need for improved diagnostic approaches.

The mNGS has emerged as a powerful, unbiased tool for detecting a wide range of pathogens directly from clinical samples without prior knowledge of the causative agent [5,6]. Unlike conventional PCR-based methods, which are targeted, mNGS enables comprehensive pathogen identification, including rare and novel organisms. Although cost remains a limitation, its application has the potential to improve aetiological diagnosis and guide targeted treatment, reducing complications associated with empirical therapy [7].

Limited studies are available from Central India evaluating the use of mNGS in ME. Previous studies have demonstrated the clinical utility of mNGS in CNS infections, including improved diagnostic yield and identification of uncommon pathogens [8]. However, most available evidence is derived from studies conducted outside India, with only limited regional data available, including preliminary reports [9].

REVIEW OF LITERATURE

The ME remains a diagnostic challenge, as conventional methods such as culture and targeted PCR often fail to identify the causative pathogen in a substantial proportion of cases [10]. mNGS has emerged as a powerful, unbiased tool capable of detecting a wide range of bacterial, viral, fungal, and parasitic pathogens directly from CSF without prior assumptions. Clinical studies have demonstrated that mNGS improves pathogen detection rates and facilitates faster, more accurate diagnosis in ME cases [8,11-14].

For instance, Wilson MR et al., reported that mNGS significantly enhanced diagnostic yield, including detection of rare and unexpected pathogens not identified by routine methods [8]. A large prospective study involving over 200 patients further supported its role as a complementary diagnostic tool in CNS infections [2]. Additionally, cohort studies have shown that mNGS exhibits higher sensitivity than culture-based methods, reinforcing its clinical utility [13].

Despite these advantages, widespread use of mNGS is limited by high cost, longer turnaround time, and the need for specialised infrastructure and bioinformatics expertise. This highlights the need for scalable and cost-effective diagnostic approaches. Peptide-based immunoassays represent a promising strategy to translate mNGS-based pathogen discovery into rapid and accessible diagnostics [14]. However, studies integrating mNGS with peptide-based immunoassay development for ME remain limited, particularly in the Indian clinical context.

The primary objective includes Illumina-based mNGS for identification of uncommon pathogens and the secondary objective is the development of immunodiagnostics for uncommon ME pathogens identified by metagenomic sequencing using synthetic peptides.

The Null Hypothesis (H0) is mNGS and peptide-based immunoassays do not signifi cantly improve the detection of uncommon pathogens in undiagnosed ME **cases compared with conventional diagnostic methods and**

Alternative Hypothesis (H1) includes the mNGS and peptide-based immunoassays signifi cantly improve the detection of uncommon pathogens in undiagnosed ME cases compared with conventional diagnostic methods.

MATERIALS AND METHODS

The study will be conducted at the Neurological Intensive Care Unit (NICU) of CIIMS Hospital, Nagpur, Maharashtra, India, a tertiary care centre in Maharashtra, India. The study will be carried out from April 2026 to April 2028 over a period of two years. This facility manages a wide range of neurological disorders and infections. All clinical sample collection, patient monitoring, and initial diagnostics will be performed at this site under the supervision of experienced clinicians and neurologists. The study was approved by the Institutional Ethics Committee of the Central India Institute of Medical Sciences (CIIMS) in Nagpur (IEC Approval No.: CIIMS/HEC/14/02/2023; Date: 14 February 2023) and conducted in accordance with the Code of Ethics of the World Medical Association Declaration of Helsinki (2013) [15]. Written consent will be obtained from all patients after an oral explanation of the study.

The present study will be a prospective observational study designed to evaluate the aetiological spectrum of ME using mNGS in comparison with conventional microbiological, molecular, and immunological diagnostic methods. In addition, the study will focus on the development of a peptide-based ELISA for the early detection of uncommon pathogens identified through mNGS.

Sample size calculation: A total of 146 participants will be enrolled and categorised into defined aetiology, undiagnosed aetiology, and non infectious control groups. The overall study workflow will include patient recruitment, CSF collection, routine diagnostics, mNGS analysis, bioinformatic interpretation, antigenic peptide selection, and development and evaluation of peptide-based immunoassays.

Patients will be recruited prospectively from the NICU at CIIMS Hospital, Nagpur. A total of 146 participants aged 18-60 years with clinically suspected ME will be enrolled in the study.

Considering the reported prevalence of ME of approximately 2-3% (~3%) based on published studies (Jayaraman Y et al., 2018) [16] and a population size of approximately 2.5 million (Nagpur district), the sample size for this diagnostic accuracy study was calculated using the method described by Flahault A et al., for estimation of sensitivity. Assuming an expected sensitivity of 95% for mNGS, a desired precision of 5%, and a 95% confidence level, the minimum number of diseased subjects required was calculated to be 73. Considering an anticipated disease prevalence of 50% among clinically suspected ME cases, the total sample size was estimated to be 146 participants, comprising approximately equal numbers of diseased and non-diseased individuals [17].

The sample size was determined based on the clinical burden of ME cases at CIIMS Hospital and the planned study duration (April 2026-April 2028). As a tertiary-care referral centre for CNS infections in Central India, CIIMS manages a substantial number of suspected ME cases annually, making recruitment of approximately 146 participants feasible for the primary objective.

This study is designed as a prospective observational diagnostic accuracy study to evaluate the diagnostic utility of mNGS and to develop peptide-based immunodiagnostic assays for detecting uncommon ME pathogens. A total of 146 participants (18-60 years) with clinically suspected ME will be enrolled in the study, as determined by diagnostic accuracy-based sample size calculation to ensure adequate precision in estimating the sensitivity of mNGS.

Likewise for Peptide based Immunoassay, Recruitment of 146 participants will be based on clinical presentation, diagnostic work-up, and classification into three groups:

- Group-1: Symptomatic undiagnosed ME cases (n=37) with no confirmatory bacterial or viral diagnosis;
- Group-2: ME cases with confirmed aetiology (n=36);
- Group-3: Non infectious controls (n=73) such as known cases of stroke, migraine, or epilepsy without any infectious aetiology.

Inclusion criteria: Participants aged 18-60 years with clinically and/or biochemically diagnosed or suspected ME presenting with one or more features such as fever, headache, altered sensorium, seizures, or neck stiffness will be included.

Exclusion criteria: Patients with known malignancies (including carcinoma, leukaemia, or CNS tumours), severely immunocompromised states, or clearly established non infectious neurological aetiologies will be excluded from the study.

Study Procedure

CSF will be collected under aseptic conditions via standard lumbar puncture. Approximately, 3-5 mL will be divided into two aliquots: one for routine clinical investigations and the other for nucleic acid extraction and quality control for mNGS. Samples will be collected in sterile, nuclease-free containers without preservatives. Fresh samples will be preferred; stored samples will be maintained at -80°C and thawed on ice prior to processing.

An aliquot (100-200 µL) will be centrifuged at 1500 × g for five minutes, and the supernatant (for sequencing) and pellet (backup) will be separated. Deoxyribonucleic Acid/Ribonucleic Acid (DNA/RNA) Shield will be added to preserve nucleic acids (1:1 for supernatant; 100-200 µL for pellet). Samples will be stored at minus 80°C or processed immediately for nucleic acid extraction [18].

I. DNA extraction:

Reagents and Chemicals:

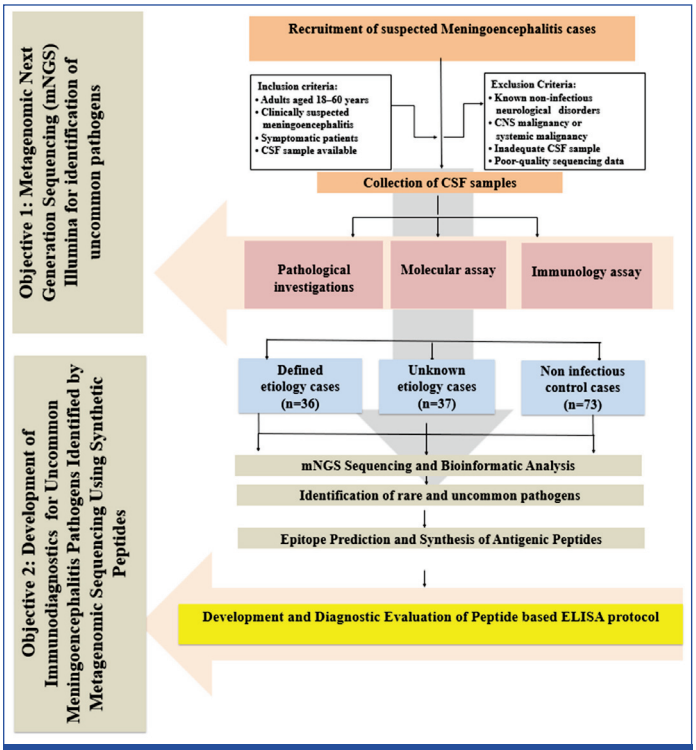
1. DNA/RNA Shield (2× Concentrate)
2. Pathogen DNA/RNA Buffer
3. ZymoBIOMICS™ Mag Binding Beads
4. MagBead DNA/RNA Wash 1

5. MagBead DNA/RNA Wash 2
6. Ethanol (95-100%)
7. Zymo BIOMICS™DNase/RNase-FreeWater
8. RNaseA (Sigma-Aldrich,USA)

Equipment's:

1. QuickSpinMini Centrifuge 6,000 rotations per minute (rpm) (make-Himedia);
2. MagneticStand (16-tube magnetic rack) (make-In-vitrogen);

Study protocol [Table/Fig-1]



[Table/Fig-1]: Schematic flowchart for evaluation of metagenomics-based approach to identify the pathogen causing ME.

Nucleic acids will be extracted from CSF samples using the Quick-DNA/RNA Pathogen MagBead Kit (Zymo Research, USA) according to the manufacturer's instructions. Briefly, CSF samples preserved in DNA/RNA Shield will undergo lysis followed by magnetic bead-based nucleic acid purification. After washing steps to remove contaminants, purified DNA will be eluted in ZymoBIOMICS™ DNase/RNase-Free Water and stored at -20°C until library preparation [19].

II. Nucleic acid Quality Control (QC) analysis:

The quantity and quality of extracted nucleic acids will be assessed prior to library preparation using the Qubit™ dsDNA High Sensitivity Assay (Thermo Fisher Scientific). Quality control will be performed after DNA extraction and at key steps of the sequencing workflow to ensure sample integrity and successful library preparation [20].

III. Library preparation and sequencing:

Reagents and Chemicals:

1. General Illumina Library Preparation Reagents
   a. Bead-Linked Transposomes (BLT)
   b. Tagmentation Buffer1 (TB1)
   c. Tagment Stop Buffer (TSB)
   d. TagmentWashBuffer(TWB)
   e. Enhanced PCR Mix (EPM)
   f. Nuclease-Free Water
   g. Index Adapters
   h. Nextera DNACD Indexes (96 Indexes-96 Samples)
   i. IDT for Illumina DNA/RNA UD Indexes
   j. Illumina Purification Beads (IPB)
   k. Resuspension Buffer (RSB)
   l. Freshly Prepared 80% Ethanol (EtOH)
2. Sequencing-Specific Reagents
   a. iSeq 100 i1 Reagent Cartridge v2 (8-pack; Illumina, USA)
   b. Qubit 1X dsDNA HS Assay Kit (for DNA quantification)
   c. ZymoBIOMICS™DNase/RNase-FreeWater *(used if referenced for elution prior to sequencing)*

Equipment's:

1. Qubit4 Fluorometer (Starter Package with Wi-Fi)-(Make:In-vitrogen-Model: Qubit 4 Fluorometer)
2. Illumina iSeq100 Sequencing System (Make: Illumina)
3. DNALoBindTubes/1.5 mL Microtubes-(Make: Eppendorf-Model: LoBind Tubes)

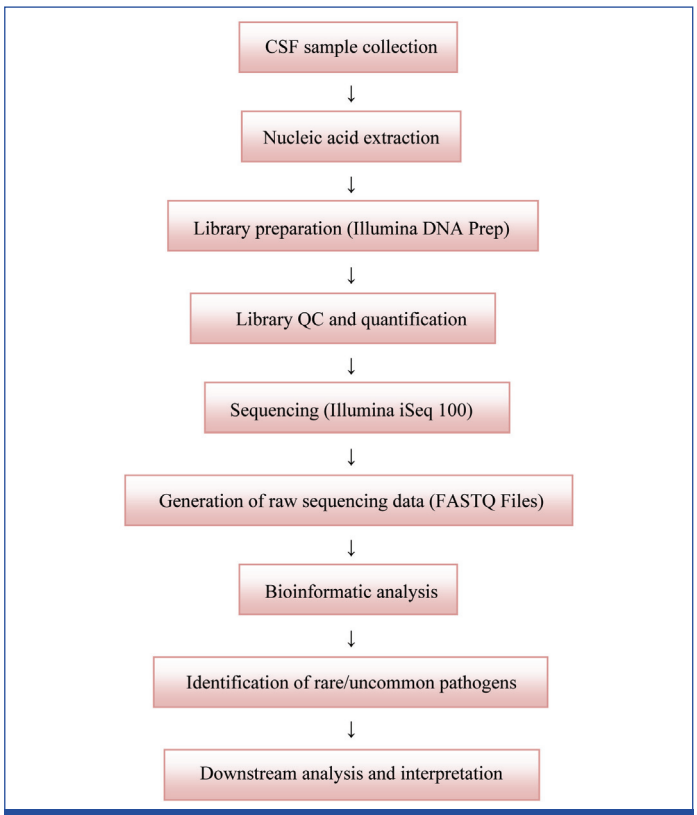
Protocol:

Metagenomic libraries will be prepared from extracted DNA using the Illumina DNA Prep workflow with Bead-Linked Transposome (BLT) technology (Illumina, USA) as per the manufacturer's instructions. This method enables simultaneous DNA fragmentation and adapter tagging, followed by PCR amplification and purification.

Libraries will be quantified using the Qubit™ Double-Stranded DNA (dsDNA) High Sensitivity Assay, normalised, and pooled prior to sequencing. Sequencing will be performed on the Illumina iSeq 100 platform using the iSeq 100 i1 Reagent Cartridge v2 in paired-end mode. Runs will be managed using Local Run Manager (LRM) software, and raw sequence reads (FASTQ files) will be generated for downstream bioinformatic analysis [21]. All steps will be performed according to the manufacturer's instructions and using the reagents and equipment specified earlier.

- Metagenomic mNGS Workflow

Step 1: Sequencing run set-up [Table/Fig-2].



[Table/Fig-2]: Workflow of run set-up in iSeq 100 and data analysis.

- The final diluted pooled library will be loaded on to the iSeq100i1 Reagent Cartridge v2.
- A sequencing run will be initiated on the Illuminai Seq100 using paired-end mode.
- The system will be set to target approximately ~4 million reads per sample (depending on platform capacity and pooling).
- Sequencing run set-up will be managed using the LRM software [21].

Step 2: Output Generation and Data Retrieval

- Upon completion, raw sequencing reads (FASTQ files) will be generated by the instrument.
- These FASTQ files will be downloaded and stored for further bioinformatics analysis.

IV. Bioinformatics Analysis of mNGS Data for Pathogen Identification

Step1: Metagenomic Data Processing and Pathogen Identification

Raw sequencing reads will be analysed using the IDseq platform (https://idseq.net) or alternatively with Geneious (version 11.1.5) and other bioinformatics tools [22,23]. To minimise background noise, environmental and commensal taxa will be filtered using negative controls, including environmental and water samples, and organisms detected in more than 50% of samples over a three-month period will be excluded.

Filtered data will be evaluated based on genome coverage, sequencing depth, and Species-Specific Read Numbers (SSRN). Putative pathogens will be identified using mNGS read metrics, confirmation by targeted Reverse Transcription-Polymerase Chain Reaction (RT-PCR) where applicable, and correlation with clinical presentation and CSF findings. Organisms meeting these criteria will be considered true pathogens, while others will be classified as commensals or contaminants. Final pathogen assignment will follow established interpretative frameworks [9,12].

Step2: Genome Annotation of Undiagnosed ME Cases Using Bakta

1. The user will open the link https://bakta.computational.bio/ in a local web browser.
2. The input file in FASTA format (from NGS output) will be uploaded.
3. Specific settings for the annotation process will be chosen (if required). Default configurations will be sufficient for most bacterial genomes. The annotation process will be initiated.
4. Once completed, Bakta will generate outputs such as annotation summaries, sequence features, and visual reports.
5. Bakta was used specifically for the annotation of bacterial genomic/metagenomic data identified through mNGS. No viral, fungal, or parasitic pathogens were identified in the analysed samples; therefore, taxon-specific annotation for these pathogen groups was not applicable in the present study.

Step3: Interpretation of Annotated Genomic Features for Comparative Analysis

Bakta will provide detailed annotation results that include the identification and classification of genes, Coding Sequences (CDS), rRNA, tRNA, and other genomic features. Functional annotations will be predicted using reference databases such as UniProt, Pfam, and InterPro. Additionally, metadata enrichment will incorporate information on functional categories, metabolic pathways, and enzyme annotations to enhance biological context. The platform will also generate visual outputs, including circular genome maps and feature plots, to support intuitive data interpretation. Annotation results will be available in multiple formats, including GenBank, Generic Feature Format Version 3 (GFF3), and tabular summaries, to facilitate downstream analyses.

Step 4: Selection of Pathogen-Specific Target Proteins Missed by Conventional Methods Following annotation with Bakta, CDS and predicted proteins will be identified. Among these, uncommon or novel pathogen-associated proteins-those detected through mNGS but missed by conventional diagnostic methods-will be shortlisted as potential targets. These target proteins will then be verified using BLAST analysis against the National Centre for Biotechnology Information (NCBI) Nucleotide/Non-Redundant (NT/NR) database to confirm their specificity and eliminate any sequences related to the human host. Verified protein sequences will subsequently be processed for further structural and functional annotation.

Step 5: In Silico Protein Annotation and Epitope Prediction for Early Immunodiagnostics

Verified protein sequences will be annotated using UniProtKB/Swiss-Protand the Expert Protein Analysis System (EXPASY) proteomics server to obtain detailed structural and functional information [24,25]. Candidate proteins will be prioritised based on functional relevance, protein abundance, and sequence conservation across pathogenic strains.

Immunodominant regions will be identified, and antigenic peptides will be predicted using the Kolaskar AS and Tongaonkar PC method, which evaluates antigenicity based on amino acid properties and surface accessibility, with ~75% accuracy [26]. The selected peptides will then be assessed for pathogen specificity and screened to ensure minimal homology with human proteins, thereby reducing the risk of cross-reactivity [27].

Step 6: Design and synthesis of peptides for ELISA-based detection of uncommon ME

Pathogens:

Final antigenic peptide candidates will be synthesised through an outsourced peptide service. The synthesised peptides will be coated onto microtiter ELISA plates for use CSF samples. These peptide-based ELISAs will serve as diagnostic tools for detecting ME pathogens identified through mNGS [28,29].

V. Peptide-Based ELISA for immunodiagnostic detection of uncommon pathogens in

ME cases:

After peptide synthesis, an indirect peptide-based ELISA will be developed to detect pathogen-specific Immunoglobulin G (IgG) and Immunoglobulin M (IgM) antibodies in CSF samples against antigens identified through mNGS. Synthesised peptides will coat microtiter plates, and detection will be performed using Horseradish Peroxidase (HRP)-conjugated secondary antibodies [30]. The assay will evaluate the diagnostic sensitivity and specificity of selected peptides using clinical CSF samples. Prior to performing the antibody detection ELISA, a checkerboard titration will be conducted to optimise the concentrations of the coating antigen (synthetic peptides), CSF sample dilutions, and HRP-conjugated secondary antibodies. This standardisation step will help determine the ideal working concentrations that yield the signal-to-noise ratio and ensure consistent assay performance. Based on the titration results, the optimised conditions will be used in the final ELISA protocol for evaluating diagnostic accuracy [31].

The performance of the peptide-based ELISA will be validated by comparison with gold-standard methods, including conventional culture, targeted PCR/RT-PCR, and mNGS. ELISA results will be compared with reference diagnostic outcomes to assess concordance and diagnostic accuracy.

Reagents and chemicals:

a. Synthesised Antigenic Peptides (outsourced)
b. 96-well ELISA Microtiter Plates (high-binding)
c. Coating Buffer (e.g., Phosphate Buffer Saline, pH7.4)
d. Blocking Buffer(e.g.,0.5%BSA)
e. CSF Samples (from ME patients and controls)
f. Wash Buffer (PBS with Tween-20)
g. HRP-Conjugated Secondary Antibodies (Goat Anti-human
h. IgG and IgM HRP Conjugate).
i. Substrate: TMB(3,3′,5,5′-Tetramethylbenzidine)
j. Stop Solution (e.g.,2.5NH_2SO_4)
k. Phosphate Buffered Saline (PBS)

Equipment's:

ELISA Plate Reader (Make-J.Mitra & Co., Ltd., Model-ER-181s)

Protocol:

Antibody ELISA will be performed to detect antibodies against uncommon ME pathogens in CSF samples. Microtiter wells will be coated with 100 µL of synthetic peptides in Phosphate-Buffered Saline (PBS) (pH 7.4) and incubated overnight at 4°C. Plates will then be blocked with 0.5% BSA for two hours at 37°C, followed by washing with PBS-T. Diluted CSF samples (1:5) will be added and incubated for one hour at 37°C. After washing, HRP-conjugated goat anti-human IgG (1:10,000) and/or IgM (1:5,000) antibodies will be added and incubated for 45 minutes at 37°C. Following washing, 3,3′,5,5′-Tetramethylbenzidine/Hydrogen Peroxide (TMB/H_2O_2) substrate will be added, and the reaction will be stopped using 2.5 Normal Sulfuric Acid solution (2.5N H_2SO_4). Absorbance will be measured at 450 nm [29].

Outcome Measures

Primary outcome: Uncommon and previously undiagnosed ME pathogens will be identified using mNGS.

Secondary outcomes: Pathogen-specific antigenic peptides will be identified through bioinformatic analyses, followed by development and evaluation of peptide-based ELISA assays. Characterisation of the aetiological spectrum of ME and generation of regional epidemiological data. The distribution of pathogens causing ME will be characterised, and regional epidemiological data will be generated.

STATISTICAL ANALYSIS

The developed peptide-based ELISA will be validated against a composite reference standard incorporating conventional microbiological methods (culture, microscopy, and antigen detection), pathogen-specific PCR assays where applicable, and relevant clinical and radiological findings. Diagnostic performance will be assessed by calculating sensitivity, specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV). Continuous data will be analysed using non-parametric tests and expressed as medians {Interquartile Range (IQR)}, while qualitative variables will be presented as percentages. Mean±Standard Deviation (SD) will be reported for normally distributed data, where applicable. ROC curve analysis will be performed to determine optimal cut-off values. All analyses will be conducted using MedCalc statistical software.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Nov 07, 2025
- Manual Googling: Jun 05, 2026
- iThenticate Software: Jun 08, 2026 (1%)

ETYMOLOGY: Author Origin

EMENDATIONS: 9

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

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