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RESEARCH ARTICLE

Identification of microbial agents in culture-negative brain abscess samples by 16S/18S rRNA gene PCR and sequencing

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ABSTRACT

Despite clinical suspicion of an infection, brain abscess samples are often culture-negative in routine microbiological testing. Direct PCR of such samples enables the identification of microbes that may be fastidious, non-viable, or unculturable. Brain abscess samples (n = 217) from neurosurgical patients were subjected to broad range 16S rRNA gene PCR and sequencing for bacteria. All these samples and seven formalin-fixed paraffin-embedded tissue (FFPE) samples were subjected to species-specific 18S rRNA PCR for neurotropic free-living amoeba that harbour pathogenic bacteria. The concordance between smear and/or culture and PCR was 69%. One-third of the samples were smear- and culture-negative for bacterial agents. However, 88% of these culture-negative samples showed the presence of bacterial 16S rRNA by PCR. Sanger sequencing of 27 selected samples showed anaerobic/fastidious gram negative bacteria (GNB, 38%), facultative Streptococci (35%), and aerobic GNB (27%). Targeted metagenomics sequencing of three samples showed multiple bacterial species, including anaerobic and non-culturable bacteria. One FFPE tissue revealed the presence of Acanthamoeba 18S rRNA. None of the frozen brain abscess samples tested was positive for 18S rRNA of Acanthamoeba or *Balamuthia mandrillaris*. The microbial 16/18S rRNA PCR and sequencing outperformed culture in detecting anaerobes, facultative Streptococci and FLA in brain abscess samples. Genetic analyses of 16S/18S sequences, either through Sanger or metagenomic sequencing, will be an essential diagnostic technology to be included for diagnosing culture-negative brain abscess samples. Characterizing the microbiome of culture-negative brain abscess samples by molecular methods could enable detection and/or treatment of the source of infection.

Keywords: Culture-negative brain abscess; 16S/18S rRNA gene; Sanger sequencing; metagenomics sequencing, bioinformatics.

INTRODUCTION

A brain abscess is a focal collection of pus in the brain parenchyma surrounded by a well-vascularized collagenous capsule in response to an infection (Brook, 2017). Other less frequent focal infections of the central nervous system (CNS) include subdural empyema and epidural abscesses (Dando *et al*., 2014). The microbiome of brain abscesses has been shown to be polymicrobial, dominated by uncultivable and anaerobic organisms of odontogenic origin (Kommedal *et al*., 2014). The conventional culture selectively isolates aerobic and facultative aerobes, which are minor constituents in abscesses and may outgrow clinically important organisms (Kozlov *et al*., 2018). In addition, absence of growth of causative agent in brain abscess might be due to delay in the transport of the sample, fastidious nature of the bacteria, nonviable bacteria in patients undergoing antibiotic therapy, low bacterial load in the sample, or viral/parasitic etiology (Lleo *et al*., 2014). Culture-independent 16S rRNA sequencing has been shown to significantly improve the yield of microbiological diagnosis of brain abscesses, especially for patients treated with antibiotics prior to sample collection (Salipante *et al*., 2013). The detection of bacterial DNA by broad-range PCR was reported to be 37% in culture-negative brain abscess samples (Bajpai *et al*., 2014). However, 16S rRNA Sanger sequencing does not discriminate between mixed flora in polybacterial samples (Stavnsbjerg *et al*., 2017). 16S rRNA metagenomic sequencing enables the identification of all bacteria present in the sample, irrespective of their relative amounts and vitality, by increasing the number of reads per sample (Kommedal *et al*., 2014; Qu *et al*., 2021).

Although bacterial infection is the usual cause of brain abscesses, fungi and viruses have also been detected in brain abscesses by next-generation sequencing (Lin *et al*., 2019). Studies have shown the presence of trophozoites/cysts and 18S rRNA sequences of neurotropic free-living amoeba (FLA), such as *Acanthamoeba* sp. and *Balamuthia mandrillaris*, in autopsy/biopsy

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brain abscess samples. They are the causative agents of chronic granulomatous amoebic encephalitis (Raju *et al*., 2022). These FLA harbour microbes, indicating FLA are competent reservoirs of pathogenic bacteria and maybe a source of infection (da Rocha-Azevedo *et al*., 2009). They are often unidentifiable in routine tissue sections under a light microscope; therefore, molecular analysis is required for confirmation (Booton *et al*., 2003). The present study was conducted to detect bacteria and FLA in smear- and culture-negative brain abscess purulent aspirate and tissue samples by direct PCR and sequencing of the V1-V2 region of 16S rRNA and species-specific 18S rRNA, respectively. For samples with mixed chromatograms in Sanger sequencing, the microbiome was characterized by targeted metagenomic sequencing of the V3-V4 region of bacterial 16S rRNA using the Illumina Mi Seq platform.

MATERIALS AND METHODS

Clinical samples

From May 2018 to December 2021, brain abscess pus/tissue samples were obtained from 217 patients after routine microbiological testing from Department of Neuromicrobiology, National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, India, which is a tertiary care hospital for neurological disorders. Samples were stored at -20°C for molecular detection of pathogenic organisms. The patients' e-records were reviewed to collect demographic characteristics, such as age, gender, symptoms, clinical history/diagnosis, abscess localization, isolated organisms, prescribed antibiotics, and treatment outcome. Seven formalin-fixed paraffin-embedded (FFPE) brain tissue samples reported positive for amoebic trophozoites/cysts obtained from Human Brain Tissue Repository were also included to identify neurotropic FLA. The study was approved by the Institutional Ethical Committee (IEC), NIMHANS (No. NIMHANS/IEC (BS & NS DIV.) 12th meeting/2018).

Microbiological investigation

Brain abscess samples were initially subjected to the following microscopic investigations: wet mount (saline and KOH), Gram staining, and Ziehl Neelsen staining. All samples were then cultured on blood agar, McConkey agar (HiMedia), and thioglycolate broth (HiMedia) for aerobic bacteria, incubated at 37°C, and observed after 24 hours. Samples were also cultured on chocolate agar and thioglycolate broth for anaerobic bacteria and incubated at 37°C in an anaerobic jar using a Gaspak system (Becton and Dickinson Company) and observed after 48 hours of incubation. The isolated colonies were identified by VITEK® 2 COMPACT system (bioMérieux, Marcy l'Etoile, France) or the MALDI-TOF MS method (Bruker Biotyper, Bremen, Germany).

Direct PCR and sequencing of the V1-V2 region of the 16S rRNA gene

Genomic DNA was extracted from brain abscess samples using Nucleospin Tissue DNA extraction kit (Macherey Nagel, Germany) according to the manufacturer's instructions. The primer sequences and the thermal cycling conditions used for amplifying the V1-V2 region of 16S rRNA are shown in Table 1. PCR amplification was performed with 25 µM forward and reverse primers, 1 µl of DNA template, and 2X PCR Master Mix (DSS Takara Bio India Pvt. Ltd.) in 25 µl reaction mixture using a Veriti thermal cycler (AB Applied Biosystems). Gel electrophoresis was performed on a 1.5% agarose gel with ethidium bromide, and bands were visualized using the Gbox gel documentation system (Syngene, India). The PCR products from smear- and culture-negative samples were sent to Madauxin, Bangalore, Karnataka, India, for purification and Sanger-based sequencing. Samples with a faint band that failed QC were excluded from the sequencing. Chromatograms were analyzed using the Ripseq software (Pathogenomix, Santa Cruz, CA, Kommedal *et al*., 2014). Species identification was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018).

Metagenomics sequencing of the V3-V4 region of the 16S rRNA gene

Three samples with mixed chromatogram in Sanger sequencing were sent for metagenomic sequencing of the V3-V4 region of 16S rRNA using specific primers, as shown in Table 1 (Medgenome,

Table 1. Primer sets and thermal cycling conditions used for PCR amplification

Gene	Primers	Size in bp	Thermal cycling conditions	Reference
Bacterial 16S rRNA (V1-V2)	F 5'- AGAGTTTGATCMTGGCTCAG-3' R 5'-CYIACTGCTGCCTCCCGTAG-3'	437	95°C for 2 min 95°C for 20 sec 54°C for 30 sec 72°C for 1 min 72°C for 5 min 29 cycles	Watts *et al.*, 2017
Bacterial 16S rRNA (V3-V4)	341F 5'-CCTACGGGNGGCWGCAG-'3 805R 5'-GACTACHVGGGTATCTAATCC-'3	465	35 PCR cycles	Garcia-Lopez *et al*., 2020
Acanthamoeba 18S rRNA	JDP1 5'-GGCCCAGATCGTTTACCGTGAA-3' JDP2 5'-TCTCACAAGCTGCTAGGGAGTCA-3'	500	94°C for 5 min 94°C for 1 min 55°C for 1 min 72°C for 1 min 72°C for 10 min 35 cycles	da Rocha-Azevedo *et al*., 2009
Acanthamoeba 18S rRNA	F 900 5´-CCCAGATCGTTTACCGTGAA-3´ R 1100 5´-TAAATATTAATGCCCCCAACTATCC-3´	180	95°C for 2 min 95°C for 15 sec 51°C for 30 sec 72°C for 30 sec 72°C for 10 min 45 cycles	Qvarnstrom *et al*., 2005
Balamuthia 18S rRNA	Bala F1451 5'-TAACCTGCTAAATAGTCATGCCAAT-3' Bala R1621 5'-CAAACTTCCCTCGGCTAATCA-3'	171	95°C for 2 min 95°C for 15 sec 52°C for 30 sec 72°C for 30 sec 72°C for 10 min 45 cycles	Qvarnstrom *et al*., 2005

Bangalore, India). The PCR products were purified and proceeded with DNA library preparation. The amplicons were end-repaired, adapters were ligated to the DNA fragments, and barcodes were incorporated, checked for fragment distribution, and loaded onto an Illumina MiSeq V2 instrument to generate 0.5 M, 250 bp paired-end reads/sample. After quality checking using the FASTQC toolkit, the sequenced reads were stitched to form long reads using the FLASH program and subjected to dereplication and chimera removal using the VSEARCH program. The reads were then mapped against the SILVA-132 database; sequences that possessed more than 97% similarity were grouped into the same operational taxonomical units (OTUs) using the RDP classifier. Taxonomy classification and relative abundance were performed using QIIME1. The raw data files are available at the Sequence Read Archive (SRA), NCBI (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA785100), under the study accession number PRJNA785100 (BioProject).

Species-specific 18S rRNA PCR for detecting neurotropic free-living amoebae

PCR for free-living amoebae was performed using species-specific primers, as shown in Table 1. Paraffin was removed from FFPE tissues with xylene, followed by alcohol wash prior to DNA extraction. A nested PCR was done to amplify 18S rRNA for Acanthamoeba (500 bp and 180 bp). Acanthamoeba (4B) T4 strain isolated from water was used as a positive control. A 18S rRNA PCR for *B. mandrillaris* was designed to amplify a 171 bp fragment. Gel electrophoresis was performed on 2.0% agarose gel with ethidium bromide, and bands were visualized using the Gbox gel documentation system.

RESULTS

Demographic characteristics

Of the 217 patients, 146 (67%) were male and 71 (33%) were female. The age of the patients ranged from 1–76 years, and the median was 37 years. The risk factors for patients for whom 16S rRNA sequencing was done in this study were postsurgical (n = 11), trauma (n = 6), chronic suppurative otitis media (CSOM, n = 3), tuberculosis (n = 2), and cyanotic heart disease (n = 1). The common symptoms in this group were headache (n = 15, 58%), fever (n = 14, 54%), vomiting (n = 7, 27%), seizure (n = 6, 23%), and lower/upper limb weakness (n = 5, 19%). All patients in this group were treated with empirical antibiotic therapy and were cured and discharged without new deficits.

Microbiological diagnosis

Of the 217 samples tested, 20 (9%) were smear-positive (Gram-positive cocci [GPC, n = 10], Gram-negative bacilli [GNB, n = 3], GPC and GNB [n = 6], and acid-fast bacilli [n = 1]). All samples were negative for motile trophozoites and one sample showed filamentous, branching, septate, hyphal structures morphologically resembling fungi in wet mount preparation. One hundred and twenty-three (56%) samples were culture positive, of which a single bacterial species was isolated in 104 samples, and two bacterial species were isolated in 19 samples (Table 2).

Direct PCR detected bacteria in smear- and culture-negative brain abscess samples

The 16S rRNA PCR was positive for 209 (96%) samples, including 67 (31%) smear- and culture-negative samples (Table 2). Twenty-seven smear- and culture-negative samples with bright V1-V2 bands in the agarose gel were qualified for Sanger sequencing. Twenty-three samples gave a single bacterial species and one sample yielded three bacterial species when analyzed by the Ripseq software. Three samples remained unidentified due to mixed chromatograms. Most of the bacterial species identified by PCR and sequencing were anaerobic/fastidious GNB (n = 10, 38%) comprising *Fusobacterium nucleatum* (n = 6), *Prevotella sp*. (n = 2), *Bacteroides haparinolyticus* (n = 1), *Ureaplasma urealyticum* (n = 1), and facultative/anaerobic GPC (n = 9, 35%), including Streptococci (n = 8) and *Parvimonas micra* (n = 1). A few nonfermenting GNB (n = 4) and Enterobacteriaceae (n = 3) were also detected by PCR (Table 3). Eight brain abscess samples were PCR-negative, and seven of these samples were negative for smear and culture as well. Only one sample with GPC in the smear and *Streptococcus* sp. in culture was negative by PCR (Table 2).

Multiple bacteria, including uncultured bacteria found in metagenomic sequencing

Three samples with mixed chromatogram in Sanger sequencing were examined by metagenomic next generation sequencing. The total sequencing reads were 455966, 345746, and 438658 for samples P32, P49, and P8, respectively; after dereplication and chimera removal, the valid reads were 447822, 336437, and 413709, respectively. Based on the OTU data, the dominant bacterial species in high relative abundance in these samples are listed in Table 4. Sample P32 had mostly Proteobacteria (80%), followed by Actinobacteria (14%) and Firmicutes (6%), of which only *Pseudomonas stutzeri* and *Actinomyces viscosus* were identified at the species level. Sample P49 had mostly Firmicutes (70%), followed by Bacteroidetes (28%) and Fusobacteria (2%). Only *Dialister pneumosintes* and *B. fragilis* were identified at the species level. Sample P8 mostly had Firmicutes (61%), followed by Fusobacteria (31%) and Epsilonbacteraeota (7%). Most of the bacteria were identified only at the genus level by metagenomic sequencing, including several uncultured bacteria. The alpha diversity indices calculated using the Chao1 and Shannon matrices were different for the samples; the highest being 462 and 4.3, respectively for P32 followed by 265 and 3.8, and 152 and 3.9, respectively for samples P49, and P8.

Free-living amoebae detected in FFPE brain tissue sample

One FFPE tissue sample showed 180 bp fragment of Acanthamoeba 18S rRNA (Figure 1, Table 5). All frozen brain abscess samples were negative for Acanthamoeba and *B. mandrillaris* by species-specific PCR.

Gram-positive cocci, the most common organism in brain abscess samples

A total of 142 bacterial species were isolated in culture. In addition, Sanger sequencing identified 26 bacterial species, and metagenomics

Table 2. Amplification of V1-V2 region of 16SrRNA detected bacteria in smear and culture-negative brain abscess samples

		Smear Positive	Culture Positive	Smear & culture negative	Total
16S rRNA PCR	Positive	20	122	67	209 (96%)
	Negative	0	1	7	8 (4%)
	Total	20 (9%)	123 (56%)	74 (34%)	217

Table 3. Bacterial identification by 16S rRNA PCR and Sanger sequencing for patients with culture-negative brain abscess

Patient ID	Age/ Sex	Symptom	Risk factors	Abscess Location	Clinical history/ diagnosis	Antibiotic T_x before /after sample collection	16S rDNA Sanger Sequencing	Outcome
P67/18	19/M	Headache, Fever; R-eye swelling	Trauma	Frontal	P. tumor	**Before:** Colistin Vancomycin	*Aeromonas hydrophila*	Discharged without deficits
P78/18	10/F	Fever Seizures	None	L-insular	Intracranial abscess & granuloma	**After:** Amikacin Ceftriaxone Metronidazole	*Streptococcus intermedius*	Discharged without deficits
P107/18	16/M	Headache Fever Vomiting	Post-surgical	L-parietal	Intracranial abscess & granuloma	**Before:** Amikacin Ceftriaxone Metronidazole	*Fusobacterium nucleatum*	Discharged without deficits
P115/18	31/F	Headache Fever Vomiting	Post-surgical	L-occipital	Intracranial abscess and granuloma	**Before:** Amikacin Ceftriaxone Metronidazole	*Klebsiella pneumoniae/ varicola*	Discharged without deficits
P158/18	35/M	Fever Vomiting	Post-surgical	L-parietal	Shunt surgery	**After:** Amikacin Ceftriaxone Metronidazole	*Prevotella oris*	Discharged without deficits
P166/18	50/M	Headache Fever	None	R-frontal	NA	**After:** Amikacin Vancomycin	*S. pneumoniae*	Discharged without deficits
P20/19	11/F	Headache Fever	Post-Surgery	Cerebello-pontine angle	P. tumor neoplasm of uncertain or unknown behavior	**After:** Amikacin Ceftriaxone Metronidazole	*S. pyogenes*	Discharged without deficits
P21/19	72/M	Headache Fever	Post-Surgery	R-subgaleal infection	NA	**After**: Amikacin Ceftriaxone Metronidazole	*F. nucleatum*	Discharged without deficits
P23/19	44/M	Seizure L-upper & lower limb weakness	Trauma	L-frontal & temporal (multiple)	P. tumor Intracranial abscess and granuloma	**Before**: Amikacin Piperacillin & Tazobactam	*S. intermedius*	Discharged with hemiplegia
P38/19	40/F	Headache Fever	None	L-subgaleal	NA	**After:** Amikacin Ceftriaxone Metronidazole	*F. nucleatum*	Discharged without deficits
P53/19	52/M	Headache L-side weakness	Tuberculosis	R-parietal	R-parietal tubercular abscess	**After:** Amikacin Ceftriaxone Metronidazole	*Bacteroides haparinolyticus*	Discharged without new deficits
P72/19	11/F	Headache Fever Vomiting	Cyanotic heart disease	L-frontal	P. tumor	**After:** Amikacin Ceftriaxone Metronidazole	*F. nucleatum S. intermedius Parvimonas micra*	Discharged without new deficits
P77/19	48/M	Headache Fever, seizure Vomiting	Trauma	L-parieto-occipital	Traumatic subdural haemorrhage	**After:** Amikacin Ceftriaxone Metronidazole	*K. pneumoniae/ quasipneumoniae*	Discharged without new deficits

P157/19	23/M	Fever Neck pain	L-ear CSOM	L-temporal	L-FTP inter dural abscess with multiple left posterior temporal abscess	**Before:** Amikacin Ceftriaxone Metronidazole	*Brucella melitensis / canis / suis / microti*	Discharged without new deficits
P32/20	20/M	Seizure L-upper & lower limb weakness	Trauma	R-subdural empyema	Head injury R-temporal contusion	**After:** Amikacin Vancomycin Metronidazole	*S. pneumoniae/ mitis*	Discharged without new deficits
P33/20	43/M	Headache	R-ear CSOM	R-temporal	P. tumor	**Before:** Amikacin Vancomycin Amoxy-clav Metronidazole	*Achormobacter xylosoxidans/ denitrificans/ insolitus*	Discharged without new deficits
P44/20	22/M	Headache Vomiting Seizure	? tuberculosis	R- parieto-occipital	Intracranial, intraspinal abscess & granuloma	**After:** Amikacin Ceftriaxone Cefixime Metronidazole	*P. conceptionensis/ loescheii*	Discharged without new deficits
P66/20	55/F	Headache Fever Altered sensorium	None	R-thalamic	Stereotactic biopsy	**Before:** INH, Rifampicin Ethambutol Pyrinzinamide	*F. nucleatum*	Discharged without new deficits
P82/20	12/F	Headache L-ear ache	L-ear CSOM	L- cerebello pontine angle	P. tumor	**After:** Amikacin Ceftriaxone Metronidazole	*S. pyogenes*	Discharged without new deficits
P92/20	60/M	Seizures R-upper & lower limb weakness	Trauma	R-frontal & L-occipital (multiple)	Head injury	**After:** Amikacin Ceftriaxone Metronidazole	*S. pyogenes*	Discharged without new deficits
P135/20	52/M	R-upper & lower limb weakness	Post-surgery	L-parietal	Burr hole for chronic subdural	**After:** Amikacin Ceftriaxone Metronidazole	*F. nucleatum*	Discharged without new deficits
P138/20	29/F	Pus discharge from wound	Post-surgery	L-frontal	P. tumor Malignant neoplasm	**After:** Amikacin Ceftriaxone Metronidazole	*Pseudomonas aeruginosa*	Discharged without new deficits
P15/21	42/M	Loss of conscious-ness	Trauma	R-fronto-temporal	Head injury, Acute hemorrhage with extradural empyema	**After** Cefixime	*Ureaplasma urealyticum*	Discharged without new deficits
P18/21	8/M	Headache Fever Vomiting	Post-surgery	L-parieto-occipital (multiple)	Burr hole for chronic subdural	**After:** Amikacin Ceftriaxone Metronidazole	*Serratia marcescens*	Discharged without new deficits

Note: M: Male; F: Female; L: Left, R: Right; P. tumor: Parenchymal tumor; CSOM: Chronic suppurative otitis media; NA: not available; T_x Treatment.

Table 4. Metagenomic sequencing of 16S rRNA for patients with culture-negative brain abscess samples (n=3)

Sample ID	Age/ Sex	Symptom & Risk factors	Abscess location & Clinical diagnosis	Metaganomic sequencing - sorted by decreasing abundance	Antibiotic T & Outcome
P32	29/F	Pus from bone flap Post-surgery	R-subgaleal Bone flap removal Intracerebral haemorrhage	**Accession number**: **SRX13271109** g_Vulcaniibacterium;s_uncultured-bacterium s_*Pseudomonas stutzeri* f_Burkholderiaceae;Other;Other s_*Actinomyces viscosus* g_Streptococcus;Other f_Sphingomonadaceae;Other;Other g_Sphingomonas;Other g_Actinomyces;Other g_Enhydrobacter; s_uncultured-bacterium g_Enhydrobacter;Other g_Cupriavidus;Other	**After:** Linezolid Metronidazole Discharged without deficits
P49	17/M	Pus from incision site Post-surgery	Frontal Craniectomy for craniosynostosis	**Accession number**: **SRX13271110** g_Peptostreptococcus;s_uncultured bacterium s_Bacteroidales-oral-clone-MCE7_164 s_Porphyromonas-sp.-HMSC077F02 g_Porphyromonas;s_unidentified g_Parvimonas;Other g_Erysipelotrichaceae-UCG-007; s_uncultured-bacterium s_*Dialister pneumosintes* s_*Bacteroides fragilis* g_Peptoniphilus;s_Peptoniphilus-sp.-KHD5 g_Bacteroides;s_Bacteroides-sp.-HPS0048 g_Streptococcus;s_uncultured-Strept. sp. g_Streptococcus;Other g_Odoribacter;Other g_Fusobacterium;s_uncultured-bacterium	**After:** Ceftriaxone Amikacin Metronidazole Discharged without deficits
P8	26/M	Pus from surgery site Post-surgery	Lumbar spine Lumbar Decompression laminectomy	**Accession number**: **SRX13295897** g_Parvimonas;Other o_Clostridiales;Other;Other;Other g_Streptococcus;other g_Fusobacterium;s_uncultured-bacterium g_Fusobacterium;Other g_Campylobacter;Other	**Before**: Ceftriaxone Amikacin Metronidazole Discharged without new deficits

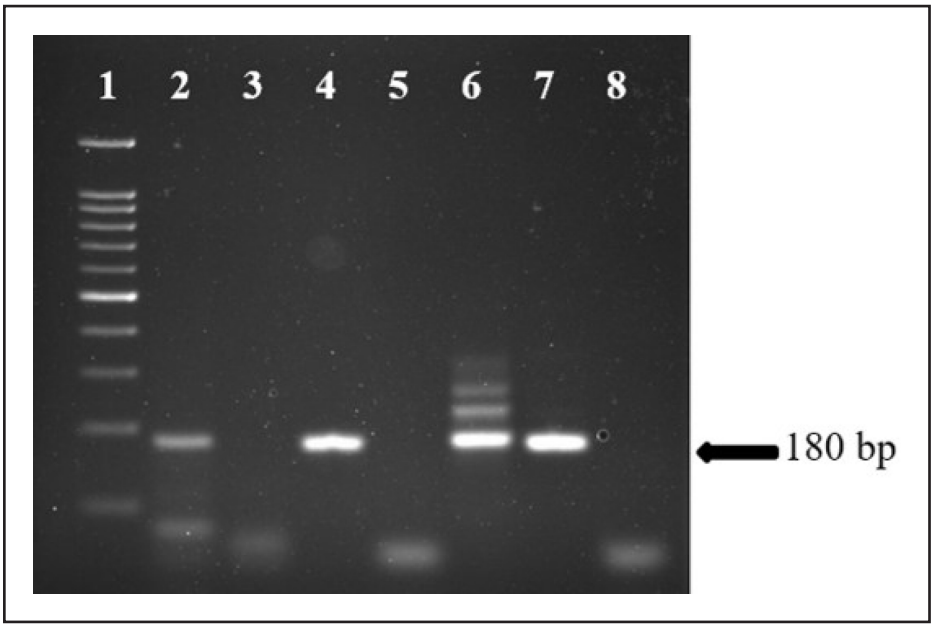

Figure 1. Agarose gel showing Acanthamoeba - specific 18S rRNA. Lane 1: DNA marker; Lane 2: Direct PCR- sample 758; Lane 3: Balamuthia primers; Lane 4: Positive control (gDNA of Acanthamoeba T4 strain); Lane 5: Negative control; Lane 6: Nested PCR - sample 758; Lane 7: Positive control, nested PCR (gDNA of Acanthamoeba T4 strain); Lane 8: Negative control.

Table 5. Microbes detected in brain abscess samples by microbiological and molecular methods

Culture Positive (n=123)	16S rRNA PCR		Total No.
	Sanger sequencing (n=24)	Metagenomics sequencing (n=3)	
Aerobic/facultative gram positive cocci (n=98, 57%) *Staphylococcus* sp.-1; *S. aureus*-14; MRSA-20; MSSA-1; *S. epidermidis*-1; MRCONS-3			**40**
Streptococcus sp.-12; *S. intermedius*-14; *S. constellatus*-9; *S. anginous*-1; *S. pyogens*-1; *S. pneumoniae*-1; *Anaerobic streptococci*-1	*S. intermedius*-3 *S. pyogens*-3 *S. pneumoniae*-2 *P. micra*-1		**48**
Enterococcus sp.-2; *E. faecalis*-3; *E. faecium*-3; *E. avium*-1; *E. raffinosus*-1			**10**
Aerobic gram negative bacilli (n=56, 32%) Enterobacteriaceae *Klebsiella* sp.-6; *K. pneumoniae*-12; *K. varicola*-1; *Escherichia coli*-8; *Enterobacter* sp.-3; *E. cloacae*-4; *Citrobacter koseri*-1; *S. marcescens*-3; *P. mirabilis*-1	*K. pneumoniae*-2 *S. marcescens*-1		**42**
Nonfermentors *Pseudomonas* sp.-1; *P. aeruginosa*-3; *Acinetobacter baumannii*-3; Unidentified GNB-1	*P. aeruginosa*-1 *A. hydrophila*-1 *A. xylosoxidans*-1 *B. melitensis*-1 *U. urealyticum*-1	*P. stutzeri*-1	**14**
Anaerobic gram negative bacilli (n=17, 10%) *F. nucleatum*-2; *P. saachrolyticus*-1; *P. melanogenica*-1; *B. fragilis*-1; *B. pyogenes*-1	*F. nucleatum*-6*; P. oris*-1; *P. conceptionensis/ loescheii*-1; *B. heparinoliticus*-1	*B. fragilis*-1; *D. pneumosintes*-1	**17**
Gram positive bacilli (n=1, 0.5%)		*A. viscosus*-1	**1**
Neurotropic free-living amoeba (n=1, 0.5%) (18S rRNA species-specific PCR)	*Acanthamoeba* sp.-1		**1**
142	27	4	**173**

sequencing identified four bacteria at the species level (Table 5). Of these, 98 (57%) were GPC, including Streptococci (n = 48; 28%), Staphylococcus (n = 40; 23%), and Enterococci (n = 10; 6%). Enterobacteriaceae (n = 42, 24%) were more common compared to nonfermenting GNB (n = 14, 8%) and anaerobic GNB (10%, n = 17). One isolate of *A. viscosus* was identified by metagenomics.

DISCUSSION

Sequencing short regions of 16S rRNA by Sanger or metagenomics is currently being used in the microbiome identification and characterization of brain abscesses (Salipante *et al*., 2013; Kommedal *et al*., 2014). This study examined single and deep sequencing-based identification of microbes in culture-negative brain abscess samples. Most of the patients in this study were between 21–60 years, the most economically productive age group, having a predominance of males, similar to other studies (Brouwer *et al*., 2014). Only 9% of the brain abscess samples were smear-positive in this study due to the low sensitivity of the microscopic technique (Keller *et al*., 2010). Among the culture-positive samples (56%), most were monomicrobial, and 19 samples (15%) had two bacterial species. Others have reported polybacterial etiology in 11–23% of brain abscess cultures (Lakshmi *et al*., 2011; Al Masalma *et al*., 2012; Brouwer *et al*., 2014). A limitation of culture-based methods is that only a small fraction of bacteria can be successfully cultured, such as Enterobacteriaceae members, which are generally a minority in polymicrobial samples. They outgrow clinically significant organisms that are slow-growing, fastidious, and difficult to preserve during transport (Kozlov *et al*., 2018).

In this study, the concordance between smear and/or culture and PCR was 69% (149/217; 142-positive and 7-negative). Rantakokko-Jalava *et al*. (2000) showed 83% agreement between PCR sequencing and the conventional microbiological method. The 16S rRNA sequencing was performed only for the culture-negative samples in this study. Since we did not sequence the culture positive amplicons, it is not possible to compute the sensitivity and specificity of PCR (Lleo *et al*., 2014; Salzberg *et al*., 2016; Stavnsbjerg *et al*., 2017). One sample was negative by PCR in this study but smear- and culture-positive (*Streptococcus* sp.). GPC are not detected as efficiently as GNB by PCR methods because of their cell wall: hence mechanical disruption by sonication and bead beating has been shown to effectively release DNA (Rantakokko-Jalava *et al*., 2000; Jenkins *et al*., 2012). Seven other PCR-negative samples were from patients with malignant neoplasm of the brain (n = 2), anti-tuberculosis treatment/prophylaxis (n = 2), one each with acute subdural hematoma, fungal elements, and cerebritis phase of the abscess (Kozlov *et al*., 2018; Bivand *et al*., 2021). In the current study, 31% (n = 67) of the samples were culture-negative but PCR-positive. Culture-negative but PCR-positive clinical samples have been reported to be 14% in earlier studies, and the authors suggested that this could be due to injured, starved, and viable but unculturable bacteria after antibiotic treatment (Lleo *et al*., 2014). Negative cultures in seven patients in Table 3 could have been due to prior antibiotic treatment. Ten samples with anaerobic bacteria were positive only by 16S rRNA (Table 3). The bacterial 16S rRNA PCR was shown to outperform culture in detecting anaerobes in brain abscess material (Kommedal *et al*., 2014). Case reports show the detection of *F. nucleatum* in brain abscess samples only by PCR, which helped

in directing targeted antibiotic treatment (Nagalingam *et al*., 2014; Chakvetadze *et al*., 2017). In addition, eight aerobic bacteria that are routinely cultivable were detected only by PCR, probably due to the low bacterial load and autolysis of *S. pneumoniae* (Jenkins *et al*., 2012). A low bacterial load could also be the reason for samples (n = 40) that remained unqualified for sequencing, since the analytical sensitivity of direct PCR sequencing is shown to be 50 to 100 target copies per ml of sample (Bivand *et al*., 2021). Single bacteria were identified in 23/27 samples sequenced in this study, and one sample showed three bacterial species (Table 3). It is unlikely that brain abscesses are the result of single bacterial seeding; the use of universal primer has limitations because DNA from all bacteria in a sample compete for the same reagents, and those present at the lowest concentrations may not be detected efficiently (Al Masalma *et al*., 2012).

Several studies show the advantage of metagenomic analysis in characterizing microbiota in clinical samples with polymicrobial etiology (Kommedal *et al*., 2014; Kozlov *et al*., 2018; Dyrhovden *et al*., 2019; Bivand *et al*., 2021). Three samples with mixed chromatograms showed multiple bacterial species, including anaerobes and several uncultivable bacteria, by metagenomic sequencing. The organisms identified in sample P32 (subgaleal) were mostly environmental bacteria belonging to the genera Vulcaniibacterium, Burkholderia, Actinomyces, Sphingomonas, Enhydrobacter, and Cupriavidus, all of which are skin commensals with low human pathogenicity (Church *et al*., 2020). Samples P49 and P8 showed several anaerobic and microaerophilic bacteria, including Peptostreptococcus, Porphyromonas, Parvimonas, Bacteroides, Dialister, Peptoniphilus, Fusobacterium, and Campylobacter, which are typical of the human oral/gut microbiota implicated as relevant organisms in brain abscess formation (Al Masalma *et al*., 2012; Salipante *et al*., 2013). It has been shown that oropharyngeal and gastrointestinal anaerobes play an important role in brain abscess formation, and *F. nucleatum*, *S. intermedius,* and *Aggregatibacter aphorphiles* are considered key pathogens in the establishment of spontaneous polybacterial brain abscesses (Kommedal *et al*., 2014). Numerous factors, including the DNA extraction method, primer sequences, PCR conditions, choice of hypervariable regions, and sequencing platform employed, might impact microbial composition data (Fouhy *et al*., 2016). The use of the V3-V4 regions previously resulted in higher richness and diversity in shrimp microbiota analysis (Garcɒa-Lɳpez *et al*., 2020). It has also been shown that smear- and culture-negative clinical samples show significantly lower microbial richness and diversity (Kozlov *et al*., 2018). In this study, samples P49 and P8 showed lower bacterial diversity than P32. Decreased microbial diversity in tracheal aspirate has been shown to correlate positively with the level of disease severity (Langelier, *et al*., 2018). The clinical significance of bacterial DNA in culture-negative samples must be correlated with clinical symptoms (Lleo *et al*., 2014; Watts *et al*., 2017).

Overall, Streptococcus (28%), Staphylococcus (23%), and Enterobacteriaceae (24%) were the most common bacterial species detected in the present study, which is similar to earlier reports (Lakshmi *et al*., 2011; Brouwer *et al*., 2014). Postsurgical brain abscesses are often associated with Staphylococcus (e.g., *S. aureus*, *S. epidermidis*) and Enterobacteriaceae members (Al Masalma *et al*., 2012). Dental manipulation, sinusitis, and ear infections predispose susceptible individuals to develop brain abscesses by *S. anginosus* group members, particularly *S. intermedius*, due to its ecological niche and the presence of specific virulence factors (Issa *et al*., 2020). *S. intermedius* was shown be the predominant pathogen of brain abscesses in a recent report (Darlow *et al*., 2020). Nonfermenting gram-negative bacilli, *Aeromonas hydrophila*, and *Achromobacter xylosoxidans*, are environmental bacteria that might have caused brain abscess in patients with head injury and CSOM, respectively (Mahabeer *et al*., 2014). Species-specific PCR confirmation is required for the sample that showed Brucella 16S rRNA. Only one solitary brain mass mimicking a brain abscess caused by *B. melitensis* was reported earlier (Erdem *et al*., 2012). One patient with a posttraumatic brain abscess showed the presence of *U. urealyticum*, which lacks a cell wall and colonizes the genitourinary tract and might have resulted from hematogenous spread following urinary catheterization (Deetjen *et al*., 2014).

One FFPE brain tissue sample showed Acanthamoeba-specific 18S rRNA 180bp fragment. *Acanthamoeba* granulomatous encephalitis (AGE), can be caused by several species of *Acanthamoeba* and are usually identified in biopsies of brain lesions during the late stage of the disease process or in autopsy (da Rocha-Azevedo *et al*., 2009). In recent years, PCR has been used to identify *Acanthamoeba* DNA in cerebrospinal fluids and brain tissue samples and has been considered as an alternative to conventional methods (Qvarnstrom *et al*., 2005). The absence of FLA-DNA in other FFPE tissue samples in this study may be due to the degradation of DNA during prolonged storage, the presence of PCR inhibitors and a low parasite load (Khan *et al*., 2006; Guyard *et al*., 2017).

Although sequencing the first 500 bp region of 16S rRNA is sufficient to identify most of the bacterial species, some clinically important bacterial families and genera display too few variations in this region (Woo *et al*., 2011; Loong *et al*., 2016). Hence, sequencing longer 16S rRNA gene (1,060 bp) and additional housekeeping genes (recA and rpoB) are recommended for better taxonomic resolution (Church *et al*., 2020; Bivand *et al*., 2021). Sequencing the entire 16S rRNA gene is now possible with the Pacbio and Oxford Nanopore sequencing platforms (Wagner *et al*., 2016). Whole-genome shotgun metagenomic sequencing allows comprehensive microbiome characterization in a target-independent manner and detects bacterial, viral, and fungal communities, including antibiotic resistance, in a single assay (Lin *et al*., 2019). This technique was used recently to diagnose *P. loescheii* and *B. mandrillaris* in patients with brain abscess and facilitated targeted treatment (Deng *et al*., 2020; Hirakata *et al*., 2021).

Although the brain is assumed to be a sterile organ in the absence of disease, deep sequencing and metatranscriptomic analysis show the presence of microbial genomes in normal brain tissues with a predominance of α-proteobacteria (50–70%) followed by Actinobacteria, Firmicutes, and Bacteroidetes, suggesting that the brain is tolerant to the presence of certain microbes and they have a beneficial organ-specific adaptation (Branton *et al*., 2013) It is believed that these commensal flora in the brain might be outcompeted by hostile/non-beneficial microbes introduced from other body sites/environments in the case of intracerebral abscesses (Al Masalma *et al*., 2012; Perz *et al*., 2019). The microbiome constitution of brain abscess changes over time. Pathogens established the infection and important for expanding the abscess at the periphery may remain at low concentrations in the pus aspirated from the necrotic, anaerobic center of the abscess (Kommedal *et al*., 2014; Dyrhovden *et al*., 2019). The synergistic molecular interactions of pathogens in the brain abscess may influence the overall pathologic process (Kozlov *et al*., 2018).

CONCLUSION

The concordance between smear and/or culture and PCR was 69% in this study. Thirty-one percent of the culture-negative samples were PCR-positive (Figure 2). The bacterial 16S rRNA PCR and sequencing outperformed culture in detecting anaerobes and facultative Streptococci (70%) in brain abscess samples. Single case of Acanthamoeba 18S rRNA was identified from FFPE tissue sample (Figure 2). Genetic analyses of 16S sequences, either through Sanger or metagenomic sequencing, will be an essential diagnostic technology to be included for diagnosing culture-negative samples. Characterizing the microbiome of culture-negative brain abscess samples could enable detection/treatment of the source of infection.

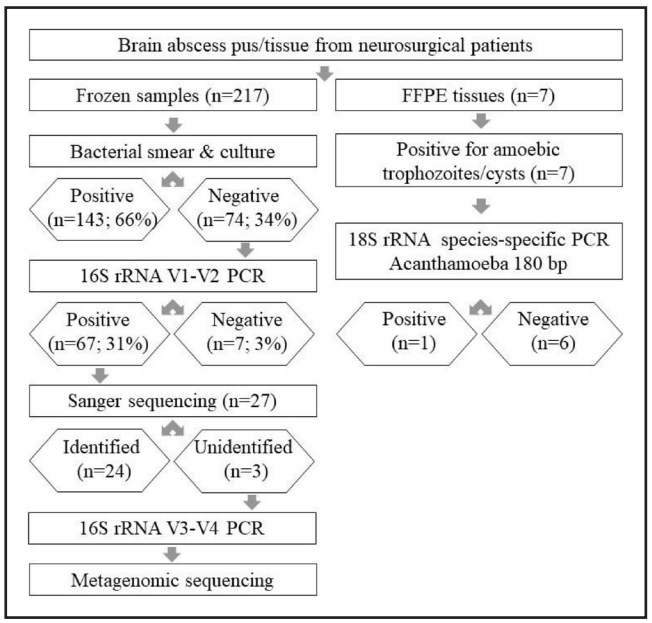

Figure 2. The study work flow.

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Conflict of interest

The authors declare that they have no conflict of interest.

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