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Diagnosis of *Balamuthia mandrillaris* Encephalitis by Thymine–Adenine Cloning Using Universal Eukaryotic Primers

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Background: Identifying the causal pathogen of encephalitis remains a clinical challenge. A 50-year-old man without a history of neurological disease was referred to our department for the evaluation of an intracranial lesion observed on brain magnetic resonance imaging (MRI) scans, and the pathology results suggested protozoal infection. We identified the species responsible for encephalitis using thymine–adenine (TA) cloning, suitable for routine clinical practice.

Methods: We extracted DNA from a paraffin-embedded brain biopsy sample and performed TA cloning using two universal eukaryotic primers targeting the V4-5 and V9 regions of the 18S rRNA gene. The recombinant plasmids were extracted, and the inserted amplicons were identified by Sanger sequencing and a homology search of sequences in the National Center for Biotechnology Information Basic Local Alignment Search Tool.

Results: The infection was confirmed to be caused by the free-living amoeba *Balamuthia mandrillaris*. Two of 41 colonies recombinant with 18S V4-5 primers and 35 of 63 colonies recombinant with the 18S V9 primer contained *B. mandrillaris* genes; all other colonies contained human genes. Pathogen-specific PCR ruled out *Entamoeba histolytica*, *Naegleria fowleri*, *Acanthamoeba* spp., and *Toxoplasma gondii* infections.

Conclusions: This is the first report of *B. mandrillaris*-induced encephalitis in Korea based on molecular identification. TA cloning with the 18S rRNA gene is a feasible and affordable diagnostic tool for the detection of infectious agents of unknown etiology.

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INTRODUCTION

Identifying the causes of encephalitis can be challenging because various bacterial, viral, fungal, and parasitic infections can manifest with overlapping symptoms and signs. Pathogen-specific serological, PCR, and culture-based analyses are expensive, time-consuming, and lack standardization [1]. Recently, metagenomic next-generation sequencing (NGS) has been used to

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identify unusual or novel pathogens, including those causing meningoencephalitis. For example, *Leptospira*, a spirochaete, and *Balamuthia mandrillaris*, a free-living amoeba, have been identified in patients with meningoencephalitis by metagenomic NGS [1, 2]. Despite the several advantages of this approach, the detection of unusual protozoan infections by NGS within a reasonable timeframe is not always feasible for routine practice owing to the need for specialized equipment, and the time and expertise required for bioinformatic analyses. As an alternative, thymine–adenine (TA) cloning is more feasible and affordable than NGS. Here, we describe a patient with encephalitis caused by *B. mandrillaris* that was identified using the TA cloning method. To the best of our knowledge, this is the first report on *B. mandrillaris* gene detection in Korea. Cloning was performed using universal 18S rRNA gene primers that have been widely applied in eukaryotic metagenomic studies [3]. We initially identified all

protozoal species in the brain sample, because *B. mandrillaris* infection was unknown in Korea at the time and was not initially considered.

MATERIALS AND METHODS

Brain biopsy sample from a patient with encephalitis

A 50-year-old man without a history of neurological disease was referred to the Department of Neurosurgery, Eulji University Hospital, Daejeon, Korea, for evaluating an intracranial lesion visible on his brain magnetic resonance imaging (MRI) scans. The patient had a three-day history of mild speech disturbance and right-sided hemiparesis after a focal seizure. He did not chew tobacco, drink alcohol, or use drugs and showed no signs of infection, such as fever, leukocytosis, and meningeal irritation. A brain MRI revealed a mass in the cortex of the left parietal lobe
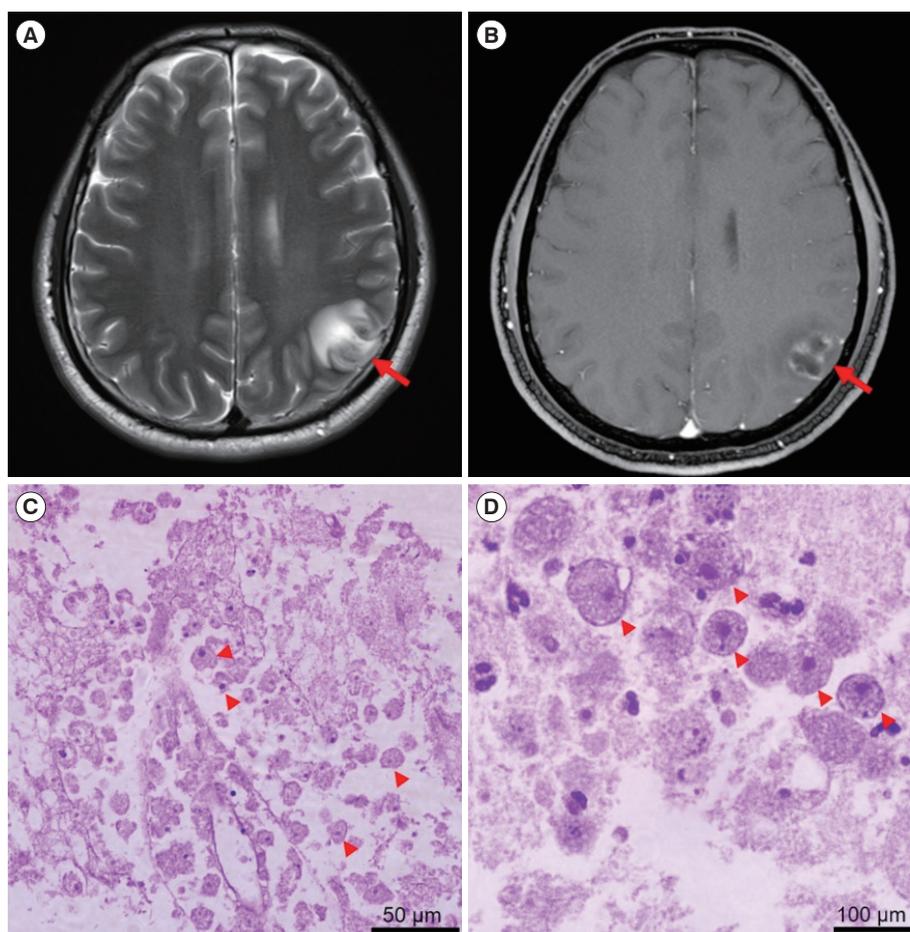

Fig. 1. Magnetic resonance image (MRI) and brain biopsy. T2-weighted MRI showing (A) a lesion (~21 mm×18 mm) with an irregular, marginated, heterogeneous dark signal and (B) marginal thin-rim enhancement of gadolinium at the left parietal cortex with surrounding edema (red arrows). (C, D) Hematoxylin and eosin staining of brain biopsy shows numerous amoebic trophozoites (20–25 μm) in the hemorrhagic necrosis background (arrowheads). Magnification: ×200 (C), ×1,000 (D).

(Fig. 1A, B). An open biopsy of the brain performed immediately upon arrival suggested amebic infection in the brain with inflammatory cell infiltration and necrotizing vasculitis. Numerous amebic trophozoites (20–25 μm) were identified by microscopy (Fig. 1C, D), and anti-*Entamoeba histolytica* and anti-*Toxoplasma gondii* IgM antibody titers were positive and negative, respectively. Laboratory findings were normal. An initial laboratory evaluation revealed a white blood cell count of 6.65×10^9/L, hemoglobin of 151 g/L, serum sodium level of 138 mmol/L, and serum glucose level of 7.05 mmol/L. A diagnosis of intracranial amebiasis was made based on the pathological findings, and the patient was treated with metronidazole, amphotericin B, and sulfadiazine/pyrimethamine. However, drowsiness and progressive hemiparesis seven days after the open biopsy indicated a deteriorated neurological status. Emergency MRI showed multiple new lesions on the opposite site of the brain and increased intracranial pressure, which was reduced by decompressive craniectomy.

The study protocol was approved by the Institutional Review Board of Yonsei University Sever-ance Hospital, Seoul, Korea (approval No. 4-2020-0201), and the patient provided written in-formed consent.

DNA extraction

A paraffin block of the sample was prepared immediately after the biopsy (February 21, 2020 at Eulji University Hospital). Two weeks after the biopsy, the paraffin block was transferred to the laboratory at Yonsei University College of Medicine, and DNA extraction, TA cloning, and PCR were performed on the same day. DNA was extracted from paraffin-embedded brain biopsy samples using the Exgene Formalin-Fixed, Paraffin-Embedded Tissue DNA Kit (GeneAll, Seoul, Korea) in accordance with the manufacturer's instructions and stored at 4°C until analyses.

TA cloning

We amplified protozoan 18S rRNA gene sequences by PCR using two sets of primers targeting the V4-5 and V9 regions of 18S rRNA, which are typically used in eukaryotic metagenomics as they amplify the 18S rRNA of nearly all types of eukaryotic species [3]. Thus, this procedure can discriminate the 18S rRNA gene sequences of patient and protozoan DNA. The 18S rRNA gene V4-5 and V9 regions of any eukaryotic species in the sample were cloned using the TOPcloner TA Kit (Enzynomics, Daejeon, Korea), in accordance with the manufacturer's instructions with the following primers: V4-5 region, V4-1 forward 5´-GCGG-TAATTCCAGCTC-3´ and V4-4 reverse 5´-GCCMTTCCGTCAA-

TTCC-3´; V9 region, 1391f 5´-GTACACACCGCCCGTC-3´ and EukBr 5´-TGATCCTTCTGCAGGTTCACCTAC-3´ [3, 4]. The reaction conditions were as follows: initial denaturation for 5 minutes at 95°C; 34 amplification cycles comprising denaturation for 30 seconds at 95°C, annealing for 30 seconds at 55°C, and extension for 45 seconds at 72°C; and final extension for 5 minutes at 72°C. The following day, recombinant colonies were cultured overnight in Luria–Bertani broth with ampicillin, and the recombinant plasmids from the recombinant clones were extracted using the Exprep Plasmid SV Mini Kit (GeneAll, Seoul, Korea). Forty-one plasmids recombinant with 18S rRNA V4-5 regions and 63 plasmids recombinant with 18S rRNA V9 re-gions were sequenced at Bionics (Seoul, Korea) using the M13F(-20) primer. Homology was de-termined using sequences from the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis

The DNA sequences were compared with nucleotide sequences available in GenBank (NCBI). A phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis version X (MEGA-X) software (The Pennsylvania State University, PA, USA) [5].

Conventional PCR for specific protozoal infection

Conventional PCR was performed to detect the protozoa *Entamoeba histolytica*, *Toxoplasma gondii*, *Naegleria fowleri*, and *Acanthamoeba* spp. based on previous studies [6-9]. *N. fowleri*-specific PCR was performed using the following primer set to amplify the amplicon of predicted size 183 bp: Nae3-F (5´-CAA-ACACCGTTATGACAGGG-3´) and Nae3-R (5´-CTGGTTTCCCT-CACCTTACG-3´) [6]. *Entamoeba histolytica*-specific nested PCR was per-formed using the following primer sets to amplify the amplicon of predicted size 270 bp: Fw1 (5´-GCAGAAAAAAATA-ATAATAAC-3´) and Rv1 (5´-TTCATTTGTTTTACTTTCA-3´) for the first PCR and Fw2 (5´-CGCCCGCCGCGCGGCCGCGGCCGGCC-GGGGGCACGCGGCGGCAGAAAAAAA-TAATAATAAC-3´) and Rv2 (5´-TTCATTTGTTTTACTTTCA-3´) for the subsequent PCR [7]. *Toxoplasma gondii*-specific PCR was performed using the following primer set to amplify the amplicon of predicted size 469 bp: Tg1 (5´-AAAAATGTGGGAATGAAAGAG-3´) and Tg2 (5´-ACGAATCAACGGAACTGTAAT-3´) [8]. *Acanthamoeba*-specific PCR was performed using the following primer set to amplify the amplicon of predicted size 463 bp: JDP1 (5´-GGCCCA-GATCGTTTACCGTGAA-3´) and JDP2 (5´-TCTCACAAGCTGC-TAGGGGAGTCA-3´) [9]. To confirm the TA cloning results, *B.*

mandrillaris-specific PCR was performed using the following primer sets to generate products of pre-dicted sizes 1075, 230, and 170 bp, respectively: 5'Balspec16S (5'-CGCATGTATGAA-GAAGACCA-3') and 3'Balspec16S (5'-TTACCTATATAATTGTC-GATACCA-3'); 5'Balspec 16S (5'-CGCATGTATGAAAGAAGA-CCA-3') and Balspec 16Sr 610 (5'-CCCCTTTTTAACTCTAGT-CATATAGT-3'); and BalaF1451 (5'-TAACCTGCTAAATAGTCAT-GCCAAT-3') and BalaR1621 (5'-CAAACTTCCCTCGGCTAATCA-3') [10-12].

RESULTS

Four days after the brain biopsy, the protozoan species in the brain lesion was identified as *B. mandrillaris* by TA cloning. However, the patient had already succumbed to brain death and died four weeks later.

The pathogen-specific PCR results ruled out *E. histolytica*, *N. fowleri*, *Acanthamoeba* spp., and *T. gondii* infections based on the absence of positive bands (Fig. 2A). Simultaneous TA cloning was performed using general eukaryotic primers to detect unexpected protozoan infections because we did not consider *B. mandrillaris* infection.

The homology analysis using NCBI BLAST showed that the 18S V4-5 and V9 regions of the protozoan pathogen in the sample exhibited 99.2% and 100% nucleotide identity with those of *B. mandrillaris* KF874819 and KT175741 in GenBank, respectively (Table 1).

The DNA of the patient and *B. mandrillaris* was detected at different rates when cloning the 18S rRNA V4-5 or V9 variable region. This is likely related to the variation in the GC content
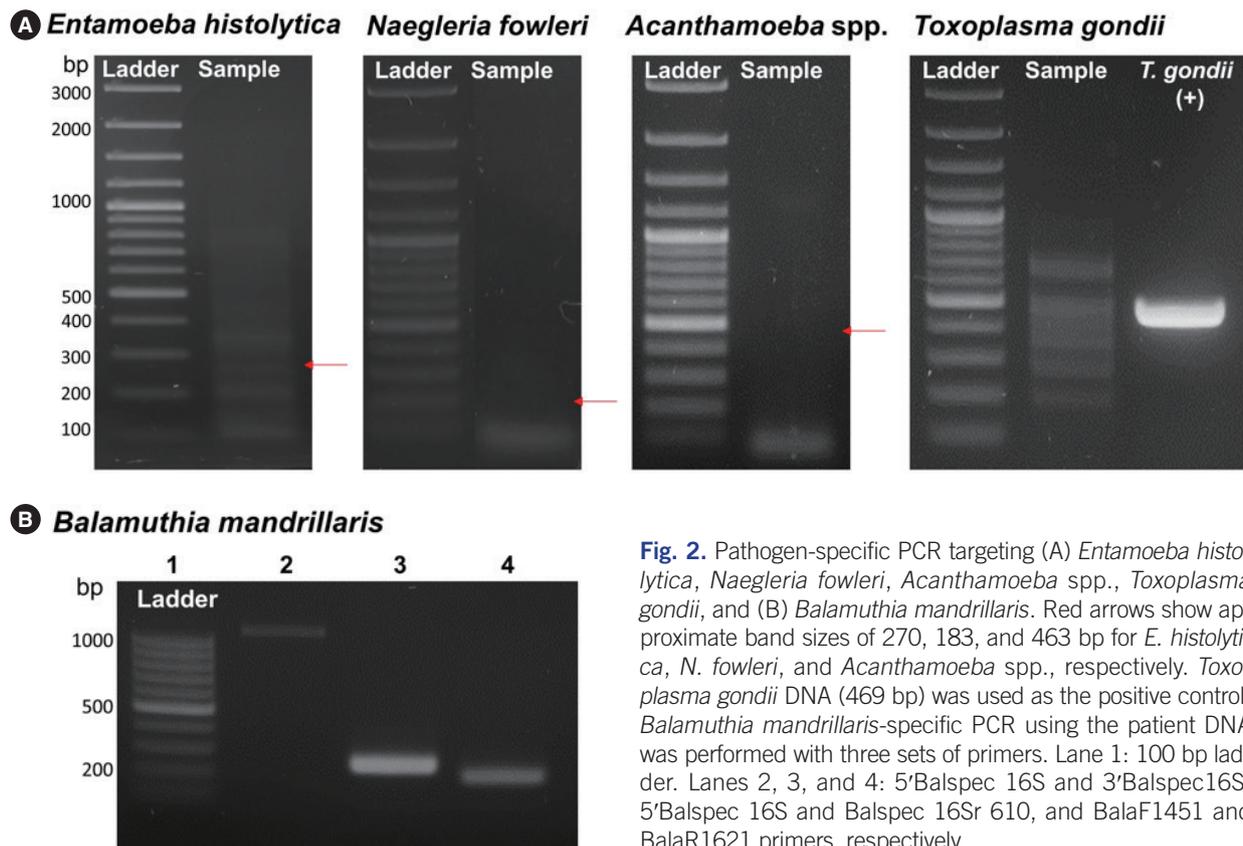

Fig. 2. Pathogen-specific PCR targeting (A) *Entamoeba histolytica*, *Naegleria fowleri*, *Acanthamoeba* spp., *Toxoplasma gondii*, and (B) *Balamuthia mandrillaris*. Red arrows show approximate band sizes of 270, 183, and 463 bp for *E. histolytica*, *N. fowleri*, and *Acanthamoeba* spp., respectively. *Toxoplasma gondii* DNA (469 bp) was used as the positive control. *Balamuthia mandrillaris*-specific PCR using the patient DNA was performed with three sets of primers. Lane 1: 100 bp ladder. Lanes 2, 3, and 4: 5'Balspec 16S and 3'Balspec16S, 5'Balspec 16S and Balspec 16Sr 610, and BalaF1451 and BalaR1621 primers, respectively.

Table 1. Molecular identification (nucleotide identity) of the protozoa from the patient and previously published protozoan DNA sequences that required differential diagnoses

18S rRNA region	Balamuthia mandrillaris		Entamoeba histolytica		Naegleria fowleri		Acanthamoeba castellanii		Toxoplasma gondii	
	Identity	GenBank N	Identity	GenBank N	Identity	GenBank N	Identity	GenBank N	Identity	GenBank N
V4-5	99.2%	KF874819	53.5%	AB426549	57.2%	U80059	70.3%	U07401	69.8%	L24381
V9	100%	KT175741	38.3%	AB426549	44.0%	U80059	35.7%	U07401	44.8%	L24381

Table 2. Number of recombinant colonies containing 18S rRNA genes (V4-5 and V9 regions) of *Balamuthia mandrillaris* and *Homo sapiens* and their GC content and DNA length

	18S rRNA V4-5 region			18S rRNA V9 region		
	Recombinant colonies (N)	GC content (%)	DNA length (bp)	Recombinant colonies (N)	GC content (%)	DNA length (bp)
B. mandrillaris	2	46.1	662	35	40.8	179
H. sapiens	32	55.2	591	8	58.6	174

*The total number of recombinant colonies examined was 41 and 63 for the V4-5 and V9 regions, respectively. Among them, 7 and 20 plasmids were found to be empty vectors.
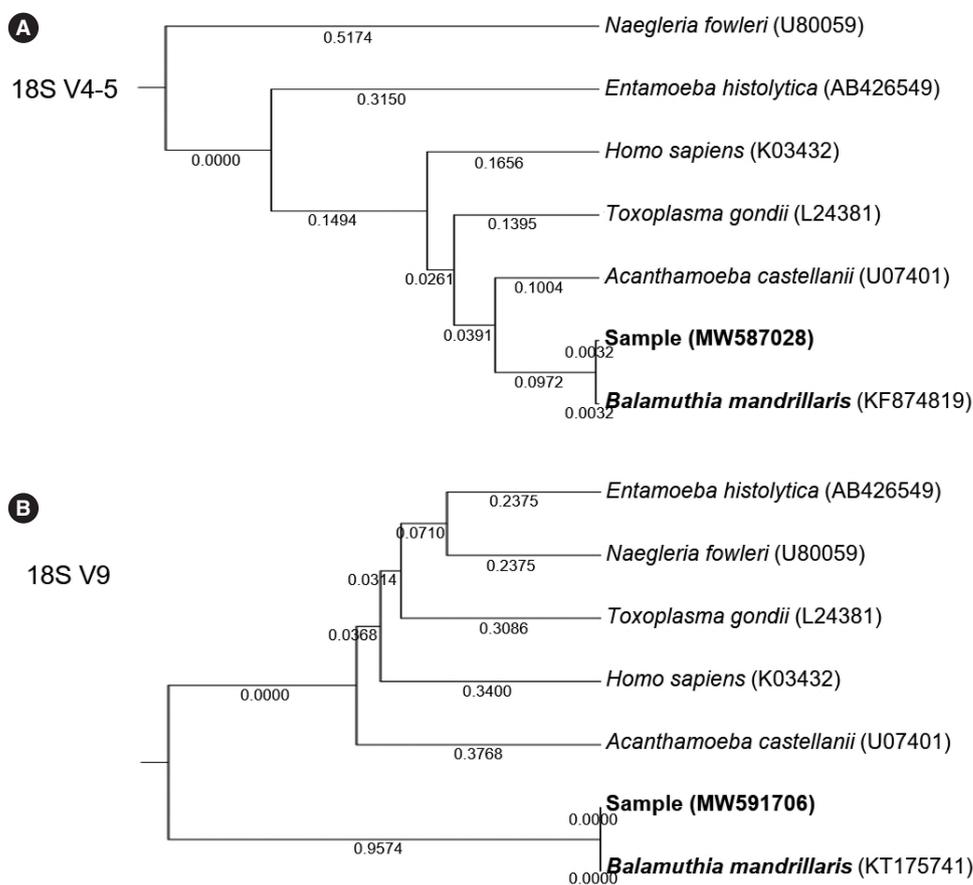

Fig. 3. Phylogenetic trees of the sequenced sample based on the (A) V4-5 region and (B) V9 region of the 18S rRNA gene. The total branch lengths of phylogenetic trees for the V4-5 and V9 regions were 0.8465 and 2.2305, respectively, computed using the maximum composite likelihood method and measured in base substitutions per site. Sequences of 18S rRNA genes of reference organisms were obtained from GenBank (National Center for Biotechnology Information).

and DNA sequence lengths in the two genes (Table 2).

B. mandrillaris infection was reconfirmed using *B. mandrillaris*-specific PCR with three primer sets. The bands resolved at the expected points in gel electrophoresis and their DNA sequences were identical to published *B. mandrillaris* nucleotide sequences (Fig. 2B).

The phylogenetic tree of 18S rRNA V4-5 and V9 sequences showed that the sequence of the sample was distant from those

of other protozoan candidates, including *E. histolytica*, *N. fowleri*, *Acanthamoeba castellanii*, and *T. gondii* (Fig. 3).

DISCUSSION

Naegleria fowleri, *Acanthamoeba* spp., and *B. mandrillaris* are established etiological agents of encephalitis. *N. fowleri* causes primary amebic meningoencephalitis with a fulminant disease

course, and *Acanthamoeba* spp. and *B. mandrillaris* cause granulomatous amebic encephalitis [13, 14]. Among approximately 150 patients with *Balamuthia* amebic encephalitis reported worldwide, only 10 have survived [15]. *B. mandrillaris* enters the host via skin wounds or inhalation and spreads through the bloodstream to the central nervous system, resulting in fever, seizures, cranial nerve dysfunction, motor deficits, increased intracranial pressure, and altered consciousness [16, 17]. The survival rate of patients with *B. mandrillaris* encephalitis can be <2% or a maximum of approximately 6.7% due to its rarity, difficulty in diagnosis, and lack of effective treatment protocols [16, 18]. The US Center for Disease Control and Prevention recommends pentamidine, sulfadiazine, flucytosine, fluconazole, clarithromycin, azithromycin, and miltefosine to treat *B. mandrillaris* encephalitis [19-23].

To the best of our knowledge, this is the first case of *B. mandrillaris* encephalitis in Korea identified using a molecular (TA cloning) approach. Although a 71-year-old man in Korea was diagnosed as having *B. mandrillaris* encephalitis in 2019 based on microscopy findings [24], including the detection of trophozoites and cysts of *B. mandrillaris* as well as areas of granuloma in host tissues, this protozoan is morphologically indistinguishable from *Acanthamoeba* spp. [13, 15, 25]. *Balamuthia* culture requires feeder cells and several weeks for cell proliferation. A diagnosis should be confirmed by PCR-based detection of *B. mandrillaris* DNA in tissues [26]. However, this technique is not widely available given the extreme rarity of *B. mandrillaris* encephalitis [1].

In the present case, pathogen-specific PCR was negative for *E. histolytica*, *N. fowleri*, *Acanthamoeba* spp., and *T. gondii*. *T. gondii* was the only positive control because protozoal and infected tissue samples were not available in our laboratory. This is likely a common situation in other research institutions, as it is difficult to maintain positive samples for all possible infectious diseases.

We applied TA cloning in this case, because it can provide results faster than metagenomic NGS and the protozoan cells in the brain tissues detected by microscopy were enough to enable confirmation using TA cloning by separating amplified protozoan and patient 18S rRNA genes. With this approach, we identified *B. mandrillaris* genes in the brain biopsy sample four days after receiving the paraffin-embedded sample. Theoretically, TA cloning can generate results within 48 hours; therefore, it should be among the diagnostic choices available to detect infectious agents of unknown etiology in clinical practice.

Cloning with the primer amplifying the 18S rRNA V9 region resulted in 35 colonies with the *B. mandrillaris* gene and eight

colonies with the patient gene. In contrast, cloning with the 18S V4-5 primer resulted in two and 32 colonies with *B. mandrillaris* and human genes, respectively (Table 2). This difference is probably due to the length and the GC content of the PCR products (Table 2). The PCR product of the 18S V4-5 region of *B. mandrillaris* was 11% longer than that of the human gene. Furthermore, the GC content of *B. mandrillaris* PCR products increased from 40.8% for the V9 primer to 46.1% for the V4-5 primer, which might have reduced PCR efficiency. Various primers can be used to amplify different 18S rRNA regions during TA cloning to increase the probability of protozoa detection.

This study is limited by the fact that we identified *B. mandrillaris* by TA cloning only in a brain lesion. As brain tissue biopsies can cause neurological disorders, diagnosing *B. mandrillaris* infection in samples other than brain tissues such as the blood and cerebrospinal fluid might be more helpful for patients undergoing clinical treatment.

In conclusion, to the best of our knowledge, this is the first study to describe the molecular identification of *B. mandrillaris* encephalitis in Korea using TA cloning. This method should be considered for detecting infectious agents of unknown etiology in routine clinical practice.

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AUTHOR CONTRIBUTIONS

Kim JY and Kim SM contributed to the conception and design of the study; Yoo HD and Yeom JS were involved in clinical evaluation; Yi MH and Kim M interpreted the results; Kim JY drafted the manuscript; and Yong TS supervised the study. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

None declared.

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