Acanthamoeba Encephalitis: Isolation of Genotype T1 in Mycobacterial Liquid Culture Medium

Rula Azzam,^a Paul R. Badenoch,^c Michelle J. Francis,^b Charles Fernandez,^b Penelope J. Adamson,^d Claire Dendle,^{a,f} Ian Woolley,^{a,f} Jenny Robson,^e Tony M. Korman,^{a,b,f} Maryza Graham^{a,b}

Monash Infectious Diseases^a and Department of Microbiology,^b Monash Health, Clayton, Victoria, Australia; Department of Ophthalmology, Flinders Medical Centre and Flinders University, Bedford Park, South Australia, Australia^c; Department of Microbiology and Infectious Diseases, SA Pathology at Flinders Medical Centre and Flinders University, Bedford Park, South Australia, Australia^d; Sullivan Nicolaides Pathology, Brisbane, Queensland, Australia^e; Department of Medicine, Monash University, Clayton, Victoria, Australia^f

We report a case of *Acanthamoeba* encephalitis diagnosed from an antemortem brain biopsy specimen, where the organism was first isolated in mycobacterial liquid medium and first identified by using a sequence generated by a commercial panfungal sequencing assay. We correlate susceptibility results with clinical outcome.

CASE REPORT

A 67-year-old man was brought in by ambulance following two generalized seizures necessitating intubation. The family reported a 1-week history of memory difficulties, 1 month of short-lived episodes of confusion and disorientation, and 7 months of worsening vision. The patient was born in Chile and migrated to Australia 40 years ago. He had a history of treated latent tuberculosis and cryptogenic multifocal ulcerating stenosing enteropathy (CMUSE) diagnosed 8 years earlier after life-threatening gastrointestinal bleeds requiring a hemicolectomy and ileostomy. Treatment of CMUSE included azathioprine for many years and more recently prednisolone (10 to 25 mg daily) for 3 months.

Investigations revealed mild pancytopenia with a hemoglobin level of 93 g/liter, white cell count of 10.7×10^9 /liter (neutrophils, 10.16×10^9 /liter; lymphocytes, 0.37×10^9 /liter), platelet count of 132×10^9 /liter, and C-reactive protein level of 32 mg/liter. Magnetic resonance imaging (MRI) on admission demonstrated a 25- by 31-mm inferolateral left occipital lobe lesion (Fig. 1a). His clinical course was complicated by further seizures during the following week, with ongoing confusion and disorientation. Repeat MRI on day 5 demonstrated rapid enlargement of the left occipital lesion and the development of a new right lentiform nucleus lesion. He commenced treatment with intravenous (i.v.) benzylpenicillin, meropenem, vancomycin, and acyclovir. A lumbar puncture was performed, and cerebrospinal fluid (CSF) analysis demonstrated 6×10^6 /liter lymphocytes, no polymorphonuclear cells, no organisms visible on Gram stain, and normal protein, glucose, and lactate dehydrogenase levels.

A brain biopsy of the left occipital cortex was performed, and histopathology revealed meningoencephalitis with a perivascular and diffuse neutrophil and macrophage infiltrate in the brain substance and leptomeninges with “rare single large round cells with very prominent nucleoli” noted by the anatomical pathologist (Fig. 2a). Periodic acid-Schiff (PAS) stain, mucicarmine stain, and Ziehl-Neelsen stain were negative. Immunohistochemistry was negative for cytomegalovirus, herpes simplex virus type 1, simian virus 40, and CD1a antigens. Biopsy tissue was cultured for bacteria (including mycobacteria) and fungi. CSF taken on day 12 was reported as positive for cryptococcal antigen (latex agglutination assay; Meridian Bioscience, Cincinnati, OH), and 5-flucytosine

and liposomal amphotericin B were added: the patient appeared to stabilize, although he remained confused and disoriented. Given that *Cryptococcus* was not isolated from culture of brain tissue after 1 week of incubation, the cryptococcal antigen test was repeated on the same CSF sample and found to be invalid due to nonspecific agglutination in the control latex.

Nucleic acid testing of CSF for *Cryptococcus neoformans*, herpes simplex virus, varicella-zoster virus, enterovirus, *Mycobacterium tuberculosis* complex, *Toxoplasma gondii*, *Legionella* spp., and *Tropheryma whippelii* was negative. Serum HIV antigen/antibody was not detected, although notably, the mild lymphopenia persisted for the first 2 months of his admission. Acyclovir was ceased, and i.v. ciprofloxacin was added.

On day 27, MRI demonstrated enlargement of the lesions and formation of a new lesion in the left parietal lobe (Fig. 1b). Mycobacterial liquid broth (BacT/Alert MP supplemented Middlebrook 7H9 broth incubated in the BacT/Alert 3D Mycobacteria detection system; bioMérieux, Durham, NC) inoculated with tissue from the patient's brain biopsy specimen persistently flagged positive, commencing on day 32 of incubation. Gram stain on the MP broth showed round objects measuring 11.5 μ m in diameter (Fig. 2b). These objects were fluoresced with a calcofluor white stain. Mycobacterial solid medium (Lowenstein-Jensen [LJ]) demonstrated brown discoloration but no distinct colonies. Gram stain from this discoloration showed similar structures to those in Fig. 2b.

Panfungal PCR using a commercial assay (MicroSEQ D2 LSU

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Address correspondence to Maryza Graham, Maryza.Graham@monashhealth.org.

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FIG 1 MRI image on hospital day 1 showing left inferolateral occipital lobe lesion (a), hospital day 27 showing a rapidly enlarging occipital lesion and new lesions in the lentiform nucleus and left parietal lobe (b), and hospital day 76 showing some improvement in the right-sided lesion and stable lesions elsewhere (c).

rDNA fungal identification kit; Applied Biosystems, Foster City, CA) performed on the LJ slope discoloration surrounding the inoculated tissue generated a 205-bp sequence. Sequence analysis using GenBank BLAST V2.0 demonstrated 83% homology with *Acanthamoeba castellanii* ([GU001160.1](https://www.ncbi.nlm.nih.gov/nuclot/GU001160.1)) as the top match. The organism demonstrated motility in the MP broth after inocula-

tion of the bottle with *Escherichia coli* ATCC 25922. Furthermore, *Acanthamoeba* was isolated from the MP broth by inoculation of nonnutrient agar with an *E. coli* overlay.

A paraffin section of the original brain tissue was subjected to multiplex real-time PCR for *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*, performed as per a previous

FIG 2 (a) Brain biopsy specimen (hematoxylin and eosin stain) demonstrating “rare single large round cells with very prominent nucleoli,” as described by the anatomical pathologist. (b) ZN stain on MP broth inoculated with tissue from the patient’s brain biopsy specimen demonstrating *Acanthamoeba* cysts (round structures measuring 11.5 μm in diameter).

report (1), and was positive for *Acanthamoeba*. The patient commenced treatment with trimethoprim-sulfamethoxazole (SXT), azithromycin, and fluconazole, as well as miltefosine, based on the encouraging results from recent cases (2, 3) and advice from the Centers for Disease Control and Prevention. Antimicrobials were eventually changed to a final regimen containing miltefosine, 5-flucytosine, fluconazole, SXT, azithromycin, and liposomal amphotericin B. Severe gastrointestinal side effects necessitated cessation of the miltefosine after only 3 weeks. Other antimicrobials were continued for the remainder of the patient’s hospitalization. Further questioning for possible exposure history was limited by the patient’s cognitive impairment, but the family reported that the patient had been using his rainwater tank to water his garden.

The patient made significant clinical improvement, with improved mental status and mobility. Repeat MRI demonstrated a small reduction in the size of cerebral lesions (Fig. 1c), and he was transferred to rehabilitation. Unfortunately, delirium and acute renal impairment developed secondary to high stoma output and decreased oral intake. MRI did not demonstrate any acute changes. He developed hospital-acquired pneumonia, continued to deteriorate over the following weeks, and died on day 107.

The *Acanthamoeba* isolate grew slowly on nonnutrient agar seeded with *Escherichia coli*, taking 7 days to spread across a standard petri dish at 28°C compared with 2 to 3 days for most *Acanthamoeba* strains. Axenic culture in PYNFH medium (peptone [1%, wt/vol], yeast extract [1%, wt/vol], yeast nucleic acid [0.1%, wt/vol], glucose [0.1%, wt/vol], folic acid [15 $\mu\text{g}/\text{ml}$], hemin [1 $\mu\text{g}/\text{ml}$], biotin [0.02 $\mu\text{g}/\text{ml}$], KH_2PO_4 [0.036%], Na_2HPO_4 [0.05%], and fetal calf serum [10% vol/vol]) was achieved after 3 months. Mature cysts showed a Pussard-Pons group II phenotype (4) having stellate walls with up to 12 arms. DNA was extracted from pelleted trophozoites using the QIAamp DNA minikit tissue protocol (Qiagen, Hilden, Germany). A fragment of the 18S rRNA gene was amplified using primers JDP1 and JDP2 (5). A 417-base consensus sequence was deposited into GenBank, and a BLASTn search demonstrated >99.5% identity at 100% coverage with submissions from three human isolates designated *Acanthamoeba* sp. genotype T1, including the type strain.

Susceptibility testing results were only available after the patient’s death (Table 1). The effect of antimicrobial agents on the isolate was tested using a published method (6). Briefly, doubling dilutions of agents in 0.1 ml PYNFH (minus added yeast nucleic acid) were prepared in duplicate in microtiter plates. Cysts (10^4 /

well) were added at a final concentration range of 0.25 to 512 $\mu\text{g}/\text{ml}$. The MIC was defined as the lowest concentration at which there was complete inhibition of trophozoite growth after 7 days of incubation at 28°C. The minimum cysticidal concentration (MCC) was the lowest concentration at which there was no growth from the wells showing inhibition when plated on *E. coli*-agar and incubated for 7 days at 28°C. The assays were repeated, and the results were averaged.

In 1965, Carter and Fowler reported four cases from South Australia of fatal acute pyogenic meningitis “probably due to *Acanthamoeba* sp.” (7). Carter later further identified this pathogenic species, which he named *Naegleria fowleri* in memory of his late coauthor (8). In 1981, Carter et al. reported a case of fatal meningoencephalitis due to another free-living amoeba, “probably *Acanthamoeba* sp.,” which was subsequently identified as *Balamuthia mandrillaris* (9), followed by other reports from Australia (10–12). There is only one previous report of confirmed *Acanthamoeba* encephalitis from Australia (13).

Acanthamoeba is an agent of corneal, brain, skin, and various disseminated infections. It is assumed all members of the genus

TABLE 1 Results of susceptibility testing of the *Acanthamoeba* isolate from this study and duration of treatment with the corresponding agents

Agent	MIC ($\mu\text{g}/\text{ml}$)	MCC ($\mu\text{g}/\text{ml}$) ^a	Treatment
Liposomal amphotericin B	>512	>512	Yes (13 wk)
Azithromycin	>512	>512	Yes (10 wk)
SXT ^b	512	>512	Yes (10 wk)
5-Flucytosine	8	128	Yes (13 wk)
Fluconazole	256	>512	Yes (10 wk)
Itraconazole ^c	>512	>512	No
Miltefosine	512	>512	Yes (3 wk) ^d
Pentamidine	256	512	No
Rifampin	>512	>512	No
Streptomycin	>512	>512	No
Voriconazole	1.0	512	No

^a MCC, minimum cysticidal concentration.

^b SXT, trimethoprim-sulfamethoxazole.

^c Solubilized in minimal ethanol.

^d Miltefosine treatment was ceased after 3 weeks due to severe gastrointestinal side effects.

can exist as free-living organisms and that human disease results from incidental exposure to environmental sources, such as dust, freshwater, and tap water. The genus is divided into 19 genotypes placed into three morphological groups. Strains of genotype T4 (group II) predominate in the environment and are responsible for most cases of encephalitis and nearly all corneal infections (14). Strains of group III genotypes tend to have a high temperature tolerance, are particularly virulent when inoculated into mice, and are also found in encephalitis and a small percentage of corneal infections. Genotype T1 strains are rare and have never been isolated from the environment. Genotype T1 strains branch with group III based on 18S rRNA gene sequence type (15) yet have a cyst morphology that is distinctly characteristic of group II. Genotype T1 isolates have been almost exclusively associated with fatal encephalitis in humans (14, 16), although there is a report of a multisystem infection in a dog (17).

Acanthamoeba encephalitis most often affects immunosuppressed or debilitated individuals. It is possible that the CMUSE was a risk factor in our patient: given that *Acanthamoeba* has been isolated from fecal samples (18), it is possible that the organism was hematogenously spread to our patient's central nervous system via his compromised intestine, assisted by iatrogenic immunosuppression.

To our knowledge, this is the first published case of *Acanthamoeba* isolation in mycobacterial liquid culture medium and detection by an automated culture system. It is interesting that the Middlebrook 7H9 broth-based medium supported the replication and/or metabolism of the organism sufficiently to allow the organism to produce low-level CO₂, which is the criterion for flagging in the algorithm for this automated instrument. *Acanthamoeba* strains can be fastidious in broth culture, and our isolate took several months to adapt to axenic growth in medium designed specifically for free-living amoebae. The ingredients that the mycobacterial and PYNFH media have in common are biotin, glutamic acid (folic acid), phosphate buffers, glucose as an energy source, and pancreatic digestion of casein (peptones). Further studies are needed to investigate whether other *Acanthamoeba* isolates are able to grow in this medium and be detected by the automated culture system. Given that this medium is readily available in many routine clinical microbiology laboratories, this information may be useful for diagnosis of this rare condition.

This is the first report in which *Acanthamoeba* was identified by using a sequence generated by a commercial panfungal sequencing assay. Although the top match in GenBank was with a sequence for an *Acanthamoeba* sp., there were no other matches with *Acanthamoeba* in GenBank. The next two matches were with partial 26S rRNA genes from unidentified or uncultured organisms. This is likely due to the infrequent use of this region of the genome for identification of *Acanthamoeba*. The GenBank accession number reference for the highest-matching sequence indicates that the *Acanthamoeba* isolate in that study had both 18S and 28S rRNA genes sequenced as part of a phylogenetic analysis of a novel genus of marine zooflagellates (19). Given the existence of shared sequences among eukaryotes using some fungal sequencing primers, microbiology laboratories need to be aware of the possibility of identification or detection of organisms from other kingdoms using these primers.

Guidelines recommend SXT plus rifampin plus ketoconazole, or fluconazole plus sulfadiazine plus pyrimethamine for the treatment of *Acanthamoeba* encephalitis (20). However, the condition

is still often fatal, and the optimal treatment is not well established. Our patient received SXT and fluconazole and also miltefosine. However, based on the susceptibility results, 5-flucytosine may have made the most significant contribution to our patient's improvement. Voriconazole was the most active agent tested for inhibition of trophozoite growth, while flucytosine was the most cysticidal. Given the generally high concentrations of agents that are required to affect *Acanthamoeba* *in vitro*, it is likely that MIC results are more relevant than amoebicidal results in the treatment of encephalitis. It is possible that the cysticidal activity may be more relevant for prediction of relapse.

Many methods have been used to assess the susceptibility of *Acanthamoeba* isolates to antimicrobial agents. There does not appear to be an association between phenotype, genotype, and susceptibility (21). The inhibitory and cysticidal concentrations will depend on variables such as the method of cell preparation, the period in axenic culture, and the assay system, but a ranking of agents according to activity should be achievable. Our MIC assay involved a large inoculum of cysts exposed to antimicrobial agents for an extended culture period (7 days). Two agents tested in this report were included in a study of 19 corneal isolates using the same method: the T1 isolate was relatively resistant to both pentamidine and amphotericin B (6). The relationship between *in vitro* susceptibility and clinical outcome is unknown, and work toward a useful animal model continues (22). Although such a relationship could not be demonstrated for the topical treatment of *Acanthamoeba* keratitis (6), the situation in the brain may be different.

There are many challenges in the treatment of *Acanthamoeba* encephalitis. In the laboratory, these include the small number of neural strains available for study, the lack of a standard method for susceptibility testing, and the time required to prepare an isolate in the face of a deteriorating clinical situation. As there does not appear to be an association between phenotype, genotype, and antimicrobial susceptibility to direct empirical treatment, an attempt should be made to test each neural strain upon isolation. However, the relationship between *in vitro* susceptibility and clinical outcome needs further investigation.

Nucleotide sequence accession number. A 417-base consensus sequence from the isolate examined in this study has been deposited in GenBank under accession no. [KM015457](https://www.ncbi.nlm.nih.gov/nuclseq/KM015457).

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