Rapid, Noninvasive Diagnosis of *Balamuthia mandrillaris* Encephalitis by a Plasma-Based Next-Generation Sequencing Test

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Granulomatous amoebic encephalitis (GAE) caused by *Balamuthia mandrillaris* is a rare subacute infection with exceptionally high mortality. Diagnosis is typically made by brain biopsy or at autopsy. Detection of *Balamuthia mandrillaris* cell-free DNA by next-generation sequencing of plasma enabled rapid, noninvasive diagnosis in a case of amoebic encephalitis.

Keywords. *Balamuthia mandrillaris*, granulomatous amoebic encephalitis, next generation sequencing, rapid diagnosis.

There are 3 main pathogenic free-living amoebae that cause disease in humans [1]. They cause primary amoebic meningoencephalitis (PAM; *Naegleria fowleri*), granulomatous amoebic encephalitis (GAE; *Acanthamoeba* and *Balamuthia*), *Acanthamoeba keratitis*, and infections in other organ systems including the skin. The usual features of GAE are nonspecific and consist of fever, lethargy, cerebellar ataxia, headaches, neck stiffness, visual disturbances, hemiparesis, aphasia, seizures, and coma [2, 3].

Balamuthia mandrillaris is a free-living amoeba that is found primarily in soil and sometimes water [3–6]. It was first isolated postmortem from an infected mandrill at the San Diego zoo in California in 1986. The first human infection was reported in 1990 [7]. *Balamuthia mandrillaris* has a 2-stage life cycle, a vegetative trophozoite stage, and a dormant cyst form. After infection (or inoculation), it is spread hematogenously. Infections affect both immunocompromised and immunocompetent individuals, progressing in a subacute to chronic course [3]. Patients have developed *Balamuthia* encephalitis months to years after developing cutaneous *Balamuthia* [8, 9]. The prodromal period can last from weeks to months [3]. Cerebrospinal fluid (CSF) findings include lymphomononuclear pleocytosis with elevated protein and a normal or low glucose [4, 10]. Imaging may reveal space-occupying and ring-enhancing lesions and can demonstrate leptomeningeal involvement [11, 12]. Lesions are characteristically seen in the frontal and parietal lobes [11]. Specific diagnosis of *Balamuthia* encephalitis is challenging, often delayed, and frequently requires brain biopsy [11, 12].

We present a case of *Balamuthia mandrillaris* brain infection where a rapid diagnosis was made by a noninvasive plasma-based next-generation sequencing (NGS) test for pathogen detection.

CASE REPORT

A 51-year-old man presented to the hospital in March 2018 with complaints of left-sided weakness, persistent seizures, headaches, confusion, incoordination, fevers, drenching night sweats, and diarrhea. His medical history was notable for hypertension, seizures, alcohol abuse, and sporadic cocaine abuse. He worked as a mason and lived in temporary housing while traveling around Florida. When at home, he lived on a farm with cats, dogs, chickens, and ducks. In the 6 months before admission, the patient had self-extracted several of his teeth using pliers.

The patient developed seizures 6 months before presentation. The seizures increased in frequency 2 weeks before presentation to a local hospital. Upon initial presentation, he had a normal head computed tomography scan and was discharged with a diagnosis of alcohol-related seizures. He re-presented to the hospital with fevers, headache, altered mental status, recalcitrant seizures, disorientation, and left-sided weakness. He was febrile to 101.5°F with otherwise normal vital signs. Routine labs were within normal limits, but brain magnetic resonance imaging (MRI) showed multiple ring-enhancing lesions in the cerebellum and cerebrum bilaterally (Figure 1A). Lumbar puncture demonstrated a CSF white blood cell count of 33 with 18% polymorphonuclear leucocytes, 60% lymphocytes, and 22% monocytes, protein of 295 mg/dL, and glucose of 43 mg/dL. CSF gram stain and herpes simplex virus (HSV) polymerase chain reaction (PCR) were negative. He received broad-spectrum antibiotic therapy with vancomycin, cefepime, and metronidazole.
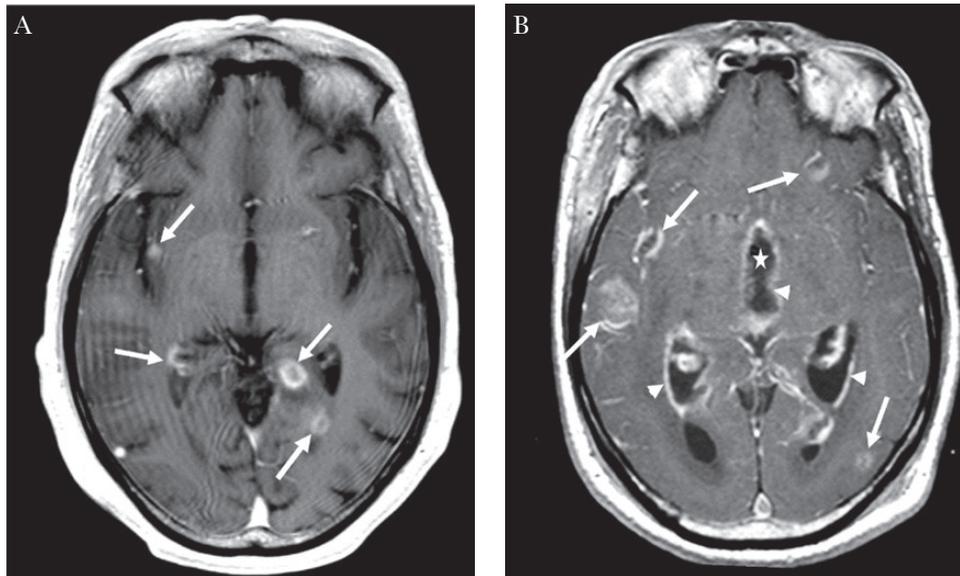
Figure 1. A, Axial T1-weighted gadolinium-enhanced magnetic resonance (MR) image obtained on initial presentation shows multiple ring-like peripheral enhancing nodular lesions (arrows). B, Axial T1-weighted gadolinium-enhanced MR image obtained on day 12 reveals interval progression of multiple ring-like peripheral enhancing nodular lesions (arrows). Hydrocephalus (star) and ventriculitis are also noted (arrowheads).

CSF cultures, cytology and flow cytometry, enterovirus PCR, Epstein-Barr virus PCR, cytomegalovirus PCR, and varicella zoster virus PCR were negative. *Toxoplasma gondii* antibody, *Blastomycosis* antibody, *Coccidioidomycosis* antibody, *Histoplasma* urine antigen, *Cryptococcus* antibody, rapid plasma reagin, *Borrelia burgdorferi* antibody, HIV, and an Interferon Gamma Release Assay test for tuberculosis were all negative. Urine *Legionella* antigen was positive, after which levofloxacin was added (subsequent repeat *Legionella* antigen was negative). A trans-thoracic echocardiogram was normal without any vegetation. The patient had a macular pigmented lesion in his right medial thigh for a year, which was not biopsied.

Two days after admission, he developed clinical deterioration requiring intubation. Repeat brain MRI showed ventricular enlargement and cerebral edema. He developed a syndrome of inappropriate antidiuretic hormone secretion with a sodium level of 119 mmol/L. Repeat lumbar puncture demonstrated an elevated opening pressure of 20 mmHg and a CSF white blood cell count of 30 cells with 34% polymorphonuclear neutrophils and 62% monocytes, markedly elevated CSF protein of 1778 mg/dL, and glucose of 28 mg/dL. Empiric antifungal coverage was initiated with amphotericin B. Preliminary results from a brain biopsy on hospital day 7 showed features of an abscess, and a frozen section revealed no evidence of malignancy.

A plasma sample sent on hospital day 6 for NGS of circulating microbial cell-free DNA (mcfDNA; Karius Inc., Redwood City, CA, USA) detected and reported *Balamuthia mandrillaris* cell-free DNA (without any other co-detection) on hospital day 8 (48 hours after sample collection). Empiric antibiotics were discontinued, and the patient was started on flucytosine (2 g intravenously [IV] Q6 hours), fluconazole (400 mg orally [PO] daily), albendazole (400 mg PO daily), clarithromycin (500 mg PO daily), pentamidine (4 mg/kg IV daily), and miltefosine (500 mg Q8 hours). Brain biopsy demonstrated an exuberant lymphohistiocytic infiltrate, granuloma formation, and rare amoebic trophozoites (Figure 2A). A wet mount was not performed on the specimen. Repeat MRI showed severe hydrocephalus, ventriculitis, and progressive lesions (Figure 1B). With further clinical deterioration, the patient's family elected to withdraw care, and shortly thereafter the patient died on hospital day 13.

CSF and brain tissue samples from the initial brain biopsy were sent to the Centers for Disease Control and Prevention (CDC) and confirmed *Balamuthia mandrillaris* by real-time PCR, histology, and immunohistochemistry (IHC) postmortem (Figure 2B). Banked, frozen plasma from the patient was unavailable for retrospective analysis to estimate the earliest time point at which Karius testing would have been positive.

METHODS

Peripheral blood plasma was sent to the Karius Clinical Laboratory Improvement Amendments (CLIA)/College of American Pathologists–accredited laboratory (Redwood City, CA, USA) for processing. McfDNA was extracted from plasma, NGS libraries were prepared, and sequencing was performed by an Illumina NextSeq 500 sequencer. Human sequencing reads were removed, and the remaining sequences were aligned to a curated pathogen database. Any of >1400 organisms in the Karius clinically reportable range found to be present above a predefined statistical threshold were reported [13–16]. The Karius commercial assay at the time of its use in this case was a qualitative test; it is now a quantitative test reporting molecules of microbial cell-free DNA/μL of plasma.
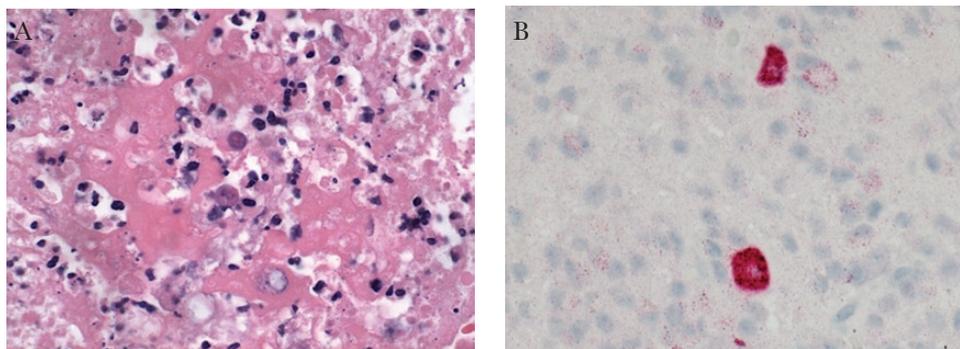
Figure 2. A, Brain, hematoxylin and eosin (HE). *Balamuthia mandrillaris* trophozoites in a background of necrotic brain tissue. Abundant neutrophils and macrophages are also identified; HE 600X magnification. B, Brain, immunohistochemical assay targeting free-living amoeba, including *Balamuthia mandrillaris*. Amebic trophozoites are stained red; 400X.

Two CSF samples collected 5 days apart, 11 and 6 days before the patient's death, were sent to the CDC's CLIA-accredited Free-Living and Intestinal Amoebas (FLIA) laboratory for detection of pathogenic free-living amoeba (FLA) DNA by multiplex real-time PCR. DNA was extracted from CSF by the Qiagen blood and tissue kit according to the manufacturer's instructions, and FLA real-time PCR was performed with the extracted DNA according to Qvarnstrom et al. [17]. Both CSF samples were positive for *B. mandrillaris* by FLA real-time PCR, and they gave almost identical computed tomography values (data not shown). Tissue blocks from the brain biopsy were sent to the CDC's Infectious Diseases Pathology Branch (IDPB) for histopathologic evaluation and immunohistochemical testing for amoeba. Immunohistochemical staining for amoeba was performed as previously described [18].

Manual Assessment of *Balamuthia mandrillaris* Detection

We manually assessed the validity of the *Balamuthia mandrillaris* detection reported with the automated Karius Test pipeline by inspecting read alignments to the *B. mandrillaris* reference genomes [19, 20]. In total, there were 649 reads with a total of 3999 alignments to 1 or both *B. mandrillaris* reference genomes. The locations of read alignments on the reference followed a uniform distribution (Supplementary Figure 1A). More than 300 of those reads had exact full-length matches with the reference. Only 9 reads had alternative alignments to other taxa across the database, and these were mostly of low quality (Supplementary Figure 1B). The remaining 640 reads aligned exclusively to *B. mandrillaris*; however, most reads had multiple alignments within the genome due to its repetitive structure (for detailed methods, see the Supplementary Methods).

DISCUSSION

In a review of *Balamuthia* cases in the United States between 1974 and 2016, the majority of patients were diagnosed by indirect immunofluorescence followed by polymerase chain reaction and histopathology, with testing mostly performed on brain tissue. Eighty-eight percent of patients with *Balamuthia* required a brain biopsy to aid in the diagnosis [12]. Common clinical features on presentation are fever, headache, vomiting, and lethargy [12]. In the case presented here, the patient's occupation as a mason (exposure to soil) and cocaine use are risk factors associated with *Balamuthia* infection [12]. Encephalitis has a very broad differential diagnosis; the diagnosis of *Balamuthia* often requires brain biopsy. Cell-free plasma NGS shows promise as a rapid noninvasive diagnostic tool.

The broad-range nature of an NGS platform for pathogen detection enables the clinician to order a single test for the potential diagnosis of many infectious agents rather than an exhaustive, costly, highly specialized battery of individual tests with long and differential turnaround times, especially where specimens are limited (CSF, brain tissue).

An advantage of NGS testing is its "unbiased" approach that does not require the clinician to suspect a specific agent and order the appropriate specific test. The approach also accommodates unexpected causes when the clinician may not have suspected the eventual diagnosis. NGS may be valuable earlier in the diagnostic journey in cases such as this, where the differential diagnosis is broad, leading to a more parsimonious and rapid means of diagnosis. Plasma-based NGS for mcfDNA will not replace the need for lumbar puncture given that the vast majority of patients with a concern for brain infection will undergo CSF sampling to assess cell counts and biochemical parameters. Indeed, "syndromic" testing is the basis for many emerging multiplex PCR platforms and has been applied to CSF with some success [21]. These panels, however, are limited and do not include uncommon or unexpected causes of brain infection. NGS is a logical extension of this approach but until recently has been limited by complex laboratory methods, the lack of a rapid, robust, and accessible bioinformatics pipeline, small-scale research deployment, and long turnaround times. NGS of CSF and brain biopsy

specimens has been used to detect *Balamuthia mandrillaris* in a case of PAM [19] and endophthalmitis/meningoencephalitis [22]; the results were not clinically actionable and were only available after the patients had succumbed to their infections. The overall utility of CSF NGS for brain infection is limited [23]. The case of *Balamuthia* encephalitis presented here was diagnosed by a plasma-based NGS test for circulating mcfDNA akin to "liquid biopsy." The case is unique in that it is the first case of *Balamuthia* diagnosed premortem using NGS technology and the first using a plasma-based NGS method; the results were available in a clinically actionable time frame. In general, the turnaround time for the Karius Test ($2000) is 1 day from sample receipt, compared with 7–10 days for CSF-based NGS testing at UCSF ($2200).

A key differentiating feature of plasma-based NGS for circulating mcfDNA is its potential ability to detect infections anywhere in the body as mcfDNA from sequestered sites of infection spill into the plasma even when the pathogens themselves are not present in the blood [24]. This approach is amenable to a wide spectrum of clinical syndromes ranging from disseminated infections to sequestered infections in localized highly privileged sites (even the central nervous system), as in this case. Plasma-based NGS for mcfDNA has been used to diagnose a range of brain infections including *Scedosporium boydii* [13], *Toxoplasma gondii* [25], and *Angiostongylus cantonensis* [26]. Its sensitivity may still be limited by the overall pathogen burden and the avidity of the pathogen's spillage of its mcfDNA into the plasma. Thus far, CSF NGS (and PCR) methods are designed to detect pathogen-associated nucleic acid and can only detect causes of infection where the offending pathogen is physically present in the analyzed sample. Although brain biopsy is still considered the gold standard for diagnosis, in selected cases (HSV encephalitis), CSF PCR may be a potentially rapid diagnostic method.

CSF NGS and PCR may have some utility in a parameningeal infection and less utility in a deep parenchymal focus. In many instances, CSF does not physically contain the pathogen that causes parenchymal brain infection, and in these cases CSF NGS and PCR modalities may be insensitive, forcing the requirement of a brain biopsy. While plasma-based NGS for mcfDNA may not replace lumbar puncture, it may offer advantages over CSF NGS and should certainly be considered before diagnostic brain biopsy. Clinical studies are still needed to assess the systematic use of plasma-based NGS of mcfDNA for localized brain infection, perhaps comparing it with the utility and additional benefits and risks of invasive brain biopsy. No specific treatment is available to treat *Balamuthia* infections. Several antibiotic combinations have been tried with minimal success. Regimens have included a combination of miltefosine, fluconazole and albendazole [9], or clarithromycin, fluconazole, sulfadiazine, pentamidine, and fluorocytosine [27]. With extremely poor outcomes, prompt diagnosis may facilitate improved patient outcomes by enabling more rapid initiation of definitive, directed treatment.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

References

1. Yamanouchi K, Arima H, Sakamoto Y, et al. First report of the isolation of *Balamuthia mandrillaris* in the northern region of Japan. Parasitol Res **2018**; 117:2895–900.
2. Siddiqui R, Khan NA. *Balamuthia* amoebic encephalitis: an emerging disease with fatal consequences. Microb Pathog **2008**; 44:89–97.
3. Visvesvara GS, Moura H, Schuster FL. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. FEMS Immunol Med Microbiol **2007**; 50:1–26.
4. *Balamuthia* amebic encephalitis—California, 1999–2007. Morb Mortal Wkly Rep **2008**; 57:768–71.
5. Finnin PJ, Visvesvara GS, Campbell BE, et al. Multifocal *Balamuthia mandrillaris* infection in a dog in Australia. Parasitol Res **2007**; 100:423–6.
6. Foreman O, Sykes J, Ball L, et al. Disseminated infection with *Balamuthia mandrillaris* in a dog. Vet Pathol **2004**; 41:506–10.
7. Visvesvara GS, Martinez AJ, Schuster FL, et al. Leptomyxid ameba, a new agent of amebic meningoencephalitis in humans and animals. J Clin Microbiol **1990**; 28:2750–6.
8. Lehmer LM, Ulibarri GE, Ragsdale BD, Kunkle J. Cutaneous *Balamuthia mandrillaris* infection as a precursor to *Balamuthia* amoebic encephalitis (BAE) in a healthy 84-year-old Californian. Dermatol Online J **2017**; 23:13030/qt8c8720qm.
9. Martínez DY, Seas C, Bravo F, et al. Successful treatment of *Balamuthia mandrillaris* amoebic infection with extensive neurological and cutaneous involvement. Clin Infect Dis **2010**; 51:e7–11.
10. Jung S, Schelper RL, Visvesvara GS, Chang HT. *Balamuthia mandrillaris* meningoencephalitis in an immunocompetent patient: an unusual clinical course and a favorable outcome. Arch Pathol Lab Med **2004**; 128:466–8.
11. Ong TYY, Khan NA, Siddiqui R. Brain-eating amoebae: predilection sites in the brain and disease outcome. J Clin Microbiol **2017**; 55:1989–97.
12. Cope JR, Landa J, Nethercut H, et al. The epidemiology and clinical features of *Balamuthia mandrillaris* disease in the United States, 1974 - 2016. Clin Infect Dis **2019**; 68:1815–22.
13. Hong DK, Blauwkamp TA, Kertesz M, et al. Liquid biopsy for infectious diseases: sequencing of cell-free plasma to detect pathogen DNA in patients with invasive fungal disease. Diagn Microbiol Infect Dis **2018**; 92:210–3.
14. Abril MK, Barnett AS, Wegermann K, et al. Diagnosis of capnocytophaga canimorsus sepsis by whole-genome next-generation sequencing. Open Forum Infect Dis **2016**; 3:XXX–XX.
15. Fung M, Zompi S, Seng H, et al. Plasma cell-free DNA next-generation sequencing to diagnose and monitor infections in allogeneic hematopoietic stem cell transplant patients. Open Forum Infect Dis **2018**; 5:XXX–XX.
16. Blauwkamp TA, Thair S, Rosen MJ, et al. Analytical and clinical validation of a microbial cell-free DNA sequencing test for infectious disease. Nat Microbiol **2019**; 4:663–74.
17. Qvarnstrom Y, Visvesvara GS, Sriram R, da Silva AJ. Multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*. J Clin Microbiol **2006**; 44:3589–95.

18. Guarner J, Bartlett J, Shieh WJ, et al. Histopathologic spectrum and immunohistochemical diagnosis of amebic meningoencephalitis. Mod Pathol **2007**; 20:1230–7.

19. Greninger AL, Messacar K, Dunnebacke T, et al. Clinical metagenomic identification of *Balamuthia mandrillaris* encephalitis and assembly of the draft genome: the continuing case for reference genome sequencing. Genome Med **2015**; 7:113.

20. Detering H, Aebischer T, Dabrowski PW, et al. First draft genome sequence of *Balamuthia mandrillaris*, the causative agent of amoebic encephalitis. Genome Announc **2015**; 3:e01013–15.

21. Leber AL, Everhart K, Balada-Llasat JM, et al. Multicenter evaluation of BioFire FilmArray meningitis/encephalitis panel for detection of bacteria, viruses, and yeast in cerebrospinal fluid specimens. J Clin Microbiol **2016**; 54:2251–61.

22. Wilson MR, Shanbhag NM, Reid MJ, et al. Diagnosing *Balamuthia mandrillaris* encephalitis with metagenomic deep sequencing. Ann Neurol **2015**; 78:722–30.

23. Wilson MR, Sample HA, Zorn KC, et al. Clinical metagenomic sequencing for diagnosis of Meningitis and Encephalitis. N Engl J Med **2019**; 380:2327–40.

24. Rossoff J, Chaudhury S, Soneji M, et al. Noninvasive diagnosis of infection using plasma next-generation sequencing: a single-center experience. Open Forum Infect Dis **2019**; 6:ofz327.

25. Zhou Y, Hemmige V, Dalai SC, et al. Utility of whole-genome next-generation sequencing of plasma in identifying opportunistic infections in HIV/AIDS. Open AIDS J **2019**; 13:7–11.

26. Melish M, Casamina C, Merrifield R, et al. Next-generation sequencing-based detection of *Angiostrongylus cantonensis* (AC) using microbial cell-free DNA sequencing of plasma in atypical cases of rat lungworm meningitis presenting with ascending paralysis. Open Forum Infect Dis **2019**; 6(Suppl 2):S513.

27. Deetz TR, Sawyer MH, Billman G, et al. Successful treatment of *Balamuthia* amoebic encephalitis: presentation of 2 cases. Clin Infect Dis **2003**; 37:1304–12.