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Diagnosing *Balamuthia mandrillaris* encephalitis with metagenomic deep sequencing

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Abstract

Objective—Identification of a particular cause of meningoencephalitis can be challenging due to the myriad bacteria, viruses, fungi, and parasites that can produce overlapping clinical phenotypes, frequently delaying diagnosis and therapy. Metagenomic deep sequencing (MDS) approaches to

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infectious disease diagnostics are known for their ability to identify unusual or novel viruses and thus are well suited for investigating possible etiologies of meningoencephalitis.

Methods—We present the case of a 74 year-old woman with endophthalmitis followed by meningoencephalitis. MDS of her cerebrospinal fluid (CSF) was performed to identify an infectious agent.

Results—Sequences aligning to *Balamuthia mandrillaris* ribosomal RNA genes were identified in the CSF via MDS. Polymerase chain reaction (PCR) subsequently confirmed the presence of *B. mandrillaris* in CSF, brain tissue, and vitreous fluid from the patient's infected eye. *B. mandrillaris* serology and immunohistochemistry for free-living amoebas on the brain biopsy tissue were positive.

Interpretation—The diagnosis was made using MDS after the patient had been hospitalized for several weeks and subjected to costly and invasive testing. MDS a powerful diagnostic tool with the potential for rapid and unbiased pathogen identification leading to early therapeutic targeting.

Keywords

Metagenomics; next-generation sequencing; primary amoebic meningoencephalitis; *Balamuthia mandrillaris*; encephalitis

Introduction

Meningoencephalitis presents significant diagnostic challenges due in part to the large number of possible underlying causes. These include infectious, inflammatory, paraneoplastic, and toxic-metabolic etiologies, many of which can present with similar clinical phenotypes. Furthermore, confirming an infectious etiology can be challenging, as many pathogen-specific serologic and polymerase chain reaction (PCR) assays are expensive, time-consuming and have poor operating characteristics. Culture-based approaches, though they can often detect multiple species, are also limited, as many infectious agents require special culture conditions and prolonged incubation times. Standard culture protocols are also not commercially available for many organisms.

Metagenomics is the study of genetic material recovered from an environmental sample, including bodily fluids and tissues. Rapid advances in sequencing technology and bioinformatics have made an unbiased deep sequencing approach to metagenomics an increasingly feasible method for rapidly interrogating all the genetic material contained in a sample. We previously reported on the ability of metagenomic deep sequencing (MDS) of cerebrospinal fluid (CSF) to identify the cause of infectious meningoencephalitis in real time, leading to a dramatic improvement in a patient's medical condition.¹ Here, we describe a case of meningoencephalitis in a 74-year-old woman that illustrates the ability of an MDS-based approach to identify an amoebic cause of meningoencephalitis. Our patient had had an extensive diagnostic workup including two brain biopsies, but a final diagnosis was not reached before she died. Shortly before her death, a sample of CSF was submitted for MDS, which led to the identification of *Balamuthia mandrillaris*, a rare and almost uniformly fatal cause of meningoencephalitis. This diagnosis was later confirmed by the Centers for Disease Control and Prevention (CDC), the California Department of Public

Health (CA DPH), and analysis of additional immunohistochemical stains and additional sections of brain biopsy tissue. MDS provides an efficient and unbiased diagnostic approach to identify culprit central nervous system (CNS) infections and may generate large cost-savings when used to improve diagnosis rates in patients with costly conditions like meningoencephalitis.² Early treatment guided by rapid and accurate diagnosis may also improve outcomes in cases such as these in which delayed diagnosis has likely contributed to historically poor outcomes.

Case Report

A 74-year old Chinese woman with a history of rheumatoid arthritis treated with hydroxychloroquine sulfate presented to our hospital with altered mental status. She had immigrated to the United States from China approximately eight years prior and had been independent in all her activities. She did not use tobacco, alcohol, or illicit drugs.

In the four weeks preceding hospitalization at our institution, she was admitted three times to two other facilities. On each occasion she was evaluated for intermittent fevers and mildly altered mental status. On the second admission she was diagnosed with endophthalmitis and treated empirically with intravitreal vancomycin, ceftazidime and voriconazole, though vitreal and blood cultures eventually showed no growth, and PCRs on the vitreous fluid for herpes simplex virus, cytomegalovirus and toxoplasmosis ultimately proved negative. In addition, she was started on therapy for *Toxoplasma gondii* in the setting of a positive serum IgM titer (see Table 1). Although her vision did not improve, she was briefly discharged but then brought back to the hospital for a third admission when a cervical spine MRI performed to evaluate spinal tenderness was incidentally noted to show a T2 hyperintensity in the pons. A subsequent brain MRI showed small areas of restricted diffusion in multiple vascular territories consistent with infarcts (see Figure 1A). A stroke work-up including a trans-esophageal echocardiogram was unrevealing except for paroxysmal atrial fibrillation. The patient was discharged a third time, now on warfarin for presumed cardioembolic strokes.

Although she had been ruled out for acid-fast bacilli during her second hospitalization, the patient presented to our institution two days later for an evaluation at the city's tuberculosis (TB) clinic given her recent history of pneumonia and demographic risk factors. At the TB clinic, it was found that her neurologic status had significantly deteriorated since her discharge two days prior. She was transferred directly to the Emergency Department where her vital signs were: temperature 103°F; heart rate 111 beats per minute; blood pressure 123/81 mm Hg; oxygen saturation 93% on room air. She moved all four extremities spontaneously but was unable to follow commands or verbalize, resulting in a Glasgow Coma Scale score of 6. At this point she was intubated for airway protection and admitted to the intensive care unit (ICU).

Initial laboratory evaluation revealed a white blood cell count of 12.6×10^3 cells/ μ L, hemoglobin of 13.7 g/dL, serum sodium of 129 mEq/L, and serum glucose of 135 mg/dL. CSF analysis demonstrated 13 red blood cells/ mm^3 , 347 white blood cells/ mm^3 (57% neutrophils, 11% monocytes, 31% lymphocytes, 1% eosinophils), glucose 30 mg/dL, and protein 71 mg/dL. Brain MRI demonstrated innumerable ring enhancing and diffusion-

restricted supra- and infratentorial lesions that were felt to be most consistent with abscesses as well as severe obstructive hydrocephalus.

On the first day of her hospitalization she was initiated on empiric treatment for bacterial brain abscesses with vancomycin, cefepime, and metronidazole. Blood, urine, and CSF bacterial and fungal cultures were eventually negative, as were CSF tests for neurosyphilis, TB, and *Cryptococcus* (see Table 1). Both CSF and serologic tests for toxoplasmosis were negative. Despite broad antibiotic coverage, she continued to deteriorate with progressive loss of motor activity and subsequently brainstem reflexes over the following 16 days. On hospital day 10, a repeat lumbar puncture showed worsening pleocytosis with a more prominent eosinophilia (see Table 1). Given these findings, broad-spectrum antibiotics were stopped on day 10, and she was initiated on anti-TB and anti-fungal therapy. A brain biopsy performed on day four was non-diagnostic, revealing only mild gliosis and no inflammatory infiltrate or microorganisms. A repeat biopsy one week later (day 11) demonstrated a severe necrotizing vasculitis of unclear etiology, with a prominent component of eosinophils, chronic inflammatory cells, and neutrophils but without a significant granulomatous component. Repeat brain MRI on hospital day 14 demonstrated an increase in the number and size of her brain lesions as well as interval development of basilar meningitis (see Figure 1B and 1C). Her worsening cerebral edema was refractory to management with hyperosmolar therapy, as well as to the extraventricular drain that was placed during her second brain biopsy. The patient ultimately became hemodynamically unstable, likely due to elevated intracranial pressure, and required multiple vasopressors. Her family opted for comfort care measures to be instituted, and the patient was extubated and expired on day 17 of her hospitalization.

Methods

On day nine of the patient's hospitalization, a sample of CSF was submitted for unbiased MDS under a research protocol for the identification of potential pathogens approved by the institutional review boards of San Francisco General Hospital (SFGH) and the University of California, San Francisco (UCSF). Samples were processed for MDS analysis as previously described.¹ Briefly, total ribonucleic acid (RNA) was extracted from CSF. Samples were randomly amplified to double stranded cDNA using the NuGEN Ovation v.2 kit (NuGEN, San Carlos, CA) for low nucleic acid content samples, and MDS libraries were constructed using the Nextera protocol (Illumina, San Diego, CA) as previously described.¹ Library size and concentration were determined using the Blue Pippin (Sage Science, Beverly, MA) and Kapa Universal qPCR kit (Kapa Biosystems, Woburn, MA), respectively. Samples were sequenced on an Illumina HiSeq 2500 instrument using 135/135 base pair (bp) paired-end sequencing. The paired-end sequences were analyzed for pathogens using a rapid computational pipeline for pathogen detection as described below.

RESULTS

Identification of *Balamuthia* sequences in CSF

The MDS of total RNA from the patient sample yielded 19,642,962 paired-end reads representing 5.2 gigabases total. The data were analyzed using a rapid computational

pipeline developed at our institution to classify MDS reads and identify potential pathogens by comparison to the entire National Center for Biotechnology Information (NCBI) nucleotide (nt) reference database. Briefly, paired-end reads were quality filtered using PriceSeqFilter, a component of the PRICE assembler, followed by alignment to the human reference genome (hg19) using bowtie2 v2.2.4.^{3, 4} Unaligned reads that were at least 95% identical were compressed by cd-hit (v4.6.1), resulting in a total of 33,093 remaining reads, representing approximately 0.1% of the original data.^{5, 6} These reads were then used as queries to search the NCBI nt database using gsnapl (v2014-10-16).⁷ Over 97% of these reads yielded gsnapl alignments to vertebrate sequences (human reads not filtered by bowtie), and known contaminants found in laboratory reagents.

After filtering, our analysis identified *B. mandrillaris* as the most highly represented organism with 81 reads mapping to the three published *B. mandrillaris* genes in the NCBI repository. Twenty-nine other individually barcoded patient CSF samples (including a water control) were included in the same sequencing run, and none contained reads that aligned to *Balamuthia*, nor had *Balamuthia* reads ever been detected in this laboratory previously. The complete pipeline run time for this analysis was 1hr 5min.

These results informed a retrospective bioinformatic analysis of the original dataset wherein the raw MDS reads were directly aligned to the three available *B. mandrillaris* gene sequences. The alignment yielded ~3500 read pairs mapping concordantly to the *B. mandrillaris* genes, representing only 0.018% of the original dataset. A random sample (30 reads) of the aligned sequences was used to seed an assembly using the targeted assembler PRICE (v1.2), ultimately generating an 806 nt contig corresponding to the *B. mandrillaris* 16S ribosomal RNA (rRNA) gene (GenBank accession number KP990616).³

Confirmatory testing for *B. mandrillaris*

The presence of *B. mandrillaris* was confirmed via multiple methods. Using a published Centers for Disease Control and Prevention (CDC) polymerase chain reaction (PCR) protocol, the 18s rRNA gene from *B. mandrillaris* was detected in the patient's formalin fixed brain biopsy tissue from the second brain biopsy, CSF, and vitreous fluid.⁸ The PCR products were Sanger sequenced (Quintara Biosciences, Albany, CA) and confirmed to be sequences from the *Balamuthia* 18s rRNA gene. Based on the findings of the CSF MDS study, material from the patient's second brain biopsy was re-evaluated with additional level sections, which revealed organisms consistent with amoeba within vessels and extending into the neural parenchyma (see Figure 1D). The Infectious Diseases Pathology Branch at the CDC performed confirmatory immunohistochemistry for free-living amoeba on the brain biopsy tissue (see Figure 1E), and the CA DPH determined that the patient had a borderline positive serology for *B. mandrillaris* (see Table 1).⁹

Discussion

We present the case of a woman with several weeks of progressive meningoencephalitis that eventually proved fatal despite a three week ICU stay, various antimicrobial regimens, multiple serological and CSF studies and two brain biopsies. This extensive testing did not reveal the cause of her syndrome; however, MDS of her CSF demonstrated the etiologic

agent, *B. mandrillaris*. This finding was subsequently confirmed by PCR and by immunohistochemistry of the brain biopsy tissue. To our knowledge, this is the first case in which unbiased MDS has proven useful for identifying an amoebic infection in the context of a critical illness. The identification of *B. mandrillaris* was particularly notable given that sequences for only three of the likely ~15,000 *B. mandrillaris* genes are publicly available in the NCBI database utilized by our bioinformatics pipeline for pathogen identification. Clearly, additional genomic information for this enigmatic pathogen would be useful in the context of future sequence-based diagnostic assays, and for the purpose of identifying new therapeutic targets.

Since it was first isolated in 1986, *B. mandrillaris* has been recognized as a rare cause of amoebic encephalitis in humans, with approximately 200 cases reported worldwide.^{10, 11} The amoeba is distributed worldwide in soil, fresh, and saltwater sources, and causes disease in both immunocompetent and immunocompromised hosts.¹⁰ In fact, heavy soil exposure has been cited as a possible risk factor for developing a *B. mandrillaris* infection.¹² Although our patient had a small garden behind her house, she was not known to be an avid gardener. She was also not heavily immunosuppressed for her rheumatoid arthritis.

The infection commonly manifests with nonspecific symptoms, including fever, headache, nausea, vomiting, seizures, and focal neurologic signs.^{13–16} As in the case presented here, imaging may reveal the presence of nodular lesions involving gray and white matter, with or without ring-enhancement; disease progression commonly leads to vasogenic edema and mass effect.¹⁰

The period of incubation at the site of primary infection is variable, as it may precede by months the development of encephalitis. From the initial site, the organisms migrate to the CNS via the blood stream, where they proliferate, inducing a granulomatous inflammation that progresses for a period of weeks. Amoebic encephalitis can show various histologic patterns, and in this case no granulomatous component was seen in the brain biopsy specimen; *Balamuthia* in particular is known to present with a range of inflammatory patterns.^{17, 18} To our knowledge, this is the first time that an ocular infection with *B. mandrillaris* has been described. An unsuccessful attempt was made to culture *B. mandrillaris* from the CSF, but no attempt at amoebic culture was made from the remaining vitreous fluid given its small volume (i.e., 30 uL) and because it had been frozen for an extended period of time in a –70°C freezer. The vitreous fluid was collected in the hospital under sterile conditions when the patient first presented with endophthalmitis. All standard precautions were taken in the laboratory to prevent cross contamination. A negative PCR water control conducted simultaneously was negative for *B. mandrillaris*. The positive vitreous fluid PCR result was repeated in triplicate and confirmed by Sanger sequencing.

There are only ten reported cases of patients surviving *B. mandrillaris* meningoencephalitis, in part because of extensive disease at the time of diagnosis and a lack of well-developed treatment protocols. Pentamidine, clarithromycin, fluconazole, and sulfadiazine have been used in patients who have survived the infection.^{19, 20} Recently, miltefosine, in combination with some of these other agents, has shown some promise and is approved by the Food and

Drug Administration as an investigational treatment of granulomatous encephalitis caused by *B. mandrillaris*.²¹

Diagnosis of *B. mandrillaris* has traditionally relied on its identification in tissue samples. CSF analysis usually reveals a mononuclear pleocytosis, high protein levels and normal to low glucose concentrations.^{22, 23} Culturing *Balamuthia* amoebae is very challenging, requiring a feeder tissue monolayer such as monkey kidney cells. Growth typically takes several weeks before the amoebae start to proliferate.²⁴ PCR and antibody detection assays have been developed as well; however, the diagnosis is not frequently entertained and extant assays are not widely available.^{8, 9}

As an alternative to conventional assays, MDS represents an unbiased and rapid diagnostic tool, especially for vexing cases, such as the one presented here. One criticism of an MDS-based approach is the perceived high-cost of the assay. However, we believe this technique represents a significant cost-savings compared to the lengthy hospitalizations, diagnostic tests, and empiric therapies frequently associated with cases of idiopathic encephalitis.² For example, during our patient's final hospitalization, we estimate the reimbursement from insurance, which, after adjustments, was only 18% of charges billed, to be over \$140,000. We calculated reimbursement for her antimicrobials alone to total \$8,000, the ICU stay over \$50,000, her laboratory testing over \$14,000, and brain biopsies \$30,000. Meanwhile, we estimate the cost of MDS for a single sample to total approximately \$2,000 for materials and labor. Given current trends in genomic technology development, this cost will likely continue to drop significantly. Furthermore, though turnaround time for MDS in this case was six days, this was largely due to staffing for this experimental protocol. We have already shown that with adequate staffing, this protocol can be completed in under 48 hours.¹ This represents a significant advantage compared to many of the culture-dependent assays mentioned above. With continued decreases in cost and time, and corresponding increases in sequencing depth and advances in sequencing library preparation techniques, it is foreseeable that MDS could be deployed as a frontline diagnostic tool for patients with difficult-to-diagnose infectious syndromes like encephalitis. Early diagnosis through MDS may eventually supplement or replace the numerous and costly diagnostic assays and procedures currently employed and, more importantly, potentially improve patient outcomes. Given that our patient's eye infection preceded her meningoencephalitis, it is tantalizing to envision how early detection of *B. mandrillaris* in the vitreous fluid via MDS may have created a treatment window that could have headed off the devastating and lethal CNS manifestations of her infection.

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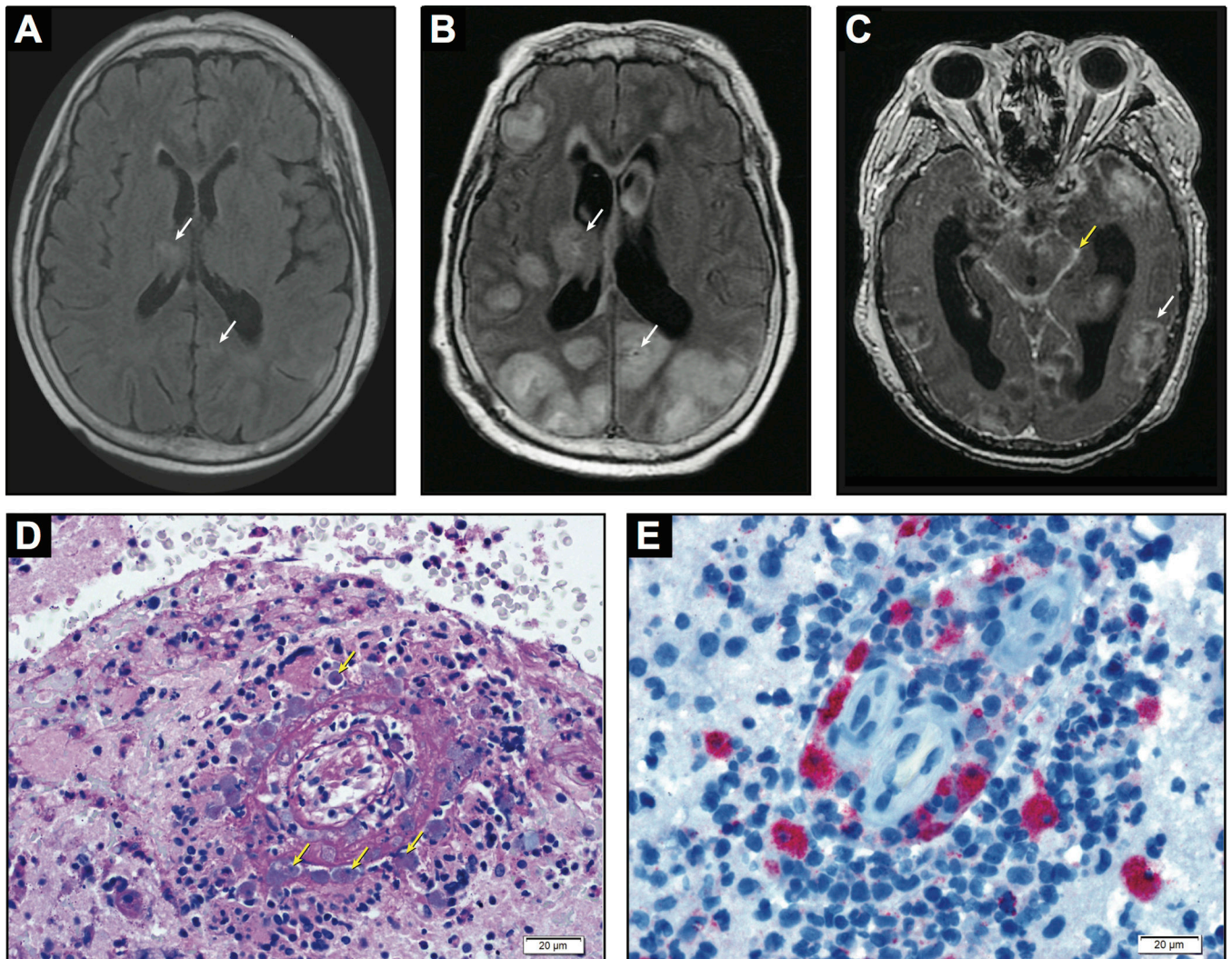


Figure 1. Neuroimaging and Neuropathology

A, Axial T2-weighted / fluid attenuated inversion recovery (FLAIR) brain magnetic resonance imaging (MRI) demonstrating multiple hyperintensities (*arrows*) which correspond to areas of restricted diffusion on diffusion weighted imaging (not shown). **B**, Axial T2-weighted / FLAIR brain MRI demonstrating progression in the size and number of hyperintensities (*arrows*) visualized in the MRI in panel A. **C**, Post-contrast axial T1-weighted brain MRI demonstrating multiple ring enhancing lesions (*white arrow*) and basilar meningitis (*yellow arrow*) as well as severe hydrocephalus. **D**, Periodic acid-Schiff stained section of brain parenchyma shows necrotizing vasculitis, chronic inflammatory cells, and a population of amoebic trophozoites that closely resemble macrophages (*arrows*), 400 \times . The majority of the tissue showed a robust necrotizing vasculitis with mixed inflammation including macrophages and numerous eosinophils. **E**, Immunohistochemistry for free-living amoebas highlights numerous *B. mandrillaris* trophozoites, 400 \times (*courtesy of M.K.K., CDC*).

Table 1

Infectious Disease Diagnostic Test Results.

Test	Site	First Hosp	Second Hosp	Third Hosp	Fourth Hosp	After diagnosis
<i>T. gondii</i> IgG Ab	Serum		Negative	Negative	Negative	
<i>T. gondii</i> IgM Ab	Serum	11.4 IU/mL (<7.9 IU/mL)		Negative		
<i>T. gondii</i> IgM ELISA	Serum		Negative			
(1–3)-Beta-D-glucan	Serum		Negative			
AFB stain and culture	Tracheal aspirate		Negative		Negative (x5)	
<i>T. gondii</i> PCR	Virreous		Negative			
HSV-1, 2 PCR	Virreous		Negative			
CMV PCR	Virreous		Negative			
VZV PCR	Virreous		Negative			
<i>T. gondii</i> PCR	Virreous		Negative			Negative
Fungal culture	Virreous		Negative			
Fungal culture	Blood		Negative (x2)			
Bacterial culture	Blood	Negative (x2)	Negative (4)		Negative (x9)	
HIV-1 Ab	Serum		Negative	Negative		
HIV-1 viral load	Serum					
Hepatitis B surface Ag	Serum				Negative	
Hepatitis B core IgM Ab	Serum				Negative	
Hepatitis C Ab	Serum				Negative	
Gram stain	CSF				Negative (x3)	
VZV PCR and IgG	CSF				Negative	
VDRL	CSF				Negative	
Cryptococcal antigen	CSF				Negative (x2)	
AFB culture	CSF				Negative (x2)	
Bacterial culture	CSF				Negative (x3)	
Fungal culture	CSF				Negative (x2)	
<i>T. gondii</i> PCR	CSF				Negative	

Test	Site	First Hosp	Second Hosp	Third Hosp	Fourth Hosp	After diagnosis
<i>T. gondii</i> IgG Ab	CSF				Negative	
<i>M. tuberculosis</i> PCR	CSF				Negative	
Bacterial culture	Urine	> 10K <i>E. coli</i>	Negative	Negative	Negative (x4); Mixed Gram+ Bacteria (x1)	
Histoplasma Ag	Urine				Negative	
<i>C. immitis</i> Ab	Serum				Negative	
Bacterial culture	Tracheal aspirate				Oral Flora (x2)	
Gram Stain	Tracheal aspirate				Negative (x1); Oral Flora (x1)	
<i>T. gondii</i> PCR	Blood			Negative		
RPR	Serum		Negative		Negative	
Bacterial Culture	Sputum, induced	Negative	Negative	Negative	Negative	
Gram stain	Sputum, induced				Negative	
First Pathology	Brain				Mild gliosis	
Second pathology	Brain				Vasculitis, Encephalitis	
Bacterial culture	Brain				Negative (x2)	
Fungal culture	Brain				Negative (x2)	
AFB culture	Brain				Negative (x2)	
Gram stain	Brain				Negative (x2)	
<i>B. mandrillaris</i> IgG (CA DPH)	Serum					Borderline Positive (1:32)
<i>A. castellanii</i> PCR (CDC)	CSF					Negative
<i>B. mandrillaris</i> PCR (CA DPH)	Brain					Positive
<i>B. mandrillaris</i> PCR (CDC)	Brain					Positive
<i>B. mandrillaris</i> IHC (CA DPH)	Brain					Positive
<i>B. mandrillaris</i> IHC (CDC)	Brain					Positive
<i>B. mandrillaris</i> 18s rRNA PCR (CDC)	CSF					Positive
<i>B. mandrillaris</i> 18s rRNA PCR (DeRisi Lab)	CSF					Positive
<i>B. mandrillaris</i> 18s rRNA PCR (DeRisi Lab)	Vitreous					Positive (raw fluid)
<i>B. mandrillaris</i> 18s rRNA PCR (CDC)	Vitreous					Negative (extracted DNA)

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Abbreviations: Hosp, hospitalization; Ab, antibody; ELISA, enzyme-linked immunosorbent assay; AFB, acid fast bacillus; PCR, polymerase chain reaction; HSV, herpes simplex virus; CMV, cytomegalovirus; VZV, varicella-zoster virus; HIV-1, human immunodeficiency virus; Ag, antigen; VDRL, venereal disease research laboratory; RPR, rapid plasma reagin; CA DPH, California Department of Public Health; CDC, Centers for Disease Control; IHC, immunohistochemistry; rRNA, ribosomal RNA.