Detection of *Balamuthia* Mitochondrial 16S rRNA Gene DNA in Clinical Specimens by PCR

Shigeo Yagi,[1]* Gregory C. Booton,[2] Govinda S. Visvesvara,[3] and Frederick L. Schuster[1]

California Department of Health Services, Viral and Rickettsial Disease Laboratory, 850 Marina Bay Parkway, Richmond, California 94804[1]; Department of Evolution, Ecology and Organismal Biology, The Ohio State University, Columbus, Ohio 43210[2]; and Centers for Disease Control and Prevention, Division of Parasitic Diseases, Atlanta, Georgia 30341[3]

Balamuthia mandrillaris **is a free-living ameba that causes granulomatous amebic encephalitis in both immunocompromised and immunocompetent individuals. Because of a lack of pathognomonic symptoms and the difficulty in recognizing amebas in biopsied tissues, most cases are not diagnosed or effectively treated, leading to a >95% mortality. We report here on five cases of balamuthiasis that were diagnosed by indirect immunofluorescence (IIF) staining of serum for anti-*Balamuthia* antibodies (titer ≥ 1:128) and confirmed by IIF of unstained brain tissue sections and/or detection of amebas in hematoxylin-eosin-stained slides. Additionally, we have used the PCR for the detection of mitochondrial 16S rRNA gene DNA from the ameba in clinical specimens such as brain tissue and cerebrospinal fluid (CSF) from individuals with *Balamuthia* encephalitis. *Balamuthia* DNA was successfully detected by the PCR in clinical samples from all five individuals. It was detected in brain tissue from three cases, in CSF from three cases, and in one of two samples of lung tissue from two individuals, but not in two samples of kidney tissue tested. One sample of unfixed brain tissue was culture positive for *Balamuthia*. In order to test the sensitivity of the PCR for detection of *Balamuthia* DNA, CSF specimens from two individuals negative for amebic infection were spiked with *Balamuthia* amebas. We found that it was possible to detect *Balamuthia* DNA in the PCR mixtures containing mitochondrial DNA from 1 to as little as 0.2 ameba per reaction mixture. A single *Balamuthia* ameba contains multiple mitochondrial targets; thus, 0.2 ameba represents multiple targets for amplification and is not equivalent to 0.2 of an ameba as a target.**

Balamuthia mandrillaris is a free-living soil ameba capable of causing encephalitis in humans and animals (22, 23). Infections are difficult to diagnose in patients because of the absence of a specific pathognomonic profile. For that reason, most diagnoses have been made postmortem based on hematoxylineosin (H&E) or immunofluorescent staining of sectioned brain tissue obtained at the time of autopsy. The amebas, because of their unfamiliar morphology, may be missed in routine histopathologic studies. The delayed diagnosis is a contributing factor to the high mortality resulting from *Balamuthia* encephalitis. More than 100 cases are on record, with only 3 known recoveries following antimicrobial treatment (6, 12). Selection of antimicrobials for treatment relies upon empirical combinations of drugs, and optimal therapy has yet to be formulated.

Over the past several years, the California Encephalitis Project (CEP) has been examining encephalitis cases in the state that have presented diagnostic difficulties (9). *Balamuthia* is one of the causal agents tested for in the program, and several cases of encephalitis have been detected by indirect immunofluorescence (IIF) assay of serum for *Balamuthia* antibodies (19). In addition to serum samples for IIF testing, the program has also received samples of unfixed brain tissue for attempted isolation of amebas, cerebrospinal fluid (CSF), and

H&E-stained and unstained sections for identification of amebas in brain and other tissues. We report here on positive identification of balamuthiasis in five patients, based on serum samples that were identified as positive for *Balamuthia* antibodies by IIF staining.

About six different *Balamuthia* clinical isolates are now in culture, and these have been studied for sequence variation of their mitochondrial small-subunit 16S rRNA gene DNA using the PCR (3, 4). Booton et al. (3) have shown that all *Balamuthia* strains isolated are members of a single species, with sequence variation ranging from 0 to 1.8% among isolates.

Because of the difficulties in diagnosing *Balamuthia* encephalitis by conventional techniques, we have explored ways of detecting the presence of *Balamuthia* DNA in clinical samples, such as unfixed tissues and CSF, using the PCR. In this report we present the results of application of PCR methodology to detection of *Balamuthia* DNA in clinical specimens. Our goal is to develop a test protocol for identification of the ameba in brain tissue obtained by pre- or postmortem biopsies, and in CSF samples from patients.

MATERIALS AND METHODS

Clinical specimens. Acute and convalescent (or early and late stage) serum samples from patients hospitalized with encephalitis were submitted to the CEP by physicians from throughout the state of California, along with a case history of the patient and pertinent medical data. One sample included in this report was submitted from Texas. As part of the CEP, serum samples were subjected to a battery of ~15 tests for viral, bacterial, or protozoal etiologic agents (9). Of the five antibody-positive *Balamuthia* samples included in this report three samples

---

* Corresponding author. Mailing address: California Department of Health Services, Viral and Rickettsial Disease Laboratory, 850 Marina Bay Parkway, Richmond, CA 94804. Phone: (510) 307-8619. Fax: (510) 307-8599. E-mail: syagi@dhs.ca.gov.

were received as part of the CEP and two samples were submitted for testing but not as part of the CEP.

Criteria for serum selection for *Balamuthia* testing. The case definition of encephalitis for specimens submitted to the CEP is encephalopathy and one or more of the following: fever, seizures, focal neurological findings, CSF pleocytosis, electroencephalographic neuroimaging findings consistent with encephalitis (9). Severely immunocompromised patients, patients with human immunodeficiency virus infection/AIDS, and patients ≤6 months of age were excluded from the CEP.

From a total of ~1,200 serum samples submitted to the CEP, ca. 250 (~20%) were selected for testing for *Balamuthia* antibodies. The criteria used in deciding which of these serum samples would be tested were (i) clinical or laboratory findings compatible with a diagnosis of *Balamuthia* encephalitis (elevated CSF protein and leukocyte counts, space-occupying lesions seen upon neuroimaging, hydrocephalus) and (ii) recreational activities (camping, swimming, or situations in which individuals were exposed to blowing soil) or occupational activities (farming or construction work) that would expose individuals to soil or water containing potentially pathogenic amebas.

Brain tissue. Unfixed brain tissue was available in three cases at the time of biopsy or autopsy and was used for attempted isolation and cultivation of *Balamuthia* amebas. Formalin-fixed brain tissue was available as H&E-stained and unstained tissue sections for all patients (Table 1). In addition to brain tissue, lung and kidney tissues were available from two cases as unstained sections for IIF (Table 1). For attempted ameba isolation, necrotic areas of tissue were macerated and the particles introduced into cultures of E6 monkey kidney cells growing in RPMI 1640 medium with 10% fetal calf serum and penicillin-streptomycin (200 U/ml each) at 37°C for outgrowth of amebas. For PCR of brain tissue, total DNA was extracted and *Balamuthia*-specific PCR targeting mitochondrial 16S rRNA gene DNA was performed.

CSF samples. Samples of CSF were submitted in cases in which a lumbar puncture was done. A total of 15 to 20 CSF samples were tested, 3 from patients diagnosed with *Balamuthia* infection (Table 1) and the remainder from patients subsequently diagnosed with other maladies (coccidioidomycosis, enteroviral or *Chlamydia* infections, tuberculosis) or never diagnosed. As for brain tissue, samples of CSF were inoculated into E6 cultures for attempted isolation of amebas. CSF samples were tested for *Balamuthia* 16S rRNA gene DNA by the PCR technique. CSF to be used for PCR was centrifuged at 1,500 × g to pellet suspended material, which was then subjected to DNA extraction and PCR testing.

Ameba DNA. When *Balamuthia* DNA was needed as a control, log phase amebas (strains V188 and V194 [Centers for Disease Control and Prevention], isolated from human biopsied brain tissue, from Georgia and Nevada, respectively) were grown axenically in BM3 medium at 33 or 37°C (20). Amebas were harvested and washed in phosphate-buffered saline (PBS) and the samples counted using a Coulter Counter (model Z$_1$).

Spiking of CSF samples. For experiments in which amebas were added to CSF, they were harvested, counted, and diluted with PBS to give the desired numbers of amebas for each sample (from ~0.05 to 100 amebas/1-μl aliquot) prior to DNA extraction. Initial counts were done with the Coulter Counter but, in cases where ameba numbers were ≤10, visual counts under a microscope were done using replicate 1-μl drops of ameba suspension (the same amount of ameba suspension added to tubes for DNA extraction) to verify the Coulter Counter and serial dilution numbers.

IIF. Sera (ca. 250 samples) from patients were tested by IIF staining for *Balamuthia* and *Acanthamoeba* antibodies as previously described (19, 20). Positive and negative controls were run along with serum samples. Patient serum samples giving elevated titers (≥1:128) were considered as positive for both *Balamuthia* and *Acanthamoeba* based upon serum samples from individuals with amebic encephalitides (G. S. Visvesvara, unpublished observations). Negative controls made use of serum samples from asymptomatic individuals in good health. Negative titers ranged from 0 (negative for ameba antibodies) to 1:32.

Positive determinations based on IIF staining of sera were corroborated with IIF staining of unstained sections of brain tissue and examination of H&E-stained brain sections for presence of amebas. Patient serum samples were also tested for *Acanthamoeba* antibodies to rule out possible cross-reactivity between the two amebas (2).

DNA extraction. DNA was extracted for amplification and sequencing from pellets of harvested amebas, sedimented CSF material, unfixed brain tissue samples, or formalin-fixed sectioned material (brain, lung, and kidney) scraped off of slides by adding lysis buffer (5) to the amebas or tissue samples, vortexing the suspension, and leaving it at room temperature for 10 min. Following addition of isopropanol to precipitate nucleic acid, the tubes were again vortexed and then centrifuged for 10 min at 10,000 × g. The supernatant was aspirated, and

TABLE 1. Clinical samples from *Balamuthia* cases: IIF and culture results and materials for PCR

Case no.	Sex/age (yr)	Location[a]	Titer[b] for: Balamuthia	Titer[b] for: Acanthamoeba	Availability[d] of: Unfixed tissue	Availability[d] of: CSF	Results[e] for: Culture attempts	Results[e] for: H&E slides	Results[e] for: Slides for IIF[c]	Results[e] for: Specimens for PCR
1	Male/64	San Diego Co., CA	128	16/32	Yes (brain)	NA	Brain, (−)	(+)	Brain, (+); kidney, (−); lung, (−)	Brain, (+); kidney, (−); lung, (−)
2[f]	Male/2.5	Bexar Co., TX	256	32	NA	Yes	CSF, (−)	(+)	Brain, (+)	CSF (+)
3[f]	Male/7	Orange Co., CA	256	2	NA	Yes	CSF, (−)	(+)	Brain, (+)	CSF (+)
4	Male/7	Santa Clara Co., CA	256	32	Yes (brain)	NA	Brain, (−)	(+)	Brain, (+); kidney, (−); lung, (−)	Brain, (+); kidney, (−); lung, (+)
5	Female/3	Sonoma Co., CA	128/256	32	Yes (brain)	Yes	Brain, (+); CSF, (−)	(+)	Brain, (+)	Brain, (+); CSF, (+)

[a] County and state from which specimen originated.
[b] Antibody levels expressed as reciprocal titers. Threshold for positive titers, 1:128.
[c] Unstained slides with tissue sections as noted.
[d] NA, not available; yes, available.
[e] (−), unsuccessful isolation and/or detection of amebas or ameba DNA; (+), successful isolation and/or detection of amebas.
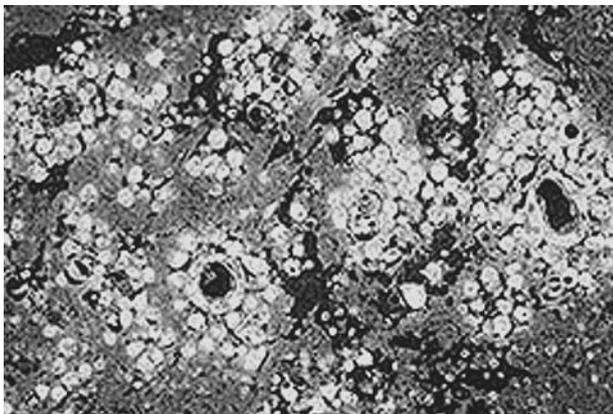[f] Not included in the CEP.

FIG. 1. Indirect immunofluorescence-stained section of necrotic brain tissue from patient 4 (Table 1). The dark circles seen in the section are the lumens of blood vessels; the bright-staining profiles surrounding the vessels and scattered throughout the tissue are *Balamuthia* amebas. In all the types of amebic encephalitides, the amebas are typically found in large numbers in the perivascular regions of the brain.

the pellet was washed with 0.75 ml of 70% ethyl alcohol, vortexed, and centrifuged. Alcohol was removed and the tubes placed in a heating block at 65°C for 10 min to evaporate the remaining alcohol, after which tubes were cooled to room temperature and stored frozen. The basic procedures employed in this and the following sections are described in Sambrook et al. (17).

DNA amplification and sequencing. PCR amplification was done with a primer set consisting of 5′Balspec16S (5′-CGCATGTATGAAGAAGACCA-3′) and 3′ Balspec 16S (5′-TTACCTATATAATTGTCGATACCA-3′) (4), which amplifies an ~1,075-bp portion of the mitochondrial 16S rRNA gene from *B. mandrillaris*. The PCR product (40 µl) was run on 1% agarose gel and purified with a Prep-A-Gene purification kit (Bio-Rad Laboratories, Hercules, Calif.). The concentration of gel-purified DNA was determined with the Low Mass DNA ladder (Invitrogen, Carlsbad, Calif.). The final elution volume was 50 µl (4).

Amplified PCR products were sequenced with the internal primer mt900 (5′-CAAATTAAACCACATACT-3′), which determines the primary sequence of the 5′ region of this amplicon (15).
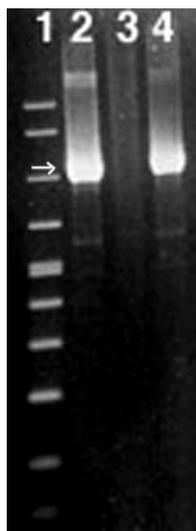

FIG. 2. PCR gel showing bands at ~1,075 bp for *Balamuthia* mitochondrial DNA. Lane 1, marker; lane 2, DNA isolated from brain tissue of *Balamuthia* patient 4; lane 3, negative control (water); lane 4, positive control (using DNA extracted from cultured *Balamuthia*). The arrow in lane 1 (marker) indicates ~1,075 bp.

RESULTS

Clinical samples. All materials used in testing for *Balamuthia* were from patients who were hospitalized with a diagnosis of encephalitis. A case history of the patient was submitted to the California Encephalitis Project (Department of Health Services) containing information on the patient (blood and CSF analyses, neuroimaging data, patient history, etc.) along with the clinical specimens. As part of the CEP, ca. 250 serum samples out of a total of ~1,200 that were submitted were tested for antibodies to *Balamuthia* by IIF, and 3 of the serum samples were found to be positive (titers of ≥1:128) for *Balamuthia* antibodies (Table 1). Two additional samples submitted for testing that were not part of the CEP, one of which was from out of state (Texas), were found to be positive for *Balamuthia* antibodies (titers of ≥1:128). The serum samples were also tested for antibodies to *Acanthamoeba*; all were negative or had low titers (ranging from no antibody response to a titer of 1:32) (Table 1). Thus, no cross-reactivity between the two genera was apparent. Formalin-fixed and paraffin-embedded tissue sections from cases that gave positive IIF titers (≥128) were tested by tissue immunofluorescence (Fig. 1) and/or examination of H&E sections to corroborate the serological diagnoses.

Brain tissue. Unfixed brain tissue, resected at autopsy, was available from three patients (Table 1). *Balamuthia* amebas were isolated from one of these samples (case 5 in Table 1) and were grown in culture (19). Repeated attempts to isolate amebas from the two other samples of brain tissue (cases 1 and 4 in Table 1) were unsuccessful.

DNA from brain tissue was extracted, amplified, and run on agarose gels. A band at ~1,075-bp for *Balamuthia* 16S rRNA gene DNA (Fig. 2) was consistent with earlier results obtained by Booton et al. (4). The bands were sequenced, and the sequences were found to be identical to those described for other cultured *Balamuthia* strains isolated from brain tissue samples (4). In one sample (case 1 in Table 1), tissue was obtained not only from necrotic areas of the brain but also from adjacent, apparently "normal" areas. The necrotic tissue gave the typical ~1,075-bp band, while the adjacent areas of the brain did not (data not included).

PCR of other tissues. DNA was extracted from unstained tissue sections that were deparaffinized and scraped from slides. These included brain, kidney, and lung tissues, the latter two from patients 1 and 4 (Table 1). All brain tissues from slides produced a band at ~1,075 bp consistent with *Balamuthia* DNA. The two kidney tissues tested negative by both PCR and by IIF. One of the lung tissues tested (patient 4) was positive for *Balamuthia* DNA by PCR. However, IIF staining of this lung tissue produced no evidence of *Balamuthia* amebas (data not included).

CSF. Initial PCR testing of CSF involved removing a sample of supernatant fluid from an agitated tube of CSF. These samples, after DNA extraction, produced either a faint band or no band at all. In subsequent testing, the CSF was centrifuged to pellet suspended debris. DNA was extracted from pelleted material and amplified. Centrifuged CSF samples produced a band at ~1,075 bp or, in some cases, multiple bands (Fig. 3). The multiple bands probably represent DNA released from leukocytes or from necrotic brain tissue present in the samples.
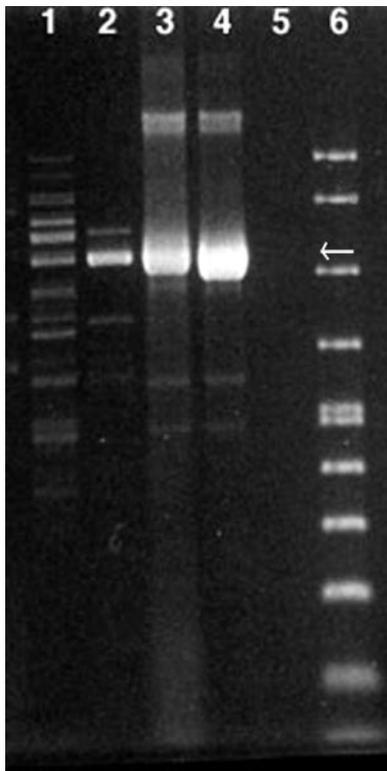
FIG. 3. Agarose gel showing banding at ~1,075 bp for *Balamuthia* DNA in CSF samples. Lane 1, CSF from patient 2 (Table 1); lane 2, CSF from patient 3; lane 3, positive control, using DNA extracted from cultured *Balamuthia*; lane 4, same as lane 3 but at 1/10 concentration; lane 5, negative control (water); lane 6, marker. The arrow in lane 6 (marker) indicates ~1,075 bp.
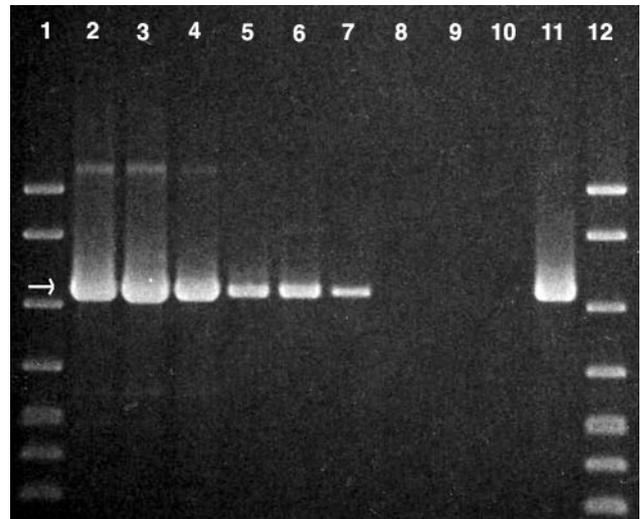

FIG. 4. Agarose gel showing CSF samples spiked with *Balamuthia* DNA. Lanes 1 and 12, markers; lane 2, CSF with DNA extracted from 100 amebas; lane 3, CSF with DNA from 50 amebas; lane 4, CSF with DNA from 12 amebas; lane 5, CSF with DNA from 3 amebas; lane 6, CSF with DNA from 0.75 ameba; lane 7, CSF with DNA from 0.2 ameba; lane 8, DNA from 0.05 ameba; lane 9, Hanks' basal salt solution; lane 10, negative control (water); lane 11, positive control (DNA from *Balamuthia* harvested from culture). Arrow in lane 1 (marker) indicates ~1,075 bp.

Ameba isolation. Attempts to isolate *Balamuthia* amebas from CSF samples were unsuccessful, even in the case of patient 5, from whose brain tissue *Balamuthia* was isolated.

Spiked samples. In several experiments, *Balamuthia* amebas grown in culture were added to samples of CSF obtained from *Balamuthia*-negative patients, based on serology or an alternative diagnosis. Of the CSF samples from *Balamuthia*-negative patients used in this study, one was submitted as a suspected *Balamuthia* encephalitis case (later diagnosed as coccidioidomycosis) and the other as a suspected West Nile virus encephalitis case with no indication of amebic encephalitis. Upon testing, these CSF samples produced no band consistent with *Balamuthia* DNA (at ~1,075 bp) on gels (data not included). In the sample illustrated, amebas in suspension were added to CSF aliquots to give, on average, final numbers of 100, 50, 12, 3, 1, 0.75, 0.2, and ~0.05 amebas per PCR mixture. All spiked samples, except the one for 0.05 ameba, gave a band at ~1,075 bp (Fig. 4). We note that there is far more than one mitochondrial target per *Balamuthia* and that "0.2 ameba" represents multiple mitochondrial targets.

DISCUSSION

In addition to disease caused by *Balamuthia*, amebic encephalitides are caused by three other species of free-living amebas: *Acanthamoeba* spp., *Naegleria fowleri*, and *Sappinia diploidea*

(21). Our survey of sera from encephalitis patients focused on *Balamuthia* as the etiologic agent but also took into account the possibility of infections caused by these other species of amebas. The CEP case definition for encephalitis excluded severely immunocompromised individuals, which includes most patients with systemic forms of acanthamoebiasis (9). It was possible, however, that by focusing on *Balamuthia*, we might have been missing cases of *Acanthamoeba* encephalitis. Thus, the testing algorithm was modified to include IIF testing for *Acanthamoeba* antibodies. No case of acanthamoebiasis was detected based on serology or IIF staining of unstained tissue sections.

Naegleria meningoencephalitis is a fulminant disease, chiefly in children or young adults, resulting in death within days following infection with amebas. It is frequently associated with a history of swimming or immersion in fresh waters, particularly those with elevated temperature conducive to the growth of *N. fowleri* (21). Its clinical course is significantly different from that of *Balamuthia* encephalitis and is typically diagnosed by finding trophic *Naegleria* amebas in the CSF. Because of its rapid onset, little or no patient antibody response is generated. Clinically, none of our cases matched the typical picture of *Naegleria* meningoencephalitis. In 10 encephalitis cases, however, there was a recent history of activity in freshwater, prompting testing for *N. fowleri* antibody; none, however, was detected. *Sappinia diploidea* as an agent of amebic encephalitis was reported from a single case (8), but, aside from histopathological demonstration of the amebas in tissue, there is no serologic test for its presence.

Balamuthia encephalitis is difficult to diagnose, in part because of the absence of unique and readily recognizable symptomatology and in part because of the lack of familiarity with

amebas in histopathologic sections. Premortem diagnoses have been made only when biopsied brain tissue was available or when patient serum had been tested for *Balamuthia* antibody by IIF (1, 6, 12). *Balamuthia* amebas were also seen in a biopsied skin nodule that was obtained premortem and later confirmed postmortem in a brain tissue smear (24). The symptoms recorded from patients with *Balamuthia* encephalitis mimic neurotuberculosis, tuberculoma, or neurocysticercosis. Neuroimaging is helpful in diagnosis by detecting space-occupying lesions, but only in the context of other supporting data (10, 13, 16). Premortem diagnoses of balamuthiasis are infrequent, and most diagnoses are made postmortem. There have been few known survivors of *Balamuthia* encephalitis, either due to extensive damage done by the amebas during the prodromal period or the use of inappropriate antimicrobial therapy (e.g., antibacterial or antiviral drugs). Even in instances where premortem diagnosis had been made, the patients were generally in an advanced state of the disease (1).

In the five cases that are referred to in this paper (Table 1), four were children <10 years of age and 1 an adult >60 years old. Four of the patients were males, and one was female. Two of the patients could be described as being immunocompromised: one was being treated for apparent kidney disease with steroids (patient 4) and a second was reportedly an alcoholic (patient 1). All of the individuals died within a period of 6 weeks following the initial hospitalization for encephalitis. Presumably there was an incubation period during which time the patients were asymptomatic. In only one case (patient 5) was it possible to pinpoint a probable source of infection (12). Four (patients 2 to 5) of the patients were diagnosed with *Balamuthia* amebic encephalitis (BAE) premortem, but at a terminal stage of the disease.

Because BAE is a chronic disease taking as long as 2 years to develop, there is ample time for generation of a humoral antibody response to the presence of the amebas. Testing for *Balamuthia* antibody in patient sera has been used as a diagnostic tool for identification of BAE (22, 23). In the CEP, ~20% of serum samples (~250 out of ≥ 1,200 samples) from patients with encephalitis were tested for *Balamuthia* antibodies by immunofluorescence assay. Three cases of BAE were diagnosed as part of the CEP in the course of the study, and two additional cases, not included in the study, were also detected, from IIF either of serum samples or of brain tissue (18). The availability of a test that could be carried out with a minimally invasive procedure (e.g., lumbar puncture versus brain biopsy) would make it easier to diagnose or confirm positive serology for BAE.

Samples of brain tissue that were tested for *Balamuthia* mitochondrial 16S rRNA gene DNA gave bands at ~1,075 bp. In one case where tissues from necrotic and nonnecrotic areas of the brain were available for comparison (right versus left temporal lobes), the former tested positive while the latter was negative. This suggests that, at least in this case, the amebas are restricted in brain tissue to the lesions and do not disseminate throughout the brain parenchyma.

In case 4, unstained lung and kidney tissue sections were stained by IIF and were negative. By PCR kidney tissue was negative, but lung tissue indicated the presence of *Balamuthia* DNA, suggesting that the respiratory tract was a portal of entry (21). This particular patient was being treated for a possible

kidney disease, diagnosed several months before he was hospitalized and before the *Balamuthia* infection was detected by IIF of serum. CSF, available from three of the five patients, was positive for *Balamuthia* DNA by PCR. *Balamuthia* DNA in the CSF was perhaps a result of extensive necrosis of brain tissue and lysis of amebas. The one case (patient 5) from which *Balamuthia* was isolated was from freshly autopsied, unfrozen brain tissue. In other cases in this series in which brain tissue was available, it had been frozen, and likely resulted in destruction of trophic amebas. Likewise, the CSF samples from which ameba isolation had been attempted had been refrigerated or frozen for periods of hours to days, even before arrival at the laboratory (California Health Services). Although it is generally believed that *Balamuthia* is not found in CSF, Jayasekera et al. (11) reported isolation of *Balamuthia* from CSF of a recent case in the United Kingdom.

PCR is a useful diagnostic tool for identifying suspected cases of balamuthiasis. The technique is sufficiently sensitive to detect mitochondrial DNA extracted from less than a single ameba (lane 7, Fig. 4). It has potential for giving relatively rapid results, allowing for early initiation of antimicrobial therapy. It requires, however, a high degree of suspicion on the part of the physician to request testing for BAE. In the CEP and other studies, indications of BAE included elevated levels of CSF protein and leukocytes, normal or decreased glucose, and hydrocephaly (1, 7, 16). Several children who developed BAE had earlier bouts of otitis media (1, 14). These signs and symptoms are generally not evident until the patient has been hospitalized for encephalitis, and by then even premortem diagnosis may be too late to effect a recovery. In three cases where premortem diagnoses were made, therapy with combinations of antimicrobials was started and the patients recovered with various sequelae (6, 12).

All of the balamuthiasis cases discussed here were in persons of Hispanic-American ethnicity. Hispanic-Americans also account for ~50% of BAE cases that have occurred in the United States, though Hispanics comprise ~13% of the population (18). In our survey of *Balamuthia* encephalitis cases in California, where Hispanics make up 32% of the state's population, sera from Hispanic-Americans (about 25% of ~1,200 CEP samples) comprised ~26% of the samples examined for *Balamuthia* antibodies but 100% of detected cases of BAE. This may reflect occupational (connection with agriculture or other work involving contact with soil), genetic (predisposition due to genetic constitution), or socioeconomic (access to medical care, pressures associated with unfamiliar life styles for recent immigrants) factors or factors yet to be defined. This association requires further study.

REFERENCES

1. **Bakardjiev, A., P. H. Azimi, N. Ashouri, D. P. Ascher, D. Janner, F. L. Schuster, G. S. Visvesvara, and C. Glaser.** 2003. Amebic encephalitis caused

by *Balamuthia mandrillaris*: report of four cases. Pediatr. Infect. Dis. **22:**447–452.

2. **Bloch, K., and F. L. Schuster.** 2005. Inability to identify *Acanthamoeba* infection premortem in a patient with fatal granulomatous amebic encephalitis. J. Clin. Microbiol. **43:**3003–3006.

3. **Booton, G. C., J. R. Carmichael, G. S. Visvesvara, T. J. Byers, and P. A. Fuerst.** 2003. Genotyping of *Balamuthia mandrillaris* based on nuclear 18S and mitochondrial 16S rRNA genes. Am. J. Trop. Med. Hyg. **68:**65–69.

4. **Booton, G. C., J. R. Carmichael, G. S. Visvesvara, T. J. Byers, and P. A. Fuerst.** 2003. Identification of *Balamuthia mandrillaris* by PCR assay using the mitochondrial 16S rRNA gene as a target. J. Clin. Microbiol. **41:**453–455.

5. **Casas, I., L. Powell, P. E. Klapper, and G. M. Cleator.** 1995. New method for the extraction of viral RNA and DNA from cerebrospinal fluid for use in the polymerase chain reaction assay. J. Virol. Methods **53:**25–36.

6. **Deetz, T. R., M. H. Sawyer, G. Billman, F. L. Schuster, and G. S. Visvesvara.** 2003. Successful treatment of *Balamuthia* amoebic encephalitis: presentation of two cases. Clin. Infect. Dis. **37:**304–1312.

7. **Duke B. J., R. W. Tysson, R. DeBiasi, J. E. Freeman, and K. R. Winston.** 1997. *Balamuthia mandrillaris* meningoencephalitis presenting with acute hydrocephalus. Pediatr. Neurosurg. **26:**107–111.

8. **Gelman, B. B., V. Popov, G. Chaljub, R. Nader, S. J. Rauf, H. W. Nauta, and G. S. Visvesvara.** 2003. Neuropathological and ultrastructural features of amebic encephalitis caused by *Sappinia diploidea*. J. Neuropathol. Exp. Neurol. **62:**990–998.

9. **Glaser, C. A., S. Gilliam, D. Schnurr, B. Forghani, S. Honarmand, N. Khetsuriani, M. Fischer, C. K. Cossen, and L. J. Anderson.** 2003. In search of encephalitis etiologies: diagnostic challenges in the California Encephalitis Project, 1998–2000. Clin. Infect. Dis. **36:**731–742.

10. **Healy, I. F.** 2002. *Balamuthia* amebic encephalitis: radiographic and pathologic findings. Am. J. Neuroradiol. **23:**486–489.

11. **Jayasekera, S., J. Sissons, J. Tucker, C. Rogers, D. Nolder, D. Warhurst, S. Alsam, J. M. White, E. M. Higgins, and N. A. Khan.** 2004. Post-mortem culture of *Balamuthia mandrillaris* from the brain and cerebrospinal fluid of a case of granulomatous amoebic meningoencephalitis, using human brain microvascular endothelial cells. J. Med. Microbiol. **53:**1007–1012.

12. **Jung, S., V. X. Schelper, G. S. Visvesvara, and H. T. Chang.** 2004. *Balamuthia mandrillaris* meningoencephalitis in an immunocompetent patient. Arch. Pathol. Lab. Med. **128:**466–468.

13. **Kidney, D. D., and S. H. Kim.** 1998. CNS infections with free-living amebas: neuroimaging findings. Am. J. Roentgenol. **171:**809.

14. **Kodet, R., E. Nohynková, M, Tichy, J. Soukup, and G. S. Visvesvara.** 1998. Amebic encephalitis caused by *Balamuthia mandrillaris* in a Czech child: description of the first case from Europe. Pathol. Res. Pract. **194:**423–430.

15. **Leedee, D. R., G. C. Booton, M. H. Awwad, S. Sharma, R. K. Aggarwal, I. A. Niszl, M. B. Marcus, P. A. Fuerst, and T. J. Byers.** 2002. Advantages of using mitochondrial 16S rDNA sequences to classify clinical isolates of *Acanthamoeba*. Investig. Ophthalmol. Sci. **44:**1142–1149.

16. **Rowen, J. L., C. A. Doerr, H. Vogel, and C. J. Baker.** 1998. *Balamuthia mandrillaris*: a newly recognized agent for amebic meningoencephalitis. Pediatr. Infect. Dis. J. **14:**705–710.

17. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

18. **Schuster, F. L., C. Glaser, S. Honarmand, J. H. Maquire, and G. S. Visvesvara.** 2004. *Balamuthia* amebic encephalitis risk, Hispanic Americans. Emerg. Infect. Dis. **10:**1510–1512.

19. **Schuster, F. L., C. Glaser, S. Honarmand, and G. S.Visvesvara.** 2003. Testing for *Balamuthia* amebic encephalitis by indirect immunofluorescence, p. 173–178. *In* F. Lares-Villa, G. C. Booton, and F. Marciano-Cabral (ed.), Proceedings of the Xth International Meeting on the Biology and Pathogencity of Free-Living Amoebae. ITSON DIEP, Ciudad Obregón, Mexico.

20. **Schuster, F. L., and G. S. Visvesvara.** 1996. Axenic growth and drug sensitivity studies of *Balamuthia mandrillaris*, an agent of amebic meningoencephalitis in humans and other animals. J. Clin. Microbiol. **34:**385–388.

21. **Schuster, F. L., and G. S. Visvesvara.** 2004. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. Int. J. Parasiol. **34:**1001–1027.

22. **Visvesvara, G. S., A. J. Martinez, F. L. Schuster, G. J. Leitch, S. V. Wallace, T. K. Sawyer, and M. Anderson.** 1990. Leptomyxid ameba, a new agent of amebic meningoencephalitis in humans and animals. J. Clin. Microbiol. **28:**2750–2756.

23. **Visvesvara, G. S., F. L. Schuster, and A. J. Martinez.** 1993. *Balamuthia mandrillaris*, N. G., N. Sp., agent of amebic meningoencephalitis in humans and other animals. J. Eukukarot. Microbiol. **40:**504–514.

24. **White, J. M. L., R. D. Barker, J. R. Salisbury, A. J. Fife, S. B. Lucas, D. C. Warhurst, and E. M. Higgins.** 2004. Granulomatous amoebic encephalitis. Lancet **364:**220.