Detection of Antibodies against Free-Living Amoebae 
*Balamuthia mandrillaris* and *Acanthamoeba* Species in a Population of Patients with Encephalitis

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**Background.** *Balamuthia mandrillaris* and *Acanthamoeba* species are 2 free-living amoebae responsible for granulomatous amoebic encephalitis in humans and animals. We have screened serum samples from hospitalized patients with encephalitis for antibodies against these 2 amoebae as a means of detecting a disease with few defining symptoms and a poor prognosis.

**Methods.** Indirect immunofluorescence antibody (IFA) staining of serum samples from patients with encephalitis was conducted over a period of 6 years to detect amoeba antibodies. More than 250 serum samples from patients hospitalized with encephalitis were screened. Most of the samples were from patients in California and were screened as part of the California Encephalitis Project, with a small number of specimens from other states.

**Results.** During the course of the study, 7 cases of *Balamuthia* encephalitis were detected; all cases were detected in Hispanic individuals, and all cases were fatal. Examination of hematoxylin-eosin–stained and immunostained sections of brain tissue obtained at biopsy or autopsy for amoebae confirmed balamuthiasis in all serum samples with positive IFA results. One case of *Acanthamoeba* encephalitis was detected in an immunocompromised individual with a normal antibody titer by identification of amoebae in immunostained brain tissue obtained at autopsy.

**Conclusions.** IFA can be successfully used in screening for balamuthiasis and acanthamoebiasis in patients whose clinical presentation, laboratory results, and neuroimaging findings are suggestive of amoebic encephalitis. Ideally, this can lead to an earlier definitive diagnosis and earlier start of antimicrobial therapy. Without IFA staining, the balamuthiasis cases in our study would have been diagnosed as neurocysticercosis, tumor, tuberculosis, or viral encephalitis or would have been undiagnosed.

*Balamuthia mandrillaris* and *Acanthamoeba* species are free-living amoebae with a pathogenic potential that cause granulomatous amoebic encephalitis in humans. Infections due to *B. mandrillaris* have been diagnosed in immunocompetent and immunocompromised individuals, whereas infections due to *Acanthamoeba* species occur almost exclusively in immunocompromised individuals. The infections may be cutaneous, nasopharyngeal, or systemic, or they may focus in the CNS.

Both infections are usually chronic and, in some instances, take as long as 2 years to develop. The extended prodromal period allows for a strong humoral response to develop, which is detectable by immunofluorescent antibody (IFA) staining. *Acanthamoeba* species are also responsible for amoebic keratitis, usually associated with contact lens wear or corneal trauma [1].

*Naegleria fowleri*, another free-living amoeba, causes primary amoebic meningoencephalitis. Infections due to *Naegleria* species occur in immunocompetent individuals, mainly children and young adults [1]. Following exposure, usually as a consequence of swimming, bathing, or playing in warm freshwater containing the amoebae, onset of disease is rapid and fatal within several days after infection. The rapid onset leaves little time for the host's immune system to mobilize an effective response, and antibody titers are low.

These amoebae are widely disseminated in soil and
water. *Acanthamoeba* species, in particular, can be readily isolated from the home environment in drains, water taps, home aquaria, humidifiers, and flowerpot soil. Thus, it is not surprising that humans are exposed to them, as indicated by the presence of antiamoeba antibodies in serum. Antibody titers have been demonstrated for *Naegleria* [2-4], *Acanthamoeba* [2, 5], and *Balamuthia* [6, 7] species in surveys of healthy humans. Similarly, amoeba antibodies have been demonstrated in domestic [8] and wild animals [9, 10]. It is not known if these antibodies afford protection against infection with these amoebae.

The presence of elevated antibody titers against these amoebae, as shown by IFA staining, has been used as a diagnostic means of detecting infection, as has indirect immunofluorescent (IIF) staining for amoebae in tissue sections [11, 12]. In this study, we tested serum samples from patients with encephalitis for antibodies to *Balamuthia, Acanthamoeba*, and, infrequently, *Naegleria* species by IFA staining to determine if any of these amoebas were the etiologic agents of the encephalitis. The amoeba antibody study is a part of the California Encephalitis Project (CEP), whose goal is to diagnose challenging encephalitis cases by testing acute-phase and convalescent-phase serum samples and other clinical samples (e.g., CSF samples, stool specimens, and nasal swabs) for a large number of infectious agents [13].

**MATERIALS AND METHODS**

**CEP.** Physicians throughout the state submitted clinical specimens from patients with encephalitis to the CEP. The CEP also received specimens from outside of California in special circumstances. Along with the clinical materials, a standardized case history form was completed with the patient’s sex, age, ethnicity, occupation, travel history, contact with animals, and medical background (e.g., blood and CSF profiles, neuroimaging findings, and medication). The criteria for inclusion in the CEP are hospitalization with encephalopathy and ≥1 of the following: fever, seizure(s), focal neurologic findings, CSF pleocytosis, and abnormal findings of electroencephalography or neuroimaging studies [13]. Severely immunocompromised patients (including patients with HIV infection or AIDS) are excluded from the project, as are infants <6 months of age. At the present time, >1800 cases have been submitted for testing. Each sample underwent a battery of laboratory tests for viral, bacterial, and protozoal agents.

**Amoeba antibody survey.** Of the samples that were submitted to the CEP, some were chosen for testing for antibodies to *Balamuthia, Acanthamoeba*, and *Naegleria* species. This represented ~20% of all samples submitted (~290 samples). Criteria for selection for amoeba antibody testing included occupation (e.g., farmer, construction worker, and other occupations that involve contact with the soil), recreational activity (e.g., swimming or camping), and clinical features (e.g., elevated CSF protein level and WBC count, hydrocephalus, ring-enhancing lesions, or a space-occupying mass visible on MRI).

*Balamuthia*-positive control samples consisted of serum samples obtained from previous patients with infection due to *B. mandrillaris*, which routinely had titers of 1:256. No positive control sample was available for testing for *Acanthamoeba* species. Negative control samples for *Balamuthia* and *Acanthamoeba* species consisted of serum samples obtained from healthy individuals (i.e., serum samples collected at the Department of Health Services for veterinarians for rabies antibody testing and an additional asymptomatic source). These titers ranged from 1:2 to 1:32 for both *Balamuthia* and *Acanthamoeba* species, a range that was defined as normal.

**IFA and IIF.** Acute-phase and (when available) convalescent-phase serum samples were used for determination of antibody titers. A mix of 3 clinical isolates of *B. mandrillaris* or *Acanthamoeba castellanii* were fixed in 1% formalin and dried on 12-well teflon-coated slides. An aliquot of patient serum (50 μL) was diluted in saline from 1:2 to 1:4096, and the diluted serum samples were added to each of the wells. Following incubation at 37°C, the slides were washed, stained with goat anti-human fluorescein isothiocyanate conjugate (FITC) and, after further washing, were mounted and viewed with a fluorescence microscope. The intensity of fluorescence was scored as +1 to +4. For IIF staining of tissue sections on slides, anti-*Balamuthia* or anti-*Acanthamoeba* serum samples from rabbits were added to deparaffinized tissue, followed by goat anti-rabbit FITC, and, after further washing, were mounted and viewed with a fluorescence microscope, as for IFA staining [14].

**RESULTS**

Table 1 provides basic information about the patient population whose serum samples were tested for amoeba antibodies and compares that information with data for the entire group of CEP patients. A more extensive profile of the entire CEP patient population can be found in Glaser et al. [13].

At the start of the amoeba project, serum samples were tested solely for antibodies to *B. mandrillaris*. Because the criteria for inclusion in the CEP study ruled out severely immunocompromised individuals, antibodies against *Acanthamoeba* species, which infect mainly individuals with impaired immune systems, were not tested for. *Acanthamoeba* encephalitis, however, has been reported in a small number of immunocompetent children and may be responsible for disease in persons with covert immune deficits. Thus, testing for antibody to *Acanthamoeba* species was later added in an effort to detect *Acanthamoeba* species as a possible cause of illness among immunocompetent patients. A small number of samples were also tested for *Naegleria fowleri* antibody, if the patient had a recent history of
exposure to freshwater, which is the archetypical source of \textit{Naegleria} meningoencephalitis [15].

Figure 1 illustrates the range of acute antibody titers for \textit{Balamuthia}, \textit{Acanthamoeba}, and \textit{Naegleria} species encountered in the survey. For \textit{Balamuthia} testing, a titer of 1:64 was used as a cutoff value (i.e., samples with titers $\geq 1:64$ were considered to be positive pending the results of additional confirmatory tests, including immunostaining of tissue samples, examination of hematoxylin-eosin–stained sections submitted by the study, all positive titer determinations were confirmed by either nonspecific cross-reactivity or exposure to \textit{Balamuthia} amoebae in soil. In the course of this survey, 7 serum samples had test results positive for antibody to \textit{B. mandrillaris}, with titers ranging from 1:128 to 1:256. Six of these samples were obtained from California residents, and the seventh sample was obtained from outside of California. Positive control samples (i.e., serum samples from patients with balamuthiasis) had titers ranging from 1:128 to 1:256, the variation in titers being attributable to subjective evaluation of immunofluorescence results or to different batches of slides being used for testing. Negative control samples (serum samples from healthy individuals) had titers of $\leq 1:32$. No cases of acanthamoebiasis or naegleriasis were detected for \textit{B. mandrillaris} that were encountered in the study, all positive titer determinations were confirmed by immunostaining of unstained tissue sections and/or by examination of hematoxylin-eosin–stained sections submitted by the patient’s hospital or by PCR of brain tissue or CSF samples for \textit{Balamuthia} 16s rRNA gene DNA [16]. In 1 case, \textit{Balamuthia} amoebae were isolated from necrotic brain tissue obtained at autopsy and established in culture [11]; a strain of \textit{Acanthamoeba} was also isolated from necrotic brain tissue obtained at autopsy [17]. Attempts to isolate amoebae from brain tissue or CSF samples obtained from other patients were unsuccessful.

A single case of \textit{Acanthamoeba} encephalitis, submitted from outside of California, was encountered [17]. The patient was a splenectomized female subject who was being treated with corticosteroids for systemic lupus erythematosus. IFA testing of serum samples revealed the patient’s \textit{Acanthamoeba} antibody titer to be 1:8 to 1:16 (i.e., essentially within the normal range; figure 1). Immunoperoxidase and FITC-staining of brain tissue, however, gave evidence of \textit{Acanthamoeba} trophozoites and cysts. Several other serum samples submitted to the CEP had elevated \textit{Acanthamoeba} titers ($\geq 1:64$), but lack of confirmatory evidence (i.e., sectioned brain tissue or CSF samples) or loss of the patient to follow-up did not allow resolution of the preliminary laboratory results.

Clinical features that were used to select serum samples for \textit{Balamuthia} antibody testing included the following: (1) MRIs of the brain showing ring-enhancing lesions or space-occupying masses, (2) differential diagnoses including neurocysticercosis or tuberculous meningitis, (3) hydrocephaly, and (4) protein and/or WBC levels in patient CSF samples. All individuals who received a diagnosis had elevated protein and WBC levels in CSF; protein levels were particularly high (table 2). Glucose levels, however, were near the lower limit of the normal range. When the CSF protein levels of individuals with test results positive for antibody to \textit{B. mandrillaris} (5 patients) were compared with the cytokine activating factor protein levels of individuals with negative test results (241 subjects), a significant difference was observed ($P<.001$, by the Mann-Whitney test). No significant difference was observed in WBC count or glucose levels between the groups. CSF samples were not available for

### Table 1. Demographic characteristics of the overall patient population in the California Encephalitis Project and the subpopulation tested for balamuthiasis and acanthamoebiasis.

<table>
<thead>
<tr>
<th>Demographic characteristic</th>
<th>California Encephalitis Project population ($n = 1453$)</th>
<th>Subpopulation tested for amoeba antibodies ($n = 290$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>805 (56)</td>
<td>189 (64)</td>
</tr>
<tr>
<td>Age, mean years (range)</td>
<td>29.1 (0.5–90)</td>
<td>32.5 (0.5–89)</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>542 (37)</td>
<td>120 (41)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>393 (27)</td>
<td>83 (29)</td>
</tr>
<tr>
<td>Black</td>
<td>124 (9)</td>
<td>24 (8)</td>
</tr>
<tr>
<td>Asian/Pacific Islander</td>
<td>180 (13)</td>
<td>37 (13)</td>
</tr>
<tr>
<td>Native American</td>
<td>8 (1)</td>
<td>1 (&lt;1)</td>
</tr>
<tr>
<td>Other/Unknown</td>
<td>206 (14)</td>
<td>21 (7)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of patients, unless otherwise indicated. Data are as of December 2004. The actual number of samples received to date as part of the California Encephalitis Project is greater.

*The subpopulation tested for amoeba antibodies was 20% of the overall patient population of the California Encephalitis Project.*

![Figure 1](https://academic.oup.com/cid/article-abstract/42/9/1260/314799/314799)
Table 2. Laboratory results for CSF samples obtained from patients with encephalitis who received a diagnosis of balamuthiasis on the basis of immunofluorescent staining of serum samples for antibodies to Balamuthia mandrillaris (balamuthiasis-positive group) and patients with encephalitis who did not receive such a diagnosis (balamuthiasis-negative group).

<table>
<thead>
<tr>
<th>Laboratory result</th>
<th>Balamuthiasis-positive group (n = 5)</th>
<th>Balamuthiasis-negative group (n = 241)</th>
<th>$p^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein level$^c$, median mg/dL</td>
<td>1247</td>
<td>93</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Glucose level$^d$, median mg/dL</td>
<td>47</td>
<td>61</td>
<td>NS</td>
</tr>
<tr>
<td>WBC count$^e$, median cells/mm$^3$</td>
<td>106</td>
<td>63</td>
<td>NS</td>
</tr>
</tbody>
</table>

**NOTE.** NS, not significant.

$^a$ CSF samples were available for only 5 of 7 patients with cases of balamuthiasis diagnosed on the basis of immunofluorescent staining of serum samples for Balamuthia antibodies.

$^b$ Statistical significance was defined as a $P$-value $<.05$, by the Mann-Whitney test.

$^c$ Normal range, 15–45 mg/dL.

$^d$ Normal range, 40–80 mg/dL.

$^e$ Normal range, 0–5 cells/mm$^3$.

all patients in the CEP, and they were available for only 5 of the 7 patients with balamuthiasis.

Antibody titers for Balamuthia and Acanthamoeba species were mutually exclusive, indicating little or no cross-reactivity between the 2 amoebae (e.g., a serum sample with a Balamuthia titer of 1:256 had an Acanthamoeba titer of 1:32). Several serum samples had titers that were borderline or above (1:64 to 1:128), but they could not be definitively identified as having been obtained from patients with balamuthiasis. Several of these samples showed a decrease in titer on subsequent IFA tests. In other cases, the patients died without autopsy, recovered to baseline without further testing, received a diagnosis involving a different infectious agent, or went into rehabilitation and/or were lost to follow-up.

Drawing on data in the published literature and from the files at the Centers for Disease Control, we have compiled a distribution of cases of Balamuthia amoebic encephalitis in the state of California, including balamuthiasis in both humans and animals (figure 2). In total, there were 12 known cases in humans in the state during 1990–2005, with 2 survivors. During the same period, there were 10 reports of disease in animals, mostly in zoos, and all were fatal. The majority of cases of Balamuthia encephalitis have occurred in the southern part of the state, both for humans and for animals.

**DISCUSSION**

The diagnosis of balamuthiasis is elusive. The importance of this article is that it describes the use of IFA to screen for amoeba antibodies as a noninvasive method for detection of the disease in patients with encephalitis. Equally important, however, is that the clinician be familiar enough with the disease to know when to consider Balamuthia encephalitis as part of the differential diagnosis. Toward that end, we present a generalized clinical profile based on the medical histories of the patients with balamuthiasis who are described in this study.

Balamuthia encephalitis is often preceded by a subacute clinical course of indeterminate duration, lasting weeks to months. At presentation, the patient may have a low-grade fever or be afebrile and may complain of headache, stiff neck, lethargy, and vomiting. Progression of neurologic symptoms ensues, including personality change, seizures, and coma. The patient may also present with hemiparesis, papilledema, meningeal inflammation, aphasia, ataxia, and cranial nerve palsies. Laboratory results include a complete blood count that is generally uninformative. CSF samples typically show marked elevation of protein levels (increasing from normal or mildly elevated early in the clinical course to $>1000$ mg/dL), pleocytosis with lymphocytes predominant, and normal or low glucose levels. Cultures of blood and CSF samples and stained slides are negative for bacteria and fungi. Neuroimaging findings are often abnormal; space-occupying masses and unifocal or multifocal
ring-enhancing lesions up to 2–3 cm in diameter may be seen in the cerebellar and cerebral hemispheres, pons, brain stem, and often near the gray-white junction. Hydrocephalus, ser-piginous enhancement, and edema may also be evident. Hemorr-hagic necrotizing vasculitis, caused by large numbers of amoebae in the perivascular spaces of brain parenchyma, can be found on brain biopsy or autopsy. A granulomatous process may be evident, though granuloma formation may be absent in immunocompromised persons. Earlier cases were thought to be due to tuberculosis, neurocysticercosis, viral encephalitis, bacterial abscess, atypical acute disseminated encephalomyelitis, and tumor. Absent early diagnosis and treatment, the prognosis is poor.

In this study, IFA staining of serum samples successfully detected 7 cases of Balamuthia granulomatous encephalitis in a population of patients with encephalitis. All antibody titers positive for B. mandrillaris (i.e., >1:64) were confirmed by immunostaining or hematoxylin-eosin staining of tissue sections to demonstrate amoebae in brain parenchyma and, more recently, by the PCR method for Balamuthia mitochondrial 16S rRNA gene DNA in patient CSF and/or brain tissue samples [16]. Unfixed brain tissue, when available, was obtained either from biopsy or autopsy for attempted isolation of amoebae.

An Australian study of antibodies to Balamuthia used fluorescent activated cell sorting (FACS) to determine titers in serum samples (including cord blood) obtained from healthy individuals [5]. Titers of 1:64 to 1:256 were detected in healthy adults and children. The reported titers are approximately the same as those obtained in the present study using IFA staining, but the titers in the present study were from serum samples obtained from patients with Balamuthia encephalitis. The FACS technique may be more sensitive (and less subjective) and therefore may result in higher titers than those obtained through estimating titers by eye with a fluorescence microscope. In our study, positive control samples (serum samples obtained from patients with clinical cases of balamuthiasis) routinely had titers of 1:128 to 1:256, but no positive control samples were tested in the Australian study for comparison. One of our positive patient samples, when tested by FACS, had titers of 1:400 and 1:800 in 2 tests, compared with our negative control (which had titers of <1:16) (A. Kiderlen, personal communication).

The single case of acanthamoebiasis encountered during the course of the study had antibody titers within the normal range, but evidence of amoebae was seen on immunostaining of autopsied brain tissue. The dampening of an effective antibody response was probably a consequence of high-dose corticosteroid administration before the patient’s death [17].

Virtualy all serum samples that were tested showed evidence of antibodies against Balamuthia and Acanthamoeba species (figure 1). The likely explanation for these antibodies in humans is contact with environmental amoebae. This conclusion is consistent with other surveys of antibody titers against Naegleria and Acanthamoeba species in humans and animals [2–5, 8–10]. None of the ~10 cases tested for antibody to Naegleria species in this study had elevated Naegleria titers or fit the clinical profile for amoebic meningoencephalitis (figure 1).

Balamuthiasis is difficult to diagnose because of a lack of specific symptoms. In various reports in the literature, neurocysticercosis and tuberculous meningitis were erroneously diagnosed [18]. All of the infections detected in this study were fatal, largely as a result of delayed diagnosis of the disease and/or lack of effective antimicrobial therapy. Not infrequently, the diagnosis was made on the basis of postmortem examination of hematoxylin-eosin–stained or immunostained sections of the brain tissue. Two cases of successful treatment and recovery have occurred in California [12], and 1 case has occurred in New York [19].

Did our survey miss any balamuthiasis cases? Approximately 1 dozen cases in our survey had borderline titers (i.e., 1:64 to 1:128), suggesting balamuthiasis or nonspecific cross-reactivity in IFA staining of serum samples. Despite multiple efforts, these preliminary findings were never resolved, either because of the death of the patient without autopsy and without the opportunity to examine hematoxylin-eosin–stained sections of brain tissue or because of patient recovery and loss to follow-up. Still open is the question of whether balamuthiasis may exist in a subacute form in the population. The presence of detectable antibody titers in our surveyed population suggests previous exposure, but we cannot determine if this was due to subacute disease.

In the population of patients tested, male subjects outnumbered female subjects, and patients ranged in age from ≥6 months to 89 years (table 1). All patients with confirmed cases of Balamuthia infection were of Hispanic ethnicity, as determined from surnames or ethnicity information reported on submitted medical history [20]. In the total CEP population, Hispanics constitute ~25% of patients with encephalitis, whereas a somewhat higher proportion of Hispanics (29%) were tested for amoeba antibodies. Hispanics are also at increased risk for several other diseases, including neurocysticercosis, entamoebiasis, tuberculosis, and coccidioidomycosis [21, 22].

We have noted that the preponderance of cases in California have been in the southern part of the state. There are several possible interpretations. First, the amoeba, which is found in soil, is more likely to be present in soil that is dry and warm, which is more typical of southern California than of other parts of the state. Second, the percentage of Hispanics, who make up a majority of balamuthiasis cases in California, is greater in the southern part of the state than elsewhere. Third, large-scale agriculture is a major industry in the southern part of the state, which increases the likelihood that more individuals are ex-
posed to soil while working in fields or as small particles carried on the wind. *B. mandrillaris* is difficult to culture from soil, mainly because of their complex in vitro growth requirements and slow rate of growth [23, 24]. Two cases of *Balamuthia* encephalitis, both occurring in young male subjects and both in southern California, involved riding in an open car across desert terrain (G. S. Visvesvara, unpublished information) and riding on the back of a motorcycle (D. J. Michelson, personal communication). Both individuals were presumably exposed to blowing soil. Three other cases have been linked to soil, one occurring in a 3-year-old girl who may have been handling flowerpot soil [24], another in a 64-year-old man after digging a shower drain in his yard [12], and a third in a 72-year-old woman who had been working with composted soil before her illness [19].

The results of this study indicate that *Balamuthia* encephalitis in California is not inconsequential [20]. Because of the difficulties in diagnosis and in initiating successful antimicrobial therapy, the disease frequently proves fatal. Initiating testing for infections due to *Balamuthia* (and *Acanthamoeba*) species requires familiarity with the disease, its symptoms, and the neuroimaging findings and CSF data typically associated with such infections. Greater awareness of the disease can lead to earlier diagnosis and intervention, thereby offering a better chance for recovery.

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**References**