GENOTYPING OF BALAMUTHIA MANDRILLARIS BASED ON NUCLEAR 18S AND MITOCHONDRIAL 16S rRNA GENES

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Abstract. Balamuthia mandrillaris is an opportunistically pathogenic ameba that causes fatal granulomatous amebic encephalitis (GAE) in vertebrates. Previous phylogenetic analyses that included the sequence of a single nuclear small subunit ribosomal RNA gene (18S or ssu rDNA) from this ameba suggested that Balamuthia is closely related to Acanthamoeba, another opportunistically pathogenic amebic genus, which includes multiple ssu rDNA genotypes. We tested whether this also is true for Balamuthia. The nuclear ssu rDNA from 4 isolates and the mitochondrial ssu rDNA from 7 isolates of B. mandrillaris have been sequenced. No variation in the nuclear rDNA sequences and low levels of variation in the mitochondrial rDNA were found. Both gene sequences were consistent with a single genotype for B. mandrillaris. The mitochondrial sequences of B. mandrillaris are unique and should be useful for development of genus-specific diagnostic probes for use with clinical, environmental, and archived specimens.

INTRODUCTION

Infections involving the small free-living ameba Balamuthia mandrillaris are emerging as a rare but serious problem worldwide. This pathogen causes granulomatous amebic encephalitis (GAE) in human and animal hosts and almost always results in death. Balamuthia first was discovered in a pregnant mandrill at the San Diego Zoo Wild Animal Park that died of meningoencephalitis and originally was identified as a leptomyxid ameba.¹ Later the organism was described as B. mandrillaris.²,³ B. mandrillaris since has been isolated from human hosts.¹ There seems to be no apparent trend among hosts because individuals of all ages and of immunocompromised and apparently immunocompetent health states have been affected. A review of the literature suggests that cases involving males are more prevalent.³,⁴–⁶

Characteristic symptoms of GAE include fever, headaches, nausea, seizures, and other focal neurologic indicators, such as brain lesions. B. mandrillaris usually is found within localized areas of the brain and clustered around blood vessels.¹,³,⁶,⁸,¹¹,¹₅,¹₆ An inflammatory response subsequently is mounted in immunocompetent hosts, and amebae can be found surrounded by immune cells, such as macrophages, lymphocytes, and neutrophils. In some cases, B. mandrillaris has been mistaken for macrophages. Infections also have caused skin lesions, and in a few cases amebae have been found in the kidneys and lungs.⁴,¹₀,¹₁,¹₅,¹₇,¹₈ Hemorrhaging of organs in many clinical cases provides evidence that B. mandrillaris had been present in these locations even when amebae were not isolated from the tissue samples. Eventually, patients die of a massive central nervous system infection. In one case in which survival of a B. mandrillaris infection has been documented, the patient was left with “severe neurologic deficits.”¹⁹ There is no current treatment of the infections, although the drugs pentamidine isethionate, propanidine isethionate, and azithromycin have been shown to be amebastatic.²⁰,²¹ In all but a few isolated cases, B. mandrillaris has been detected only postmortem.⁹,²¹,²²

GAE caused by B. mandrillaris appears clinically similar to GAE caused by Acanthamoeba spp.; however, Acanthamoeba may affect only immunocompromised hosts. The amebae of these 2 genera are morphologically similar, both having trophozoite and cyst forms with a thick double wall. The primary distinguishing features between these 2 genera are that Balamuthia trophozoites are slightly larger (12–60 μm) and can have >1 nucleolus in the nucleus, whereas Acanthamoeba typically has a single nucleolus.¹,³,⁶,¹₀,¹₅–¹₇,²³ Currently an immunofluorescence test using species-specific sera is the most reliable means to distinguish between the 2 amebae. In contrast to Acanthamoeba, which has been found readily in soil, water, and sewage, B. mandrillaris has never been isolated from the environment. The 2 genera are believed to share a similar environmental niche, however, which is presumed to be the most likely source of infections in both cases. B. mandrillaris also has been difficult to culture. Until more recently, growth required culture on mammalian cells. A cell-free growth medium now has been developed, however.²¹ The difficulty in initial attempts to grow this species axenically may be the reason why many attempts to culture B. mandrillaris from the environment were unsuccessful, rather than because amebae were not present. There are only isolated cases in which B. mandrillaris has been identified as the causative agent of encephalitis antemortem. More sensitive diagnostic tests are needed that would identify B. mandrillaris in patient specimens in a timely fashion. This diagnosis would facilitate earlier therapeutic intervention.

In addition to the morphologic similarity of B. mandrillaris and Acanthamoeba, a previous study that examined a single nuclear 18S rDNA sequence from Balamuthia showed that the gene from this organism was relatively closely related to the comparable gene from Acanthamoeba spp.²⁴ A more recent study has supported the close relationship of Acanthamoeba and Balamuthia based on this gene.²⁵ Extensive studies in the laboratory at Ohio State University and elsewhere have revealed many distinct Acanthamoeba genotypes of nuclear 18S rDNA.²₄,²₆–²₈ They also have shown, however, that most Acanthamoeba keratitis cases are associated with a single sequence type.²₄,²₆ It is not known whether there is any correlation between sequence types and the pathogenic potential of Balamuthia.

In this study, we applied techniques used in studies of Acanthamoeba to the B. mandrillaris cultures that were available from the Centers for Disease Control and Prevention (CDC). We examined specimens isolated from human, macaque, and equine hosts. These represent isolates from 2 different continents. We investigated the level of variation in...
the nuclear and mitochondrial (mt) rRNA genes of *Balamuthia* and to determine how they relate to *Acanthamoeba* genes and whether there are any significant correlations between sequence types and geographic sources or patient characteristics.

**MATERIALS AND METHODS**

The isolates of *B. mandrillaris* listed in Table 1 were obtained from the CDC and used for sequence analysis of the nuclear 18S and mt 16S rDNA.1,13,23,29 DNA extractions were performed directly on *B. mandrillaris* isolates as received at Ohio State University from the CDC without further culturing. An aliquot of 3 ml of the liquid culture was removed and pelleted at 3,000Xg in 2 separate microfuge tubes containing 1.5 ml each. Both pellets were resuspended in 200 μl of UNSET buffer.30 DNA extraction was continued from this point following the protocol of the commercially available DNA extraction kit DNeasy (Qiagen, Inc, Valencia, CA). DNA was eluted in 100 μl of the elution buffer supplied with the kit and quantified by spectroscopy. Approximately 100–200 ng of genomic DNA was used for polymerase chain reaction PCR amplifications. Nuclear 18S rDNA amplification was done using primers CRN5 (5′-ttggtgatctctgagcag-3′) and SSU2 (5′-ccggcgcggatcctgatccctccgcaggttcac-3′), which span approximately 99% of the gene. PCR products of nuclear 18S rDNA were prepared for automated sequencing using the QIAquick PCR purification kit (Qiagen Inc, Valencia, CA). Multiple pooled PCR products were sequenced directly using automated fluorescent sequencing protocols on an ABI 310 automated sequencing system (Applied Biosystems, Foster City, CA) employing primers previously used in *Acanthamoeba* studies.24

The protocol used for the amplification and sequencing of the mitochondrial 16S rDNA in *B. mandrillaris* was different from previous work. Repeated attempts to amplify the mt 16S rDNA locus using primers that we had used formerly in *Acanthamoeba* studies proved unsuccessful in repeated attempts. These primers apparently were too specific to amplify this gene in *B. mandrillaris*, so a different set of primers was developed. This new set was based on a mt 16S rDNA alignment of a more distantly related set of taxa. These sequences were available from GenBank (reference numbers following species name) and included *Acanthamoeba castellanii* plus *Cafeteria roenbergensis* NC 000946 (heterotrophic microflagellate), *Phytophthora infestans* NC 002387 (fungus), *Chlorarachnion* sp. U36908 (filose ameba), *Sphagnum palustre* AF 058671 (bog moss), *Nephroselmins olivacea* AF 110138 (green algae), *Prototheca wickerhamii* NC 001613 (green algae), and *Tetrahymena thermophila* NC 003029 (ciliate). This alignment was used to investigate whether relatively conserved regions that would be suitable for primer development could be found in the gene. One region was found near the 5′ end of the gene, and another was found near the 3′ end. Two primers, MT16sUNIV5′ (5′-ngaatntngcaatnngng-3′) and MT16sUNIV3′ (5′-ccctacnntaacnntgcc-3′), which included some redundant sites, were developed from these regions and amplified all but 400 bases at the 5′ end of the 16S gene. A sufficient number of informative sites were encompassed by the primers to permit the unambiguous identification of *B. mandrillaris* and firm conclusions regarding the relationship of *B. mandrillaris* to other genera. PCR conditions included an initial denaturing step of 7 minutes at 95°C, 40 cycles of a 1-minute denaturing step at 95°C, a 2-minute annealing step at 45°C, a 3-minute extension step at 72°C, and a final extension step of 15 minutes at 72°C and a 4°C soak. PCR products were run out on a 1% agarose gel, and products of the expected size (approximately 1,100–1,200 bp) were observed. Finally, because direct sequencing of the product would not work using *Acanthamoeba* primers, we cloned the *B. mandrillaris* 16S mt rDNA product and sequenced it using primers located in the vector. Previously purified pooled PCR products of *B. mandrillaris* 16S rDNA, 1 μl of a 1:10 or 1:100 dilution, were cloned into a plasmid vector using a T/A cloning kit (Qiagen Inc, Valencia, CA). Positive clones were identified and cultured overnight at 37°C in Luria-Bertani medium containing 50 μg/ml of kanamycin. DNA was isolated and purified using the QIAprep miniprep kit (Qiagen Inc, Valencia, CA). A restriction digest was performed with restriction endonuclease EcoRI according to the Qiagen protocol. The clones were run out on an agarose/synergel gel (1.6%) to select for those of the desired size. The putative positive clones were sequenced using the primers M13 forward (5′-gtgtagctggggaaa-3′) and M13 reverse (5′-cggagaaagatcag-3′). Sequencing using these primers permitted us to sequence the entire PCR product with overlap in the central region of the amplifier.

The nuclear 18S and mt 16S rDNA sequences of the *Balamuthia* isolates V039, V188, V194, V416, V426, V433, and V451 were aligned in the sequence alignment editor ESEE and compared with one another and with other sequences in our database (www.biosci.ohio-state.edu/tybers/tybers.htm; DR Ledee, unpublished data).21 Phylogenetic trees were constructed using the phylogenetic analysis program MEGA2.32 Sequence dissimilarities between *Balamuthia, Acanthamoeba*, and other genera were calculated using the Kimura 2-parameter model in MEGA2 employing the sequence regions also used for phylogenetic gene tree reconstruction in the nuclear (1,625 sites) and mt (722 sites) rDNA.32 The sequence dissimilarities within the *Balamuthia* mt 16S rDNA presented in Table 2 were calculated using the entire alignment of 1,109

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**Table 1**

*Balamuthia mandrillaris* isolates obtained from the Centers for Disease Control and Prevention.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host, age, geographic location</th>
<th>Location of amoeba</th>
<th>Previous health status</th>
</tr>
</thead>
<tbody>
<tr>
<td>V039</td>
<td>Female mandrill, 8 mo, San Diego, California</td>
<td>Brain</td>
<td>Pregnant</td>
</tr>
<tr>
<td>V188</td>
<td>Male human, 59 yr, Georgia</td>
<td>Brain, skin lesion</td>
<td>Traumatic leg amputation</td>
</tr>
<tr>
<td>V194</td>
<td>Male human, 60 yr, Las Vegas, Nevada</td>
<td>Brain</td>
<td>Chronic alcoholic, seizures, hemiparesis</td>
</tr>
<tr>
<td>V416</td>
<td>Female human, 10 yr, S. Brisbane, Australia</td>
<td>Brain</td>
<td>NA</td>
</tr>
<tr>
<td>V426</td>
<td>Male human, 17 yr, California</td>
<td>Brain</td>
<td>NA</td>
</tr>
<tr>
<td>V433</td>
<td>Horse, 20 yr, California</td>
<td>Brain</td>
<td>Normal</td>
</tr>
<tr>
<td>V451</td>
<td>Female human, 6 yr, New York</td>
<td>Brain</td>
<td>Normal</td>
</tr>
</tbody>
</table>

NA, data not available.

* Atlanta, Georgia.
sites because all sites were aligned reliably between these closely related isolates.

In the nuclear 18S rDNA analysis, *B. mandrillaris* sequences were aligned to 11 of the 12 *Acanthamoeba* genotypes available in our database. The mt sequences of *Acanthamoeba* genotype T6 was not available, and it was excluded from the nuclear 18S rDNA analysis so that comparisons of trees would be comparable. *Diaphanoeca grandis* (GenBank accession L10824) was added to the nuclear rDNA alignment as an outgroup genus for phylogenetic comparisons.

For the mt rDNA analyses, *Balamuthia* sequences were aligned to the mt rDNA sequences from a subset of *Acanthamoeba* species representing the same 11 *Acanthamoeba* genotypes used in the nuclear analyses plus *Tetrahymena*, *Prototheca*, and *Chlorarachion*. All ssu rDNA sequences of *Balamuthia mandrillaris* obtained in this study have been deposited in GenBank, and are available using the accession numbers AF477012-AF477018 for the mt ssu rDNA and AF477019-AF477022 for the nuclear ssu rDNA data.

### RESULTS

**Nuclear rDNA.** Nuclear small subunit (ssu) rDNA was amplified and sequenced from 4 *B. mandrillaris* isolates: V039, V451, V188, and V433. The primary sequence for isolate V039 was determined previously but was reexamined here using a new sample of this specimen. The new and old sequences of V039 agreed completely with each other and with the sequences of the other 3 specimens. Because the 4 obtained sequences were identical, a single representative sequence was used in the phylogenetic reconstruction. *Diaphanoeca grandis* was used in this analysis as an outgroup genus for the *B. mandrillaris* and *Acanthamoeba* genotypes examined here. The sequence alignment permitted inclusion of 12 homologous regions, which included 1,625 sites, of which 341 were variable and 118 were phylogenetically informative. The sequence dissimilarities between *B. mandrillaris* and the 11 *Acanthamoeba* genotypes examined using these regions were similar and ranged from 6.5–7.5%. Phylogenetic reconstruction analysis using neighbor joining methods produced the same results as previously shown by Stothard et al.24 that is, the lineage leading to the extant *B. mandrillaris* diverged before all of the *Acanthamoeba* species.

**Mitochondrial rDNA.** The primary sequence of the mt ssu rRNA gene was determined for 7 isolates of *B. mandrillaris*: V039, V451, V188, V433, V416, V426, and V194. The aligned data set that was obtained using the newly developed primers was 1,109 bp, representing approximately 75% of the entire gene. We also had obtained nuclear ssu rDNA data from 4 of these isolates. Two sets of mt ssu rDNA sequences were identical. Isolate V039 was identical to V194, and isolate V416 was identical to V426. Pairwise sequence dissimilarities across the entire data set for the 7 sequences ranged from 0–1.8% (Table 2). Isolate V451 was the most divergent of the isolates examined, differing from the other isolates by 1.6–1.8%. When V451 is excluded, the remaining isolates differ from one another by only 0–0.5%. The primary sequences of the *B. mandrillaris* mt ssu rDNA were aligned with a representative of each of 11 *Acanthamoeba* mt *rns* genotypes and with the mt ssu rDNA sequences of *Tetrahymena thermophila*, *Prototheca wickerhamii*, and *Chlorarachnion* sp. Using this data set, an alignment was produced that contained 7 conserved regions of homology (722 sites, 408 variable, 223 phylogenetically informative) that were used for phylogenetic gene tree reconstruction. *T. thermophila* was designated as the outgroup in these analyses. The neighbor-joining tree with its various lineages is shown in Figure 1.

### DISCUSSION

These results showed no variation among *B. mandrillaris* isolates in the nuclear rDNA gene. Phylogenetic analysis using *Diaphanoeca grandis* as an outgroup taxa supports the phylogenetic position of *Balamuthia* as a sister genus to *Acanthamoeba* (tree not shown). We found no evidence to support the possibility that *Balamuthia* is a new species of *Acanthamoeba*. The most divergent of the *Acanthamoeba* genotypes, *A. astronyxis* (T7), *A. tubiashi* (T8), and *A. comandoni* (T9), clearly diverged after the lineage leading to *Balamuthia*. The nuclear 18S results continue to support the hypothesis that *Balamuthia* is an independent genus, closely related to the genus *Acanthamoeba*, and that *Acanthamoeba* is a monophyletic lineage.

Sequence variation was found in the mt 16S rDNA genes of the 7 *B. mandrillaris* isolates, but the range of dissimilarity (0–1.8%) was low across the entire gene (Table 2). This range is comparable to that observed for rDNA sequences of the 2 most invariant *Acanthamoeba* spp., *A. lenticulata* and *A. mauritianensis*, and is much less than between *Acanthamoeba* genotypes. We previously used a 5% sequence divergence to discriminate genotypes. Application of this principle to the *B. mandrillaris* mt data would identify clearly all of these samples as members of a single genotype and justifies their inclusion in a single species.

Although the level of variation in the mt rDNA among *B. mandrillaris* isolates is low, the sequences are substantially different from other related taxa, including *Acanthamoeba*. Using the conserved dataset of 722 sites, the sequence dissimilarity between the *Balamuthia* isolates ranges from 0–1.0%, whereas *B. mandrillaris* V039 differs from the *Acanthamoeba* ssu mt rDNA *rns* genotypes by 17.9–21.1% (average 18.9%). The sequenced dissimilarity value is similar between *Balamuthia* and all of the *Acanthamoeba* genotypes examined. Previous studies that analyzed the nuclear ssu rDNA and more recent analysis of mt ssu rDNA have identified the *Acanthamoeba* genotypes T7, T8, and T9 as the most distantly related to the other *Acanthamoeba* genotypes (DR Lede, unpublished data).24 An examination of mt rDNA sequence dissimilarity between *Acanthamoeba* genotypes T7, T8, and T9 and the remaining *Acanthamoeba* genotypes using these 722 sites reveals a range of 7.7–9.2%, far
lower than the 17.9–21.1% range between *Balamuthia* isolates and the *Acanthamoeba* genotypes.

Phylogenetic analyses of the mt data presented in Figure 1 show that *B. mandrillaris* forms a monophyletic clade distinct from all of the *Acanthamoeba* genotypes whose mt ssu rDNA sequences have been determined. There is no support for the appearance of the *Balamuthia* lineage after the divergence of *Acanthamoeba* genotypes T7, T8, and T9 and before the branching of the lineage leading to the remaining *Acanthamoeba* genotypes; this is in agreement with the nuclear 18S analyses and supports *Acanthamoeba* as a monophyletic clade including all *Acanthamoeba* genotypes and excluding *Balamuthia*.

The low level of sequence variation observed here indicates that the lethal infections caused by *B. mandrillaris* are due to a single species with an intercontinental distribution. We find no apparent correlation between a particular mt sequence and the genus of vertebrate infected. The mt sequence obtained from the *Balamuthia* isolate from the mandrill is identical to that obtained from a male human. *Balamuthia* seems to be single lineage of ameba that can infect lethally various vertebrates including apparently healthy humans of various ages and both sexes.8,10–12 The sequence information obtained here, especially for mt 16S rDNA, will permit future development of primers specific for amplification of *B. mandrillaris*. Although not specific for *Balamuthia*, the amplification primers used in the current study are diagnostically useful. They amplify the mt ssu rDNA of *Acanthamoeba* spp. and *B. mandrillaris*. We are unaware of any case in which both genera have been found in a single infection. Nevertheless, the amplicons obtained from the 2 genera differ in size by at least 50 bp. This size difference could be used as an initial screen to determine whether either genus is present in fresh clinical specimens or even in archived encephalitis specimens in which the cause of disease has not been determined. Sequencing of the amplimers could provide a more definitive diagnosis.

Received February 14, 2002. Accepted for publication June 20, 2002.

Acknowledgments: G.S.V. would like to acknowledge Sara Wallace for help in culture of *Balamuthia mandrillaris*.

Financial support: The work of G.C.B., J.R.C., T.J.B., and P.A.F. was funded by Public Health Service grant EY09073 awarded to P.A.F. by the National Eye Institute.

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