

## Identification of *Balamuthia mandrillaris* by PCR Assay Using the Mitochondrial 16S rRNA Gene as a Target

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***Balamuthia mandrillaris* is an opportunistic pathogen that causes granulomatous amebic meningoencephalitis in animals, including humans. Based on sequence analysis of mitochondrial small-subunit-rRNA genes, we developed primers that amplify a *Balamuthia*-specific PCR product. These primers will be useful for retrospective analyses of fixed tissues and possible identification of *Balamuthia* in vivo.**

Meningoencephalitis caused by the ameba *Balamuthia mandrillaris* is a recently recognized, and nearly always fatal, infection of the brain in mammals, including humans (3, 10, 13–15). Most infections have been identified postmortem by histological examination of an infected individual's tissue samples. While infections caused by *B. mandrillaris* are relatively rare, the lack of rapid identification and diagnosis of the causative agent is likely to have had serious implications regarding the outcome of disease progression (5). *Balamuthia* infections are further complicated by the morphological similarity of *Balamuthia* in tissue sections to *Acanthamoeba*, another amebic genus involved in encephalitis infections. Therefore, we employed a simple PCR-based method for the rapid identification of *B. mandrillaris* based on DNA sequence information of the *B. mandrillaris* mitochondrial small-subunit-rRNA gene (*rns*).

The use of PCR as a potential method for the rapid identification of disease organisms in a clinical setting has expanded recently (4). In the case of encephalitis, PCR is used in organism-specific procedures to identify herpes simplex encephalitis (6). PCR methods are also used to identify numerous other pathogens, including human immunodeficiency virus, hepatitis B and C viruses, various bacterial pathogens, and *Acanthamoeba* (1, 7, 8, 9, 11). In this study we describe a PCR-based test to specifically identify *B. mandrillaris*. Recently, we examined DNA sequences of nuclear small-subunit-rRNA genes (*rns*) from a number of *B. mandrillaris* isolates and found no sequence variation among them (2). However, this study also examined the mitochondrial *rns* from seven isolates of *B. mandrillaris* and found interisolate variation. The genus-specific PCR target that we have identified here is a portion of the mitochondrial rRNA gene (*rns*). Future application of this PCR test should facilitate examination of archived or clinical samples.

The sequence alignment of *rns* from seven isolates of *B. mandrillaris* (GenBank *rns* accession numbers AF477012 to AF477018) was used to identify potential PCR primers. The

seven isolates were very similar across the aligned region of 1,109 sites. Our previous study of *rns* variation in *B. mandrillaris* found low levels throughout the gene but no variation in the region of our putative *Balamuthia*-specific primers (2). Therefore, we predicted that this primer set would work satisfactorily if *B. mandrillaris* DNA was present. The *rns* 5' and 3' primers chosen were designated 5'Balspec16S (5'-CGCATGT ATGAAGAAGACCA-3') and 3'Balspec16S (5'-TTACCTAT ATAATTGTCGATACCA-3'). These primers were predicted to yield a PCR product of 1,075 bp in *Balamuthia*. A GenBank search using these primers failed to identify any significant sequence similarities among other organisms. As mentioned previously, the genus *Acanthamoeba* has been identified as a sister genus to *Balamuthia* (2). Further, *Acanthamoeba* has also been observed in amebic encephalitis infections. Therefore, we examined the differential amplification of *Balamuthia* and *Acanthamoeba* by using the *Balamuthia* primers and two *Acanthamoeba*-specific mitochondrial 16S rRNA primers. The latter yield a PCR product of ~950 bp from *Acanthamoeba* (7a).

The DNA samples used in this study were from previously obtained extractions of *B. mandrillaris* and *Acanthamoeba* sp. isolates (1, 11). Approximately 10 to 200 ng of genomic DNA was used for PCR amplifications. Putative positive *Balamuthia* mitochondrial 16S ribosomal DNA products were sequenced by automated fluorescent sequencing. The primer used for sequencing of the *Balamuthia* PCR products was mt900, previously used to confirm that PCR products were *Balamuthia rns* (2).

The initial screening of 5'Balspec16S and 3'Balspec16S used two *B. mandrillaris* isolates, CDC V039 (ATCC 50209, a female mandrill brain isolate) and CDC V188 (ATCC 50605, a male human brain isolate), and three very distinct *Acanthamoeba* genotypes, T4 (*Acanthamoeba mauritaniensis* ATCC 50253; type strain), T5 (*Acanthamoeba lenticulata* PD2S; ATCC 30841; type strain), and T10 (*Acanthamoeba culbertsoni* 409C) (12). This amplification gave products of the expected size (1,075 bp) only from *B. mandrillaris*. PCR amplicons from *B. mandrillaris* V039 then were pooled and sequenced, confirming that the fragment was the *rns* from this strain.

Next we performed a multiplex PCR that contained a mix-

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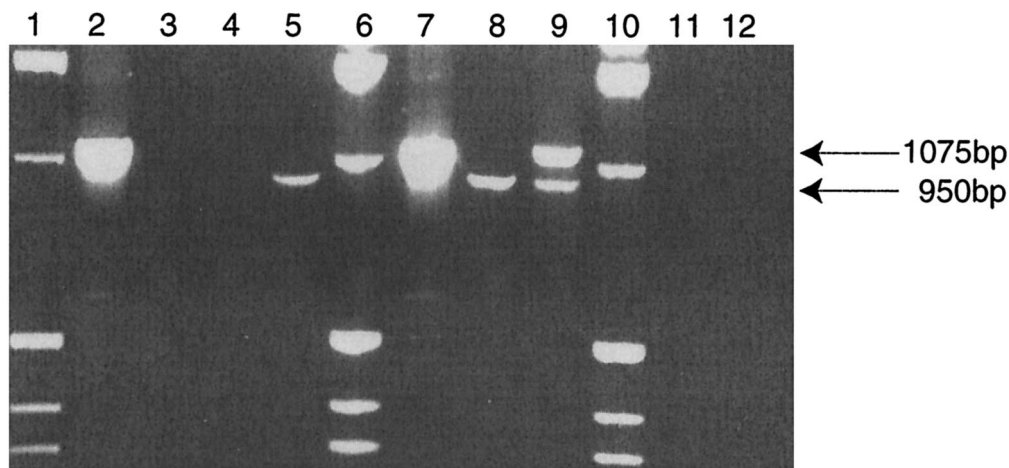


FIG. 1. *B. mandrillaris* multiplex *ms* PCR. Amplification was performed in 50- $\mu$ l reaction mixtures with 2.5 mM MgCl<sub>2</sub>; cycle conditions were 40 cycles of 1 min at 94°C, 2 min at 48°C, and 3 min at 72°C followed by a 15-min final extension at 72°C. Lanes 1, 6, and 10, 1-kb marker; lane 2, *Balamuthia* DNA with *Balamuthia*-specific primers; lane 3, *Balamuthia* DNA with *Acanthamoeba*-specific primers; lane 4, *Acanthamoeba* DNA with *Balamuthia*-specific primers; lane 5, *Acanthamoeba* DNA with *Acanthamoeba*-specific primers; lanes 7 to 9, *Acanthamoeba* and *Balamuthia* DNA with *Balamuthia*-specific primers (lane 7), with an *Acanthamoeba*-specific primer set (lane 8), and with both genus-specific primer sets (lane 9); lane 11, human DNA with both specific primer sets, lane 12, negative control. Amplicons produced by these mitochondrial 16S genus-specific primer sets are indicated on the right.

ture of ameba DNA and different primer combinations (Fig. 1). Multiplex PCR used DNA from *A. mauritaniensis* (genotype T4) and *B. mandrillaris* isolate V039. Lanes 1, 6, and 10 contain a 1-kb DNA ladder, which includes a fragment of 1,018 bp. This fragment aids in rapid identification of PCR products of the two genera because it migrates on gels between the 1,075-bp *Balamuthia* amplicon and the 950-bp amplicon of *Acanthamoeba*. Lane 2 contains *B. mandrillaris* DNA, incubated with the specific *Balamuthia* *ms* primers, which produced an amplicon of the expected size for this genus. Lane 3 contained *B. mandrillaris* DNA incubated with the *Acanthamoeba*-specific primers. No amplification was observed in this lane. Lane 4 contained *A. mauritaniensis* DNA incubated with the *B. mandrillaris*-specific primers, and as expected, no amplification was observed. Next, in lane 5, *Acanthamoeba* DNA incubated together with the *Acanthamoeba*-specific primers gave the expected *Acanthamoeba* product. Lanes 7 through 9 show multiplexing with multiple DNAs (*A. mauritaniensis* and *B. mandrillaris*) and multiple primer sets. Lane 7 used the two DNAs with the *B. mandrillaris*-specific primer set, and only the expected amplicon (1,075 bp) was observed. Lane 8 had DNA from the two species incubated with the *Acanthamoeba*-specific primer set, and only the PCR product of the predicted size of 950 bp was observed. Most significantly, lane 9 combined genomic DNA from *B. mandrillaris* and *A. mauritaniensis* with both primer sets. In this multiplexed experiment, the respective bands of 950 bp for *Acanthamoeba* and 1,075 bp for *B. mandrillaris* were observed. Lane 11 has human DNA treated with both primer sets, and no amplification was observed. Finally, no amplification was observed in the negative control (lane 12), which consisted of both primer sets and no DNA.

In this study we demonstrate the ability of primers based on the sequence of the mitochondrial *ms* to specifically amplify a product from whole-cell DNA of *B. mandrillaris*, even in the presence of DNA from *Acanthamoeba*. Also, this primer set

does not amplify products from human DNA, which would be a potential source of contaminating DNA in a clinical setting. Future application of this very sensitive diagnostic test will be in the retrospective analysis of archived encephalitis samples and in the screening of clinical specimens in an effort to detect these organisms in early stages of infection, when therapy may be possible.

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