Balamuthia mandrillaris: Identification of Clinical and Environmental Isolates Using Genus-Specific PCR

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The protist genus *Balamuthia* was first described as the causative agent of a fatal case of encephalitis in a mandrill [8]. Originally classified as a leptomyxid ameba, it was eventually given genus status as *Balamuthia mandrillaris* [8,9]. Since the original isolation, numerous isolates have been found in granulomatous amebic encephalitis (GAE) cases in a number of vertebrates, including humans, almost all of which have been fatal [3–6]. Diagnosis of a *Balamuthia* infection has been very difficult and the source of the infection is often not identified prior to autopsy examination. Furthermore, until recently, *Balamuthia* has been difficult to grow in the laboratory in cell-free media [7]. Morphologically, *Balamuthia* is somewhat similar to *Acanthamoeba*, however the trophozoite is larger than that of *Acanthamoeba*. Also, *Balamuthia* shares the biphasic life style of *Acanthamoeba* consisting of a mobile trophozoite form, and a resistant cyst form. Despite these similarities, the size difference and unique shape of the trophozoites of *Balamuthia* make it distinguishable from *Acanthamoeba* by trained observers using microscopy. Because of the similarities between these two genera, *Balamuthia* has been hypothesized to share common ecological habitats with *Acanthamoeba*, including being found naturally in soil. Despite this, until recently isolation of *Balamuthia* from the environment has been very difficult, if not impossible [7].

The goals of these *Balamuthia* studies are to determine the phylogenetic placement of *Balamuthia* relative to other free-living ameba, to determine the levels of genetic variation within isolates of *Balamuthia*, to develop genus-specific identification methods for *Balamuthia*, and finally, to apply these genus-specific identification methods to archived and live samples. This will lead to the eventual goal of more rapid and robust identification methods for putative *B. mandrillaris* opportunistic infections, which continue to increase in frequency and worldwide distribution in an expanding number of vertebrates.

MATERIALS AND METHODS

The 7 isolates of *Balamuthia mandrillaris* used in the phylogenetic and variability studies were obtained from cultures maintained by G. S. Visvesvara at the Center for Disease Control and Prevention (CDC) and are designated *B. mandrillaris* V039, V188, V194, V416, V426, V433, and V451. The clinical and environmental isolates analyzed in the application portion of these experiments were obtained by one of us (F. L. S.) from the California Dept. of Health Services and from the New York University Medical Center. The phylogenetic placement of, and levels of genetic variation within *B. mandrillaris* isolates has been done by the sequence determination of the nuclear small subunit ribosomal RNA gene (*Rns*) and the mitochondrial small subunit ribosomal RNA gene (*rns*). The data from the sequences of these 2 genes have been used to determine the phylogenetic placement of *B. mandrillaris* isolates, and to develop genus-specific PCR primers for screening clinical and environmental samples. The *Rns* of 4 isolates of *B. mandrillaris* were amplified by PCR using primers CRN5 (5′-tggttgatcctgccagtag-3′) and SSU2 (5′-ccgcggccgcggatcctgatccctccg-caggttcac-3′) [1]. PCR primers that amplify the mitochondrial *rns* of *B. mandrillaris* were developed from our own *Acanthamoeba rns* database, and GenBank. These were designated MT16sUNIV5′ (5′-nngaatnttngncaatnngng-3) and MT16sUNIV3′ (5′-cccntacnnntaccntg-tnnc-3′) [1]. The mitochondrial *rns* was amplified and sequenced from 7 isolates of *B. mandrillaris*, which included the 4 isolates that were analyzed for the nuclear *Rns*. *Balamuthia* genus-specific primers were developed from the mitochondrial data (5′Balspec16S [5′-cgcatgtat-gaagaagacca-3′] and 3′Balspec16S [5′-ttacctatataattgtcgatacca-3′]). This PCR primer set produces a PCR product of 1,075 bp [2]. 5′Balspec16S was used in conjunction with a different 3′ primer, mt900 (5′-caaattaaaccacatact-3′), in PCR reactions done on the DNA extracted from the formalin-fixed brain tissue sample. This was done when the original *Balamuthia*-specific primer set produced no apparent amplimer following PCR on this sample. This primer set (5′Balspec16S/mt900) produces a PCR product of approximately 500 bp. The sequences of the *B. mandrillaris* nuclear *Rns* and mitochondrial *rns* data are available in GenBank (AF477019-AF477022 for the *Rns* data and AF477012-AF477018 for the *rns* data).

RESULTS AND DISCUSSION

The nuclear *Rns* was amplified and sequenced from 4 isolates of *B. mandrillaris*, all from infections from human, and other vertebrate hosts. The gene is approximately 2,000 bp in *Balamuthia*. All 4 isolates were identical in primary sequence for the *Rns*, indicating there is no variation in this gene in these isolates. Phylogenetic analysis of the *Rns* sequence of *B. mandrillaris* reveals that the sister genus of *Balamuthia* is *Acanthamoeba*, which supports the close relationship of these 2 genera previously suggested by morphology. Next, an examination of the primary sequences of 7 isolates of *B. mandrillaris* was performed using the mitochondrial *rns* as a target. Using the *rns*, variation within the *B. mandrillaris* isolates was observed, however the levels of variation were low. Levels ranged from 0% (in two sets of isolates that were identical to one another) to 1.8% (in one human infection-derived isolate versus another human infection-derived isolate). This particular isolate was unique in being the most divergent from the others. More typically, most isolates were approximately 0.3–0.4% different from one another. Together, the data suggest the close relationships within the isolates examined, and certainly supports *B. mandrillaris* as a single species based on the sequence data of these isolates. Phylogenetic analysis was also done using the sequenced data from the mitochondrial *rns*, and as in the analysis based on the nuclear *Rns* data, the phylogenetic gene tree again supported *Acanthamoeba* as the sister-genus of *Balamuthia*.

The observed levels of variation in the mitochondrial *rns* analysis were low, however, there was sufficient data to permit the development of a *Balamuthia* genus-specific PCR primer set for this gene. This primer set produces a PCR amplimer of 1,075 bp. These primers do not produce products from the closely related genus *Acanthamoeba*, or from humans, which is important since these primers will be used in situations where human DNA may be a contaminant. Further, these primers can be successfully used in concert with *Acanthamoeba*-specific mitochondrial *rns* primers in multiplex PCR experiments [2].

Following the development of this *Balamuthia* genus-specific primer set, it was used in 2 initial applications. In the first application this primer set was used on 2 putative *B. mandrillaris* isolates. The first isolate was derived from a fatal GAE case. The second was

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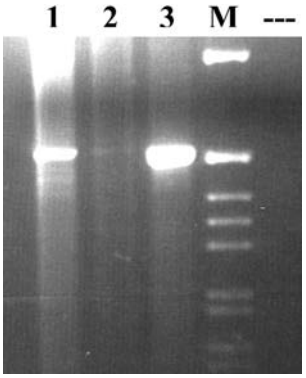

Fig. **1**. PCR results on formalin-fixed material. Lane 1, *B. mandrillaris* semi-nested PCR; lane 2, standard PCR; lane 3, *B. mandrillaris* positive control; M, 1 kb marker; - - -, negative control.

a putative *B. mandrillaris* culture derived from an environmental source, the soil of a potted plant, from this same patient's home. The DNA from both isolates was extracted, and DNA from both isolates was used as templates in PCR reactions using the *Balamuthia*-specific primer set. Both produced a PCR amplimer of 1,075 bp. Sequence analysis was performed on these 2 PCR products. Both sequences were identical to one another and also identical to other *B. mandrillaris* isolates previously sequenced. These results support other morphological and biochemical data that had determined that these isolates were *Balamuthia* [7]. This also supports the hypothesis that the environmental sample, the soil of a potted houseplant, was the source of this infection.

In the second application of this method, these primers were used on total DNA extracted from a necrotic formalin-fixed brain tissue sample from a putative case of amebic encephalitis. Initial amplification using the *Balamuthia* genus-specific primer set did not produce a visible band of the expected size, 1,075 bp. This was not completely unexpected since amplification of larger fragments (over 1,000 bp) is often difficult from formalin-fixed samples. In order to circumvent this problem, PCR was performed using the 5′ *Balamuthia* genus-specific primer in conjunction with another 3′ primer known to hybridize to the *Balamuthia* mitochondrial *rns* target, but which produces a smaller amplimer of ~500bp. This primer set was used on the DNA from the original extraction, and in a semi-nested PCR reaction on the initial PCR reaction that did not produce a visible band using the genus-specific primer set (Fig. 1). In Fig. 1, lane 1 shows that the semi-nested PCR on the original PCR product produces a strong band of the expected size using this new primer set. Lane 2 shows that this new primer set is also able to amplify a product, albeit lightly, from the original DNA extraction. These PCR products were sequenced, and they were determined to be *Balamuthia* following sequence analysis. The results led to the conclusion that the amebic encephalitis case of unknown origin was caused by *Balamuthia*. This application of the *Balamuthia* genus-specific primer set shows that with modification, it can be applied to formalin-fixed tissue samples. This should greatly expand the utility of this type of methodology if, as in this case, it can now be broadened to screen archived samples. This application also illustrates one of the current problems in diagnosis of a *B. mandrillaris* infection. The identification of *B. mandrillaris* as the source of the infection in these experiments was determined long after it could have been of use to the clinicians or patient involved in this case. Therefore, in future studies we envision the application of fluorescent in situ probes or quantitative PCR methods for meaningful real-time identification of the causative agents of amebic infections, such as those caused by *B. mandrillaris*.

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