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Genetic analysis among environmental strains of *Balamuthia mandrillaris* recovered from an artificial lagoon and from soil in Sonora, Mexico

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HIGHLIGHTS

- Description of new isolation technique for *Balamuthia mandrillaris* yielding 7 water isolates.
- This was only the second time that *B. mandrillaris* has been isolated from water samples.
- Comparison with DNA sequences of previous isolates confirmed all isolates are *B. mandrillaris*.
- Genetic variation in *B. mandrillaris* was assessed by Western Blots and DNA sequence comparisons.
- *B. mandrillaris* contains much less genetic variation than its sister taxa, Acanthamoeba.

GRAPHICAL ABSTRACT

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ABSTRACT

Since the first report of *Balamuthia mandrillaris* as a causative agent of granulomatous amoebic encephalitis in humans, the environmental niche of this amoeba was assumed to be restricted to soil and dust. A single isolation from water was recently made independently by us from Northern Mexico. Now we report the isolation of 8 new strains of *B. mandrillaris* from Mexico. This continues the pattern of an excess of isolates from North America, compared to other parts of the world. All of the new isolates are environmental isolates, 7 from water samples and one from soil. The identity of each isolate was confirmed by PCR and by examining the sequences of the mitochondrial 16S-like rRNA gene. Success in amplification was determined using comparisons of amplifications of DNA from the strain CDC: V039 and the water strain (ITSON-BM1) as positive controls. The DNA sequences of the new isolates were compared to older strains from clinical cases using phylogenetic analysis, showing very high sequence similarity. The similarity among the new isolates and with previous clinical and environmental isolates of *B. mandrillaris* was also examined using biochemical and immunological studies. High homogeneity of total protein products, and similarity in antigenic moiety among the eight new isolates and two controls was found. Taken together, the molecular and biochemical studies indicate very low levels of genetic variation within *B. mandrillaris*.

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1. Introduction

Balamuthia mandrillaris is a free-living amoeba that causes infectious diseases in humans and other mammals. Infections may affect the central nervous system (CNS) and other organs like liver, lungs, and skin lesions among others. The amoeba can infect both immuno-compromised and immuno-competent individuals (Visvesvara et al., 1993). The infection of the CNS is known as granulomatous amoebic encephalitis (GAE) (Marciano-Cabral and Cabral, 2003). This is a chronic usually fatal disease with difficult diagnosis. Infections seem to have no age, seasonal or distinct geographical specificity. Nevertheless, in a recent review (Bravo and Seas, 2012), it has been reported that these infections may be more common among warm climates around the world. More than 150 cases have been reported worldwide (Bravo and Seas, 2012; Cary et al., 2010). The higher rate in the Americas may be due to greater initial research interest on the part of investigators in this region. As physicians and researchers elsewhere become more familiar with this pathogen, patterns may change in the future (Lorenzo-Morales et al., 2013). Mortality rates have been estimated to approach 98%, and no specific successful treatment has yet been reported for these amoebic infections (Schuster et al., 2009).

The environmental source of *B. mandrillaris* is not well understood, since worldwide it has been isolated only a few times, with only 4 published isolations made from soil and dust samples (Dunnebacke et al., 2004; Niyyati et al., 2009; Schuster et al., 2003). It is presumed that the most common habitat of *B. mandrillaris* is in soil, associated with elevated amount of nutrients that allow *Balamuthia* to feed on other amoebae. Unlike most of the other amoebae, it does not feed on bacteria (Visvesvara et al., 2007).

In a recent study we accomplished an environmental isolation of *B. mandrillaris*, but unlike other published isolations, it was the first one made from a water sample (Lares-Jiménez et al., submitted). Pursuing the question of whether water could serve as major habitat for *Balamuthia*, we extended our examination of water sampling within freshwater bodies, in order to corroborate our previous finding. Sampled sites included different random locations with different depths in a freshwater lagoon in Northern Mexico. This was the same site from which our initial isolation of *Balamuthia* had been made. We also analyzed different nutrient-rich soil samples from a plant nursery.

2. Material and methods

2.1. Sampling

Ten water samples were taken using sterile one liter plastic bottles, from different random spots of the Nainari lagoon. The Nainari lagoon is an artificial lake of ~2 km of diameter, created in the west side of Ciudad Obregon (Mexico). The lagoon is a closed drainage basin, with inflow from a Canal Bajo which is the main route for agricultural water in the area. The city has semi-desert environmental conditions and is located in the Northwestern region of Mexico. The physicochemical parameters (salinity, dissolved oxygen) indicate a healthy water body with adequate oxygen levels to support aquatic life and conditions consistent with low salinity water for agricultural irrigation (García-Hernández et al., 2011). The lagoon has a basic pH (average pH ~9.0 in summer) because of runoff from surrounding areas. The concentration of coliform bacteria within the lagoon is low and does not exceed limits for recreation. Nitrate and phosphate flow into the lagoon is within acceptable thresholds and there is no evidence of algal blooms or eutrophication. The water temperature at the time of sample collection (July) ranged between 29.3 and 31.3 °C.

An additional 10 samples were taken from different soil sources from a commercial plant nursery within Ciudad Obregon. The amount of soil in each sample was approximately 5 grams collected into sterile centrifuge tubes. Samples were transported to the lab at room temperature and were processed within the first 2 h after sampling.

2.2. Sample processing

Flasks containing water samples were agitated and 50 mL from each was decanted into a sterile centrifuge tube, then centrifuged at 1700 rpm for 15 min on a Labnet Hermle Z383K (Woodbridge, NJ) refrigerated centrifuge. The supernatant was discarded and the bottom was resuspended. Soil samples were mixed with 5 mL distilled sterile water to produce a slurry mud-like mixture.

2.3. Amoeba isolation

Each processed sample was seeded onto the center of a Petri plate with non-nutrient agar (NNA) covered with *Escherichia coli* (NNE) taking care not to disperse the sample from the center. Samples were left to be absorbed for 10–20 min. After the samples were absorbed, the perimeter around the sample was cut and the center portion that had been covered by the sample was removed and reseeded onto a new NNE plate making an "Agar Sandwich". This step was performed in an attempt to reduce fungal dissemination throughout the plate, allowing motile amoebae to crawl out of the cut agar. To further reduce fungal growth, plates were incubated at 37 °C as well.

2.4. Primary cultures

Using an Axiolab (Carl Zeiss, Germany) inverted microscope, plates were checked after 24 h of incubation looking for amoebae coming out of the cut agar and that appeared to be under the surface within the agar, which is a characteristic feature of some amoebae including *B. mandrillaris*. Fungal-free spots with such suspicious amoebae among other amoebae were excised from the agar cut and reseeded onto new NNE plates until fungal-free cultures were obtained.

2.5. Subsequent cultures

It has been reported that *B. mandrillaris* does not feed on bacteria, leading to the assumption that other amoebae in the same sample which do feed on bacteria would have served as food source for *Balamuthia* in early transfers (Cuevas et al., 2006). Since *B. mandrillaris* does not feed on bacteria, but feeds instead on other amoebae or on cells in culture, segments of NNE plates that contained suspicious amoebae were excised and seeded into flasks with cells in culture, providing a first attempt of axenization. Material was transferred into flasks with VERO cell-line monolayer in DMEM Advanced with 10% fetal calf serum and 200 mg/mL of streptomycin and 200 UI/mL of penicillin. This procedure would act to eliminate bacteria, but provide a food source on which the possible *B. mandrillaris* could feed (Dunnebacke et al., 2004; Matin et al., 2008).

Flasks were incubated at 37 °C with a 5% CO_2 atmosphere. After 3–5 days of incubation, culture media was removed and placed carefully in a new flask, in case amoebae may be floating in the old media. Cell-free BM-3 medium (Schuster and Visvesvara, 2004) was added to the first flask. All samples were kept incubating at 37 °C with 5% CO_2 atmosphere and checked under inverted microscope daily looking for *Balamuthia*-like floating forms, *Balamuthia*-like amoebae could be attached to the surface of the flask or could be feeding on VERO cells. If *Balamuthia*-like amoebae were

observed attached to the surface of the flasks with old medium, the media was replaced with BM-3 medium. Flasks were kept incubating until massive growth was obtained.

2.6. Comparative tests – SDS–PAGE electrophoresis and Western Blot

To determine similarity or difference between any *Balamuthia*-like suspicious strains and a confirmed strain of *B. mandrillaris* (CDC: V039), comparative tests were made to examine the protein profile of each strain by using SDS–PAGE protein electrophoresis and immune response against hyper-immune mouse sera and normal human sera by Western Blotting.

A 1.5 mm 12% polyacrylamide gel was prepared according to standard protocols. 50 μg of *B. mandrillaris* extract in loading buffer were loaded into gel wells. Gel was run at 110 V for ~90 min in an electrophoresis chamber with running buffer. Nitrocellulose membrane, pads and the gel were soaked in transfer buffer for 5–10 min, and a "transfer sandwich" was built and placed in a semi-dry transfer chamber. Transfer was run at 120 mA for 35 min. Transfer was confirmed by Ponceau red staining. Membrane strips were cut and blocked with PBS with 5% non-fat milk for 1 h to reduce background. Membrane was twice briefly washed with PBS with 0.05% Tween 20 (PBS-T). A normal human serum with high titers against *B. mandrillaris*, and a negative human serum were added to the membrane strips at a 1:50 dilution in PBS with 0.1% non-fat milk, and incubated for 2 h at room temperature. Membrane strips were vigorously washed 5 times with PBS-T. Anti-human HRP conjugated antibody was added to membrane strips at a 1:10,000 dilution, and incubated for 2 h at room temperature. Membrane strips were again vigorously washed 5 times with PBS-T. Detection of protein bands was done by exposing membranes to Pierce ECL Western Blotting Substrate for 7 min and then exposed to film for 30 s–5 min.

2.7. DNA extraction and PCR

Flasks containing massive growth of suspected *Balamuthia* were chilled and agitated to remove amoebae from the flask surface. DNA was extracted from pellets by using the DNeasy kit (Qiagen, Inc., Valencia, CA). Following DNA extraction, PCR amplification was performed with the primer set Balspec16S 5′-CGCATGTATGAAGAAGACCA-3′ and Balspec16S 5′-TTACCTATATAATTGTCGATACCA-3′ (Booton et al., 2003a) which amplifies the mitochondrial 16S-like ribosomal RNA gene from *B. mandrillaris*. For each gene, 20% of the PCR product (25 μL) was electrophoresed on a 1% agarose gel followed by staining with ethidium bromide to confirm the success of PCR and determine whether the product was of the size expected. DNA from the original *B. mandrillaris* strain isolated from a baboon in San Diego (Schuster et al., 2003) was used as the positive control.

2.8. DNA sequencing

PCR products were prepared for automated sequencing using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). The amplification primers 5′Balspec16S and 3′Balspec16S also served as sequencing primers for the mitochondrial 16S-like gene. Sequencing was done using automated fluorescent sequencing protocols on an ABI 310 automated sequencing system (Applied Biosystems, Foster City, CA). Full length sequences were generated by concatenating the sequences from the two primers using the alignment tool in MEGA5 (Tamura et al., 2011). Full length sequences were aligned using CLUSTALW, as implemented in MEGA5, with previous sequences of the *Balamuthia* 18S rRNA gene (most from our lab) that have been deposited in Genbank. The sequences that were obtained were aligned with each other and with previous sequences of the *B. mandrillaris* mitochondrial 16S-like rRNA gene (most from our lab) that have been deposited in Genbank. Alignment was accomplished using CLUSTALW, as implemented in MEGA5. Phylogenetic relationships among sequences were inferred by neighbor-joining method (Saitou and Nei, 1987), (using the maximum composite likelihood method) and by maximum likelihood analysis, both implemented in MEGA5, using the best-fit model of nucleotide substitution as determined by the best-fit model option in MEGA5. The best-fit model for the data was determined to be the Tamura 3-parameter model with non-uniformity of evolutionary rates among sites modeled by using a discrete Gamma distribution (+*G*) with 5 rate categories (T92 + G). The reliability of data was tested by generating 100 bootstrap replicate data sets. The root of the tree was established using an outgroup.

3. Results

3.1. Primary cultures

From the 20 samples seeded on the NNE plates, 7 of the water samples and 1 sample from soil from a nursery showed suspicious positive amoebic growth under the surface within the agar. The isolates were labeled ITSON-2S (the soil sample) and ITSON-3W–ITSON-9W (the water samples. Isolate ITSON-1 is an isolate from a water sample that had been previously described (Lares-Jiménez et al., submitted). Unfortunately we missed the opportunity to correlate the depth of water and the putative positive samples. Soil samples consisted in typical agricultural soil consisting of 90% clay soil mixed with humidified sawdust as rudimentary compost. Those samples were examined daily, with only a few plates showing any fungal contamination due to incubation at 37 °C instead of room temperature. Contaminated cultures were cleared of fungal contamination by serial transfers to new plates, taking care to minimize the possibility of including any fungus in the piece of excised agar being used to seed the new plate.

3.2. Subsequent cultures

After 3–5 days of incubation, and following a transfer from DMEM medium into cell-free medium BM-3, surprisingly, all 8 cultures (7 from water and 1 from soil) showed *Balamuthia*-like floating forms in both types of culture, the flasks with cell cultures, and cultures with the remaining DMEM medium which were changed into BM-3 as long as the amoebae were seen to be attached to the bottom of the flask. For all 8 cultures, adaptation to BM-3 medium was achieved, and massive growth was obtained in 4–6 weeks of weekly changes of medium and incubation. Each culture of suspected *Balamuthia* reached confluency at different rates depending on their doubling times (Matin et al., 2008).

3.3. Comparison of protein profiles

SDS–PAGE was performed and repeated several times, using a silver staining technique for visualization. Results showed high molecular homogeneity among all 8 new environmental isolates. The isolates were also very similar in profile to that seen in the reference strain CDC: V039 (Fig. 1). Similarly, when protein profiles are examined by Western Blotting, the immune profiles against hyper-immune mouse sera (Fig. 2a), and normal human sera (Fig. 2b), produced highly homogenous results in comparisons of isolates. Again, similarity with the reference strain was high, but not necessarily complete. These results indicate a high level of similarity among all strains. Our results are consistent with previous

studies on other strains of *B. mandrillaris* isolated from either the environment or from clinical cases (Kucerova et al., 2011).

3.4. PCR confirmation

Comparison of PCR products between the 8 isolates, as well as with the positive control strain CDC: V039 (Schuster et al., 2003), confirmed that an rRNA gene amplimer consistent with that expected for *B. mandrillaris* was present in all eight strains. Putative identification as *B. mandrillaris* was possible, since the primer sets used are species specific (Booton et al., 2003a), and only the expected amplimer (1075 bp) was observed for each isolate.

3.5. DNA sequencing

DNA sequencing of PCR products from the mitochondrial 16S-like rRNA gene confirmed the identity of all the eight isolates as being representatives of *B. mandrillaris*. Sequences for 7 of the 8 samples were ~1000 bases in length. Sequencing of the sample labeled ITSON-9W was unsuccessful for the 3′ end of gene, probably due to interfering DNA from another organism (either a second *Balamuthia* that differed by an insertion/deletion change or another eukaryote whose gene could be amplified in part by the primers being used). None of the isolates differed from each other by more than six nucleotides in ~1000 bases of sequence. The partial sequence from ITSON-9W differed by 2–3 nucleotides from the remaining isolates. The sequences showed some additional slight variation from isolated previously reported in GenBank from our lab and by others (Booton et al., 2003b; Niyyati et al., 2009; Schuster et al., 2003). The maximum difference seen was 23 nucleotide differences (including in/del positions) for comparisons with the CDC: V451 isolate (Booton et al., 2003b).

The results of the phylogenetic analysis using MEGA5 is shown in Fig. 3. Both neighbor joining and maximum likelihood analyses produced similar results, and only the ML tree is presented. The isolates from Ciudad Obregon form a unique grouping. The sequences share three changes that differentiate them from the other reported sequences. We hesitate to make too much of this difference, since it is possible that one or more of the differences could represent sequencing error (either in these or previous sequences). However, each site was examined carefully on electropherograms and was unambiguous.

The sequences reported here expand the known genetic diversity of *B. mandrillaris*. Nevertheless, the species still remains highly homogeneous, especially in contrast to its putative nearest relatives, members of the genus *Acanthamoeba*. Comparisons with diversity that exist within a single species of *Acanthamoeba* is problematic, since the relationship of sequence similarity between Acanthamoeba isolates, and species names within *Acanthamoeba*

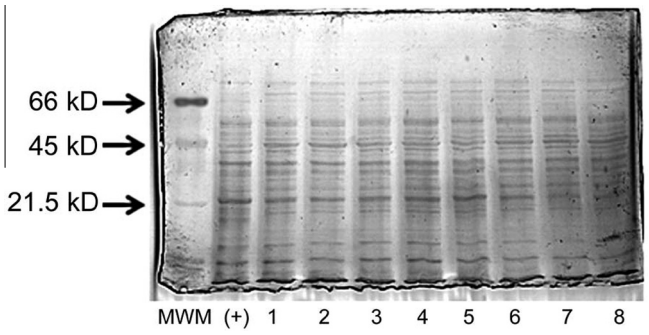

Fig. 1. SDS PAGE Electrophoresis gel of *Balamuthia mandrillaris* total proteins. (MWM) Molecular weight marker, (+) positive control CDC: V039; (1–8) environmental isolates.

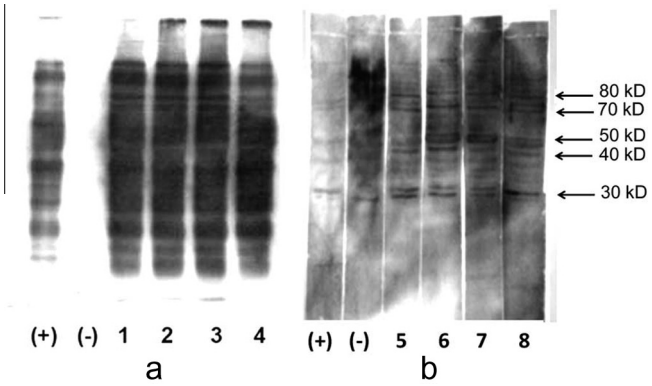

Fig. 2. (a) Western Blotting using anti-*B. mandrillaris* hyper-immune murine sera. (+) *B. mandrillaris* positive control CDC: V039, (−) negative control, (1–4) environmental isolates. (b) Western Blotting using anti-*B. mandrillaris* human sera. (+) *B. mandrillaris* positive control CDC: V039, (−) negative control, (5–8) environmental isolates.

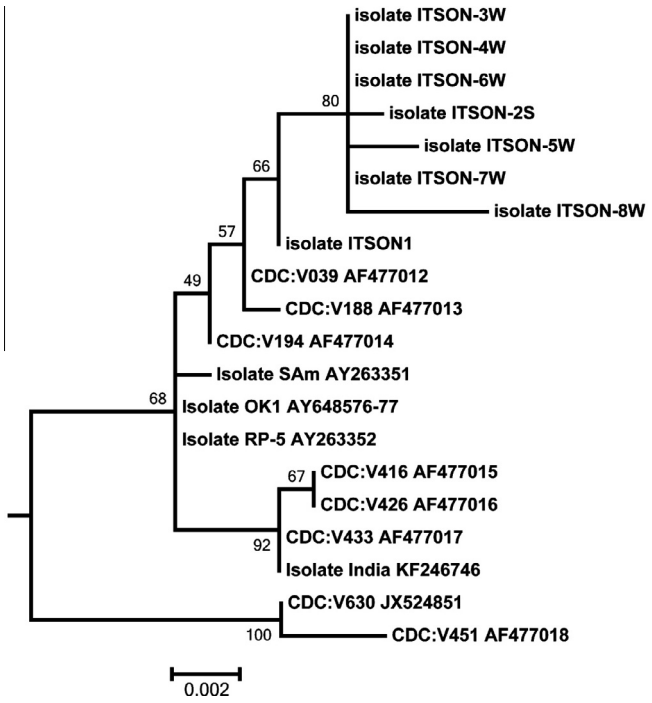

Fig. 3. Phylogenetic relationships for the sequence of the mitochondrial small subunit rRNA gene between the isolates of *B. mandrillaris* identified in this study and previous isolates of *B. mandrillaris* deposited in Genbank. Tree was constructed by maximum likelihood, using MEGA5. Only bootstrap values exceeding 50% are shown.

seems to much less clear than for *Balamuthia* (Fuerst et al., in press). The sequences have been deposited in Genbank with accession numbers KF896587–KF896594.

4. Discussion

In our studies reported here, fungal-free cultures of amoebae were obtained after just 1 or 2 passes onto new plates. Fungal contamination has been a major problem for the isolation of *Balamuthia* because of the low density of amoebae in the initial samples. The low impact of fungal contamination in our study was achieved due to the variation from the previous sampling procedure accomplished with the "agar sandwich". We feel that this allows motile

amoebae to move more rapidly apart from fungal contamination into clean agar. The low impact of fungal contamination appears to be a major factor in the success we found in isolating *Balamuthia* compared to previous attempts by ourselves and others.

The results presented here represent the first set of multiple sequences obtained in Mexico, and expand the knowledge concerning free-living amoeba in Mexico (Lares-Villa, 2001). Our results expand our knowledge concerning variation within *B. mandrillaris*. The species remains highly homogeneous, especially in comparison to levels of variation in other genera of free living amoebae such as *Acanthamoeba* and *Vermamoeba* (formerly *Hartmanella*) *vermiformis*. To illustrate this point, sequences that have been deposited in Genbank and associated databases for 98 *Acanthamoeba* sequences of the mitochondrial small subunit rRNA gene have been examined. These sequences show an average of 46.4 nucleotide differences from each other. By contrast, the 22 *Balamuthia* mitochondrial small subunit rRNA sequences that have been determined show an average of 4.54 differences between pairs of sequences. When quantifying genetic diversity, one measure that has been used is that of nucleotide polymorphism, θ, as defined by Nei and Li (1979). The estimates of nucleotide polymorphism for the comparisons among mitochondrial small subunit rRNA genes within *Balamuthia* and within *Acanthamoeba* were found to be 0.0079 and 0.0863, respectively. This again indicates that *Balamuthia* displays an order of magnitude less variation that observed in *Acanthamoeba*. It should be noted that the variation in *Acanthamoeba* represents that found in a multi-species genus, while *Balamuthia* is currently a monospecific genus. A similar comparison of variation with other free living amoebae cannot be performed at this time because there is no equivalent data set for the mitochondrial small subunit rRNA gene for any other taxa.

5. Conclusion

The success in obtaining seven new isolates from water confirms the presence of *B. mandrillaris* in aquatic habitats, expanding the spectrum of its ubiquity.

The genetic similarity among the new isolates, and their relationship with previous clinical and environmental isolates of *B. mandrillaris*, examined by using electrophoresis and Western Blot studies, showed very high homogeneity of total protein products and similar antigenic moiety among the eight new isolates and two controls. There was very high similarity of sequence among the eight new isolates and with previous strains. This is consistent with low levels of variation in both the nuclear 18S rRNA gene and the mitochondrial 16S-like rRNA gene. In general, *Balamuthia* shows significantly lower levels of genetic variation. The ecological niche occupied by *Balamuthia*, proposed to be that of an apex predator of the microscopic world, may be a major factor contributing to the low levels of genetic variation and lack of differentiation so far observed. Apex predators typically have smaller population sizes and may be under more severe natural selection to maintain competitive advantages. Both these factors will act to reduce the levels of genetic variation in a population compared to prey species.

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