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Short communication

In vitro culture, serologic and molecular analysis of *Acanthamoeba* isolated from the liver of a keel-billed toucan (*Ramphastos sulfuratus*)

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Abstract

Members of the genus *Acanthamoeba* are usually free-living amebae and are found in a variety of ecological niches including soil, fresh and brackish water, dust in air, filters of heating, ventilating, and air conditioning units, swimming pools and hot tubs, etc. Occasionally, they are also known to cause central nervous system infections in humans and other animals. We isolated into culture an amoeba from the liver tissue of a keel-billed toucan and identified it as *Acanthamoeba* sp. based on culture characteristics and immunofluorescent analysis. Further, we characterized the cultured amoeba and also the amoeba in the liver tissue as *Acanthamoeba*, genotype T4, by sequencing a diagnostic region of the nuclear small subunit ribosomal RNA gene.
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Keywords: *Acanthamoeba*; Keel-billed toucan; Indirect immunofluorescence; PCR; SSU rRNA,

1. Introduction

Members of the genus *Acanthamoeba* are well known as agents of diseases in humans as well as other animals (Martinez and Visvesvara, 1997; Schuster and Visvesvara, 2004). They are known to cause infections of the human cornea, *Acanthamoeba* keratitis, infections of the human skin and infections of the central nervous system (CNS) and lungs in humans and other animals including lower primates, dogs, horses, ovines, bovines, birds, reptiles, fishes and even invertebrates (Dyková et al., 1999; Martinez and Visvesvara, 1997; Rideout et al., 1997; Visvesvara and Stehr-Green, 1990). In this report we discuss the isolation of the amoeba from a ~6-year-old-keel-billed toucan (*Ramphastos sulfuratus*) that was found dead in the San Antonio, TX, Zoo and identify it as *Acanthamoeba* based on immunofluorescence and molecular assays.

2. Materials and methods

2.1. Necropsy

The bird was apparently fine until the day before death. A necropsy was performed on the toucan. Liver, spleen, thyroid, gall bladder, pancreas, intestine, heart,

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lungs, adipose and kidneys were removed and fixed in 10% buffered formalin. Portions of liver and spleen were also frozen and shipped to the Centers for Disease Control and Prevention (CDC).

2.2. Culture

Pieces of frozen liver tissue were triturated and inoculated into two non nutrient agar plates coated with a layer of bacteria as described before (Schuster, 2002) and one plate was incubated at 37 °C and the other at 44 °C. Pieces of frozen liver tissue were also inoculated into monkey kidney tissue culture (E6) monolayer with antibiotics and incubated at 37 °C as described previously (Visvesvara and Stehr-Green, 1990). Amoebae that grew out in the agar plates were also inoculated into liquid culture medium containing fetal bovine serum but no bacteria (Schuster, 2002).

2.3. Indirect immunofluorescence (IIF)

IIF was performed on formalin-fixed, paraffin-embedded sections of liver by deparaffinizing and covering the sections with several different rabbit antisera made against *Acanthamoeba castellanii, A. polyphaga, A. culbertsoni, Balamuthia mandrillaris* and *Naegleria fowleri* as described previously (Visvesvara, 1987).

2.4. DNA extraction and sequencing

DNA was extracted from two samples, a sample of toucan liver tissue and an *Acanthamoeba* sp. culture (CDC: V459) derived from the liver tissue. These samples were designated OSU: 04–020 and OSU: 04–23 respectively. Total DNA was extracted from tissue sample OSU: 04–020 and culture sample OSU: 04–023 using the DNeasy kit (Qiagen, Inc., Valencia, CA). Following DNA extraction, PCR was used to amplify the *Acanthamoeba* nuclear SSU rDNA (*Rns*) sequences using genus-specific primers JDP1 (5′-GGCCCAGATCGTTTACCGTGAA-3′) and JDP2 (5′-TCTCACAAGCTGCTAGGGGAGTCA-3′), which amplifies a region of the *Rns* that permits genotypic identification of an *Acanthamoeba* isolate following sequence analysis. PCR conditions for this reaction were as follows: initial denaturing step of 95 °C for 7 min, followed by 35–40 cycles of 1 min at 95 °C, 1 min at 60 °C, and 2 min at 72 °C (Booton et al., 2004; Ledee et al., 2003; Stothard et al., 1998). Ten microliters of a 50 μl PCR reaction were visualized on a 1% agarose gel. The remainder of the PCR product was prepared for sequencing using the Qiaquik PCR clean-up kit (Qiagen, Inc.). Three microliters of Qiaquik prepared PCR product was used directly in subsequent sequencing reactions.

Direct sequencing reactions were performed on these amplified *Rns* fragments using the ABI fluorescent automated sequencing system (Applied Biosystems). Direct sequencing suggested a mixed culture due to the presence of multiple peaks throughout the electorpherogram. Therefore, these products were subsequently cloned using a T/A vector cloning system. Specifically, 1 μl of a 1:10 dilution of the Qiaquik elution product was used in a T/A cloning system (Invitrogen, Inc., Carlsbad, CA) following manufacturer's instructions. Antibiotic selection produced putatively positive clones. Multiple clones were selected, grown in Kanamycin/LB media overnight, and prepared using the Qiaprep plasmid preparation kit (Qiagen, Inc.). Three microliters of resuspended plasmid DNA was then directly sequenced as described above using M13 forward primer located in the T/A cloning vector.

DNA sequencing of the cloned sequences was done with an ABI 310 automated fluorescent sequencing system using vector primers and a set of conserved primers and methods that have been used previously in our phylogenetic studies (Booton et al., 2004; Ledee et al., 2003; Schroeder et al., 2001; Stothard et al., 1998). Sequences obtained were aligned to other *Acanthamoeba* spp. sequences in our *Rns* database using the alignment program XESEE (Cabot and Beckenbach, 1989). *Acanthamoeba* sp. *Rns* sequences obtained in this study have been deposited in GenBank under the following accession numbers, DQ451160 (genotype T4 sequence 1), DQ451161 (genotype T4 sequence 2) (from toucan liver, OSU: 04:020) and DQ451162 (genotype T4 sequence 2) and DQ451163 (genotype T4 sequence 3) (from *Acanthamoeba* sp. culture, OSU: 04–023; CDCV459). *Note*: sequences DQ451161 and DQ451162 are identical to one another and referred to as genotype T4 sequence 2 in the remainder of this manuscript; however, since they were obtained from two different sources they must be deposited as two separate sequence submissions to the GenBank database.

3. Results

The body of the toucan appeared to be in good condition. The liver was a tan/pale brown in color, mottled, and appeared somewhat larger than expected with rubbery consistency. The gall bladder was full, and hemorrhage was present throughout the abdomen. Kidneys were large and slightly pale. The pancreas was firm and pale. The spleen was large and red. Based

on these findings a presumptive diagnosis of hemochromatosis and coelomic hemorrhage was made.

3.1. Histopathology

On microscopic examination the liver exhibited diffuse granulomatous inflammation and multifocal discrete granuloma formation (Fig. 1A). The granulomas were comprised predominantly of epithelioid macrophages and multinucleated giant cells with fewer mononuclear cells and heterophils. Occasional spherical amoeboid structures were detected within the cytoplasm of macrophages as well as free in the sinusoids. Granulomatous inflammation with amoebic trophozoites and cysts were also noted throughout the red pulp in the spleen (Fig. 1A, inset). Both the trophozoites and cysts exhibited a vesicular nucleus with a centrally placed large nucleolus. The thyroid had diffuse hyperplasia of follicular epithelium, reduced luminal diameter, and reduced colloid content. Kidney, intestine, heart, pancreas, adipose, and lung were histologically within normal limits.

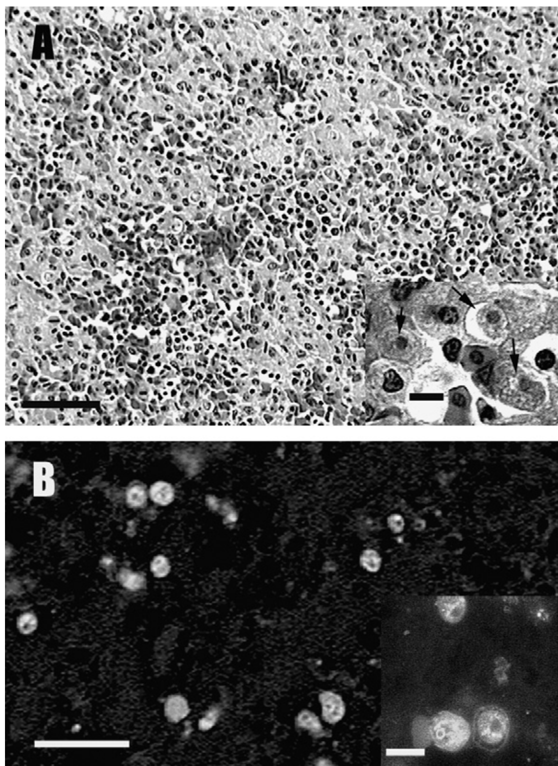

Fig. 1. (A) A low power view of a section of the liver showing intense inflammation with granulomatous changes, H&E, bar = 100 μm. *Inset*: A higher power view of the liver section showing 3 trophozoites (arrows) with a centrally placed large and round nucleolus within the nucleus, H&E, bar = 10 μm. (B) A section of the liver reacted with rabbit anti-*Acanthamoeba castellanii* serum. Note the positive reaction of the amebas in the section, bar = 50 μm. *Inset*: A higher magnification showing a brightly reacting trophozoite and a cyst, bar = 10 μm.

The final diagnosis was severe granulomatous hepatitis and splenitis with intralesional amoebae in the lesions and hyperplastic goiter. Inflammatory changes in the liver and spleen were probably due to the presence of *Acanthamoeba* and were the likely cause of hemorrhage and death.

Amoebic organisms, only in those tissue sections that were covered with the anti-(*A. castellanii*, *A. polyphaga* and *A. culbertsoni*) sera, reacted and produced bright apple green fluorescence (Fig. 1B). They did not show any reactivity with any of the other sera indicating that the amoebae belonged to the genus *Acanthamoeba*.

3.2. In vitro culture

Amoebae grew out on agar plates incubated at both 37 °C and 44 °C. The growth of the amoebae however was luxurious at 44 °C. The amoebae completely covered the agar plate within 24 h and thereafter they began to encyst. The trophozoites measured around 10–20 μm and possessed a large nucleus with a centrally placed large and round nucleolus (Fig. 2A). The trophozoites were also characterized by the presence of thorn-like filamentous projections, acanthopodia, emanating from their surface. The cysts were round to oval and measured around 8–14 μm. The cysts had double walls with the outer cyst wall being wrinkled and the inner cyst wall was either round or oval (Fig. 2B). The amoebae were identified as *Acanthamoeba* and the isolate was labeled as CDC: V459. Amoebae also grew out in the cell culture flasks although it took several days before they became discernible. However, the cell cultures were badly contaminated with fungi and were therefore discarded.

3.3. PCR and sequencing

PCR amplification using *Acanthamoeba* genus-specific *Rns* primers JDP1 and JDP2 produced a single band of the expected size from DNA extracted from OSU: 04–020 and OSU: 04–023 (CDC: V459). Direct sequencing of these products produced multiple peaks in the electropherogram suggesting the possibility of multiple sequences in the sample. Therefore, these PCR products were cloned and positive clones were sequenced. Analysis of positive clones resulted in the determination of a total of three different sequences from these two samples. Sequence 1 was found only in

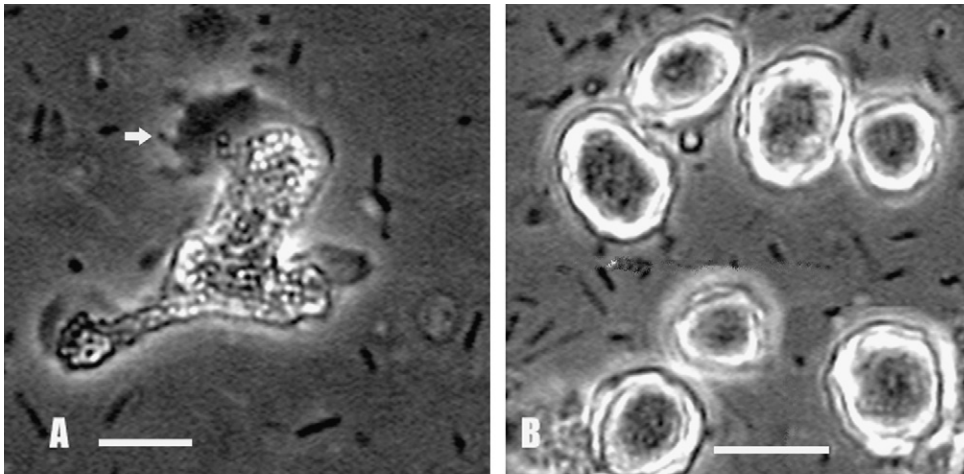

Fig. 2. (A) A trophozoite with acanthopodia at arrow, bar =10 μm; (B) cysts with double wall, an outer wrinkled wall and an inner oval or polygonal wall, bar = 10 μm.

the liver tissue sample OSU: 04–020. Sequence 2 was found in the liver sample, OSU 04–020, and also was obtained from the cultured *Acanthamoeba* sp. sample OSU: 04–023. The third sequence, sequence 3, was only obtained from the cultured *Acanthamoeba* sp. sample OSU: 04–023. Comparative sequence analysis of these *Rns* sequences to other sequences in our databases determined all were genotype T4 acanthamoebae. Sequence 1 from OSU: 04–020 (liver tissue) was identical to *Rns* sequence from *Acanthamoeba* sp. Rodriquez. Sequence 2 obtained from OSU: 04–020 and OSU: 04–023 was a novel genotype T4 *Rns* sequence. Sequence number 3, not found in OSU: 04–020 was obtained from OSU: 04–023. This sequence was also genotype T4, and also represents a novel *Rns* sequence not obtained from any previous isolates (Stothard et al., 1998).

4. Discussion

Small free-living amoebae belonging to the genera *Acanthamoeba*, *Balamuthia* and *Naegleria* have been known as soil dwellers and feed on bacteria and detritus (Martinez and Visvesvara, 1997; Schuster and Visvesvara, 2004). Several species of *Acanthamoeba* (*A. castellanii*, *A. culbertsoni*, *A. healyi*, *A. polyphaga*, *A. rhysodes),* the only known species of *Balamuthia*, *B. mandrillaris*, and only one species of *Naegleria*, *N. fowleri* have been known to cause central nervous system (CNS) infection in humans (Martinez and Visvesvara, 1997; Schuster and Visvesvara, 2004; Visvesvara and Stehr-Green, 1990). Further, *Acanthamoeba* is also known to cause a sight-threatening infection of the human cornea, *Acanthamoeba* keratitis (Martinez and Visvesvara, 1997). Additionally, *Acanthamoeba* spp. and *B. mandrillaris* have also been known to cause CNS infections in primates such as gorilla, baboon, gibbon, monkeys and other mammals including dogs, cattle, buffalo, horses and kangaroo (Martinez and Visvesvara, 1997). *N. fowleri* also has been found recently to cause infections in a South American tapir and cattle (Daft et al., 2005; Lozano-Alarcon et al., 1997). A recent study, based on sequencing of the SSUrRNA gene, has shown that several *Acanthamoeba* isolates from fish and those associated with human infection, *Acanthamoeba* keratitis, belong to the same T4 phylogenetic grouping, suggesting that features that enable these amoebae to infect animals may also help these amoebae to infect humans (Dyková et al., 1999). These amoebae have been described as amphizoic amoebae (Page, 1988) because of their ability to live within animal tissue and exist as free-living organisms in nature.

A total of three unique *Rns* sequences were obtained in this study. These three sequences were compared to an *Rns* sequence alignment of ~70 isolates of *Acanthamoeba* to determine genotype. All were identified by sequence comparison analysis as genotype T4. Genotype T4 acanthamoebae is a large assemblage of *Acanthamoeba* isolates from both clinical and natural environments. This is a phylogenetically closely related genotype (<5% sequence dissimilarity within genotype T4 using reliably aligned regions of the entire *Rns* gene) that contains nearly 10, or more, species of *Acanthamoeba*. We have previously obtained multiple *Rns* sequences from clonally isolated *Acanthamoeba* strains derived from infections (Booton et al., 2002). The two most likely

explanations for this observation are the presence of multiple strains in a single sample, or the presence of alternate *Rns* genes in a single strain. We have investigated this by repeated clonal isolation of *Acanthamoeba* strains that produced multiple *Rns* sequences. These analyses repeatedly resulted in obtaining the same multiple sequences (Booton et al., 2002; Booton, unpublished data). These investigations have led us to believe that we are amplifying alternative *Rns* sequences present in single strains of *Acanthamoeba*. This phenomenon awaits further investigation with respect to the possible reasons for the maintenance of alternative alleles and the apparent lack of concerted evolution of these sequences to a single allele in some strains.

In the current toucan *Acanthamoeba* infection analysis one of the genotype T4 sequences (sequence 1 of OSU:04–020 from toucan liver tissue) was identical in this *Rns* region to another sequence previously sequenced: *A*. sp. Rodriguez. In our phylogenetic analyses the sequence of *A*. sp. Rodriguez is found within a sub-clade of seven isolates of *Acanthamoeba* genotype T4 (designated T4-6), which is strongly supported by bootstrap analysis (G. Booton, unpublished data). The seven isolates of T4-6 are phylogenetically very closely related; the average dissimilarity within T4-6 is 0.5%. The remaining two sequences obtained in this study were new, unique genotype T4 sequences. Due to the high sequence variability within part of this diagnostic region, reliable alignments to other T4 isolates was not robust. Nonetheless, they were identifiable as genotype T4 *Acanthamoeba* based on sequences flanking these highly variable regions. Whether or not these new sequences represent potential new subclades of T4 awaits the acquisition of more sequence data. In summary, we have shown that *Acanthamoeba* strains with the same genotype capable of causing infections in humans (T4) can also be responsible for fatal infections in other animals, in this case a toucan.

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