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Isolation, morphologic, serologic and molecular identification of *Acanthamoeba* T4 genotype from the liver of a Temminck's tragopan (*Tragopan temminckii*)

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ABSTRACT

Members of the genus *Acanthamoeba* are usually free-living amoebae that are found in a variety of ecological niches including soil, fresh and brackish water, dust in the air, heating, ventilating, and air conditioning filters, swimming pools and hot tubs. Occasionally they are also known to cause central nervous system infections in humans and animals. We isolated into culture an amoeba from the liver of a Temminck's tragopan (horned pheasant) (*Tragopan temminckii*) that died of amoebic infection. We identified the infecting amoeba as *Acanthamoeba* sp. based on culture characteristics, cyst morphology and immunofluorescence assays. Additionally, we identified the amoeba as *Acanthamoeba*, genotype T4, by sequencing a diagnostic region of the nuclear small subunit ribosomal RNA gene.

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1. Introduction

Members of the genus *Acanthamoeba* are well known opportunistic agents of diseases in humans and animals (Martinez and Visvesvara, 1997; Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004; Visvesvara et al., 2007). Acanthamoebae can infect and cause severe lesions in the central nervous system (CNS) as well as infections of lung, skin and nasal sinus and eye in humans. Additionally, they have caused infections with severe disease or death in other animals including lower primates, dogs, horses, ovines, bovines, birds, reptiles, fishes and invertebrates (Dyková et al., 1999; Martinez and Visvesvara, 1997; Rideout et al., 1997; Walochnik et al., 1999; Visvesvara et al., 2007). In this report we discuss the isolation of an amoeba that caused the death of a 5-mo-old-female Temminck's tragopan (*Tragopan temminckii*), commonly called horned pheasants, in a zoo in OH. We identified it as *Acanthamoeba* based on cyst morphology, immunofluorescence and molecular assays.

2. Materials and methods

2.1. The case

A 5-mo-old-female Temminck's tragopan (*T. temminckii*) and a sibling were received by a zoo in OH from another institution and were placed into a routine 30-day quarantine. Prior to shipment, a physical examination, complete blood count, and biochemical profile were within normal limits except for elevated creatine phosphokinase. Direct and flotation examination of the feces revealed no

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parasites or ova. Both birds appeared to be doing well until the bird of the current report was found dead without any prior clinical signs 11 days after arrival.

A post-mortem examination was conducted within 12 h after death. Aerobic and anaerobic cultures of the bile and aerobic culture of the lungs were obtained. Representative samples of all organs were fixed in 10% neutral buffered formalin, processed routinely, sectioned on a microtome at 5.0 μm thickness, mounted on frosted glass slides, and stained with hematoxylin and eosin (HE) for histologic examination. Representative samples of intestines, lungs, kidney, liver, and spleen were frozen. Portions of frozen liver and spleen were shipped to the Centers for Disease Control and Prevention (CDC).

2.1.1. In vitro culture

The frozen liver was thawed in a 37 °C water bath, cut into small pieces, minced and divided into three portions. One portion was inoculated into a non nutrient agar plate coated with a layer of *Escherichia coli* and the second portion was inoculated into a human lung fibroblast (HLF) cell line containing 100 μg/ml gentamicin as described before and both incubated at 37 °C (Schuster, 2002). Amoebae that grew in the agar plates were also inoculated into liquid culture medium (PYG) containing 5% fetal bovine serum and gentamicin but no bacteria (Schuster, 2002)

2.1.2. Indirect immunoflourescence

Indirect immunoflourescence (IIF) was performed on formalin-fixed, paraffin-embedded sections of liver by deparaffinizing and covering each section with a 1:200 dilution of one of three different rabbit antisera made against *Acanthamoeba castellanii*, *Balamuthia mandrillaris* and *Naegleria fowleri* as described previously (Visvesvara, 1987)

2.1.3. DNA extraction, PCR, and sequencing

DNA was extracted from an *Acanthamoeba* sp. culture (CDC: V601) derived from the liver. This sample was designated OSU: 08-016. Total DNA was extracted from the culture sample OSU: 08-016 using the DNeasy kit (Qiagen, Inc., Valencia, CA). Following DNA extraction, PCR was performed to amplify the *Acanthamoeba* nuclear SSU rDNA sequences using genus-specific primers JDP1 (5′-GGCCCAGATCGTTTACCGTGAA-3′) and JDP2 (5′TCTCACAAGCTGCTAGGGGAGTCA-3′), which amplifies a region of the SSU rDNA that permits genotypic identification of an *Acanthamoeba* isolate following sequence analysis (Booton et al., 2002). This *Acanthamoba* genotype identification method is based on comparing the new sequence to a large database of sequences obtained from our lab and others. The identification of different rDNA genotypes was originally developed by Stothard et al. (1998). This method placed isolates in different genotypes if their full rDNA sequences differed by 5% or more in sequence alignment comparisons. This resulted in 12 different *Acanthamoeba* genotypes at that time (Stothard et al., 1998). Other genotypes using this methodology have been identified since that time (Horn et al., 1999, 2002; Gast, 2001; Hewitt et al., 2003). Comparison of new sequences obtained from this genotypically informative rDNA region to these genotype reference sequences allows *Acanthamoeba* researchers to quickly determine genotype.

PCR conditions for this reaction were as follows: initial denaturing step of 95 °C for 7′, followed by 35–40 cycles of 1′ @ 95 °C, 1′ @ 57 °C, and 2′ @ 72 °C (Booton et al., 2002, 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., Valencia, CA). Three microliters of Qiaquik prepared PCR product was used directly in subsequent sequencing reactions.

Direct sequencing reactions were performed on these PCR amplified fragments using the ABI fluorescent automated sequencing system (Applied Biosystems). DNA sequencing of the PCR amplimers was done with an ABI 3100 automated fluorescent sequencing system using vector primers and a set of conserved primers and methods as described previously (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 database using the alignment program MEGA 4 (Tamura et al., 2007). The *Acanthamoeba* sp. sequence obtained in this study has been deposited in GenBank under the following accession number, GQ889265 from *Acanthamoeba* sp. culture, OSU: 08-016 (CDC: V601).

3. Results

On post-mortem examination, the bird was noted to be thin and had lost approximately 20% of its body weight (from 825 to 650 g). Gross examination revealed splenomegaly and the presence of diffuse 1–2 mm white nodules throughout the hepatic parenchyma. The ceca were bilaterally swollen and the right cecum was filled with caseous material. The abdominal air sacs contained brown fluid and both kidneys were diffusely pale, swollen, and friable.

Aerobic culture of the bile yielded a heavy growth of non-hemolytic, rough *E. coli* while the anaerobic culture was negative. Aerobic culture of the lung yielded heavy growths of a non-hemolytic rough *E. coli*, *Kelbsiella pneumoniae* ss. *pneumoniae*, and *Enterococcus faecalis*.

3.1. Histopathology

The cecal lumina contained inflammatory pseudomembranes comprised of heterophils, fibrin, and histiocytes. The underlying mucosa was ulcerated, and the wall had a transmural infiltrate of mixed inflammatory cells admixed with bacteria and amoebae. Inflammation extended onto serosal surfaces and into the air sacs. Numerous coalescing foci of necrosis and heterophilic to granulomatous inflammation were present throughout the hepatic parenchyma and associated with large numbers of amoebae.

Moderately increased numbers of histiocytes and plasma cells were in the red pulp of the spleen, and some of the histiocytes contained phagocytized cell debris or hemosiderin. Some areas of hemorrhage and necrosis associated with few amoebae were noted in the red pulp. The pulmonary parenchyma was congested, the intersti-