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First time identification of *Acanthamoeba* genotypes in the cornea samples of wild birds; Is *Acanthamoeba* keratitis making the predatory birds a target?

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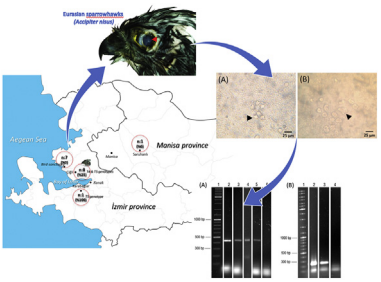
HIGHLIGHTS

- *Acanthamoeba* infection was detected in wild bird cornea samples with/without keratitis for the first time in the world.
- *Acanthamoeba* can be a cause of keratitis in wild birds of Turkey.
- Predator birds can be a target of other wild animals due to loss of sight ability caused by *Acanthamoeba*.

GRAPHICAL ABSTRACT



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ABSTRACT

Acanthamoeba is a free-living amoeba which can be isolated from environment and among others well known as an opportunist protozoan parasite causing infections in humans and animals. Eyes are extremely important for the wild birds and losing sight ability due to Acanthamoeba can be dangerous.

The studies on *Acanthamoeba* infection in wild birds is very few in world and Turkey therefore we aimed to screen deceased wild birds found in İzmir and Manisa provinces located in western Turkey using PCR and non-nutrition agar (NNA) plate method.

Cornea samples were obtained from 18 deceased wild birds. During the external examination, signs of keratitis were observed in two Eurasian sparrowhawks *(Accipiter nisus)*. All of the corneal samples were analyzed by two PCR methods and NNA plate.

According to results, the *Acanthamoeba* positivity in corneal samples was 16.6% and 5.5% by PCR and plate method, respectively. According to sequencing data, two of isolates belonged to genotype T5 and one was genotype T4.

In conclusion, *Acanthamoeba* infection was detected in wild bird cornea samples with/without keratitis for the first time in the world. The result of this study also show that *Acanthamoeba* can be a cause of keratitis in wild birds of Turkey and thus these predator birds can be a target of other wild animals due to

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loss of sight ability. In terms of public health, these results show the importance of wild birds as a source of *Acanthamoeba* infection in nature.

1. Introduction

Free-living amoebae (FLA) belonging to genus *Acanthamoeba* is one of the most common protozoan worldwide. *Acanthamoeba* is resistant to environmental conditions and have been isolated from soil, beach sand, drinking water, lakes, swimming pools, wastewater, seawater, bottled mineral water, distilled water bottles, air, ventilation systems, dialysis systems, hydrotherapy areas in hospitals, dental irrigation systems, insects, vegetables, and surgical instruments (Carlesso et al., 2010; Caumo and Rott, 2011; Landell et al., 2013).

Some species of the *Acanthamoeba* genus lead to opportunistic and non-opportunistic infection in humans and animals (Khan, 2006; Marciano-Cabral and Cabral, 2003; Visvesvara et al., 2010). *Acanthamoeba* infection is frequently detected in hosts with poor immune status. *Acanthamoeba* infection can also be detected in healthy people (Rocha-Cabrera et al., 2015). *Acanthamoeba* can cause granulomatous amebic encephalitis (GAE), amebic keratitis (AK) and disseminated amebic disease (e.g., pulmonary infections, sinusitis and dermatitis) in humans (Schuster and Visvesvara, 2004; Visvesvara et al., 2007, 2010). *Acanthamoeba* can also cause lethal encephalitis, dissemination or keratitis in various animals such as birds, dogs, horses, sheep, reptiles, fishes, and insects (Valladares et al., 2015).

Currently, *Acanthamoeba* species pathogenicity has been associated with genotypes. Genotyping of *Acanthamoeba* species is based on the 18S rRNA gene and 20 different genotypes (T1-T20) have been identified. Recently, two new genotypes, named T21 and T22 have been identified but not published yet (Genomes of Acanthamoeba, 2017). The most frequent genotype in clinical specimens was reported to be the genotype T4, which can be obtained from humans and animals (Behera et al., 2016; Lorenzo-Morales et al., 2005; Qvarnstrom et al., 2013). In addition, genotypes T1, T2, T3, T4, T5, T6, T10, T11, T12, T13 and T15 may cause encephalitis and keratitis in humans (Omana-Molina et al., 2016; Siddiqui and Khan, 2012).

In wild birds, *Acanthamoeba* may cause keratitis which may affect their feeding habit and survival. There is limited number of *Acanthamoeba* studies related to birds in literature. In these studies, *Acanthamoeba* T4 genotype has been isolated from the liver tissues of a keel-billed toucan (*Ramphastos sulfuratus*) and Temminck's tragopan (*Tragopan temminckii*) that died of amebic infection (Visvesvara et al., 2007, 2010). This study is the first time that the presence of *Acanthamoeba* has been investigated in wild birds' eyes worldwide. We aimed the study of cornea samples from deceased wild birds, evaluating the presence of *Acanthamoeba* at culture and genotyping level.

2. Material and methods

2.1. Ethics statement

Deceased wild birds were provided by the İzmir Natural Life Park and İzmir Bird Sanctuary (i.e. İzmir Bird Paradise). Deceased wild birds were found in İzmir Bird Sanctuary or brought to İzmir Natural Life Park Clinics from various districts of Izmir province and Manisa province located in western Anatolia. All experiments were performed under the instructions and approval of the Institutional Animal Care and Use Committee (IACUC) of Ege University for animal ethical norms (Permit number: 2014–016).

2.2. Samples

The deceased birds were found in İzmir Bird Sanctuary (n: 7) or were brought to İzmir Natural Life Park Veterinary Clinics from districts of Çiğli (n: 8), Karabağlar (n: 1) located in İzmir Province and from Saruhanlı (n: 1) located in Manisa Province. In addition, there was a bird with unknown origin (Table 1) (Fig. 1). As the animals arrived to the laboratory, eyes were externally examined for sings of keratitis such as opacity in the cornea. After the systemic evaluation, the corneas of all the birds were removed and divided in equal two pieces under sterile conditions. Each half cornea piece from two eyes was inoculated to two different non-nutrition agar plates and remaining half pieces were used during DNA isolation.

2.3. In vitro culture

In vitro culture of *Acanthamoeba* was performed as described (Khan, 2006; Marciano-Cabral and Cabral, 2003). Briefly, cornea samples were transferred centrally onto petri dish containing 1.5% non-nutrient agar (NNA) covered with an inactivated *Escherichia coli* (non-mucous bacteria) suspension. NNA agar plates were prepared with mixing 1.5% (w/v) Oxoid no.1 agar in Page's amoeba saline (2.5 mM NaCl, 1 mM KH_2PO_4, 0.5 mM, Na_2HPO_4, 40 μM $CaCl_2$-$6H_2O$ and 20 μM $MgSO_2 \cdot 7H_2O$) and then by adjusting pH to 6.8–6.9 with KOH. The mixture was autoclaved and dispensed to 80 mm wide petri dishes. As the cornea samples were inoculated to the petri dishes, they were sealed with Parafilm® (Pechiney Plastic, Chicago, US) and incubated at 30 °C for 15 days. Each plate was examined daily under inverted microscope (at ×100) to check the presence of genus *Acanthamoeba*.

2.4. Molecular detection

The samples obtain from 18 birds were investigated for the presence of *Acanthamoeba* DNA using the two different PCR reactions targeting 18S rRNA gene (Pasricha et al., 2003; Schroeder et al., 2001; Mathers et al., 2000; Nelson et al., 1999).

DNA isolation from cornea samples was performed using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol with minor modifications as described (Döşkaya et al., 2011). Briefly, 200 μl ATL buffer, 50 μl proteinase K, 150 μg zirconia beads (2.0 mm) and 50 μg glass beads (0.1 mm) (BioSpec Products) were added to corneal samples and incubated overnight in a thermomixer (Lab4You) with 1400 rpm at 56 °C. Thereafter, 200 μl AL buffer was added to the melted tissue, mixed vigorously and incubated at 70 °C for 10 min. After incubation, 200 μl ethanol was added to the mixture and transferred to filter tubes. Next, the filter tubes were washed and DNA sample was obtained by adding 200 μl elution buffer. The DNA samples were kept at −20 °C until the PCR experiments.

PCR targeting ~463 bp product of the *Acanthamoeba* 18S rRNA gene (GenBank accession no. U07413) was performed as described

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