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VERSITA

First detection and genotyping of human-associated microsporidia in wild waterfowl of Slovakia

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Abstract

A total of 47 avian faecal samples of wild waterfowl (great cormorant – *Phalacrocorax carbo*, great crested grebe – *Podiceps cristatus*, white stork – *Ciconia ciconia*) trapped in the eastern Slovakia were screened for the presence of human pathogenic microsporidia by microscopy and real-time SYBR Green PCR method using species primers and sequenced. Microscopic analysis showed presence in 32 samples (29 cormorants, 3 dippers). Microsporidial DNA (*Encephalitozoon cuniculi* genotype I) was identified in 19 faeces samples (40.4%) namely cormorants in 17 out of 40, one dipper of 5 and a stork out of 2. The present work describes three new host species of the bird population in microsporidium *Encephalitozoon cuniculi* genotype I which confirms the theory of low specificity of this species. .

Keywords

Waterfowl, SYBR Green PCR, microscopic method, *Encephalitozoon cuniculi*, genotype I

Introduction

Microsporidia are small obligate spore-forming intracellular parasites that infect a broad range of invertebrates and humans. They were recognized as a causative agent for many hosts including insects, fishes, birds, rodents and primates. They are classified as category B organisms on the NIAID Category A, B and C Priority Pathogens List. More than 1200 species of microsporidia belonging to ~ 150 genera have been reported (Didier 2005). At least 14 microsporidian species have been identified as opportunistic or emerging human pathogens (Didier *et al*. 2004). The most common microsporidial infections in humans and animals are caused by *Enterocytozoon bieneusi* and *Encephalitozoon* spp. (Didier *et al*. 2004, Luptáková and Petrovová 2011). Most at risk are immunodeficient or immunosuppressed individuals, old and young people, whose immune system is either weak or not sufficiently developed (Gamboa-Dominquez *et al*. 2003, Didier 2005). Microsporidia in vertebrates most commonly infect intestinal tract and can cause severe, persistent diarrhea (Didier 2005). However, the spores were isolated from virtually all organ systems. Routes of infection transmission in most cases are by uro-oral, and fecal-oral route, but can also cause infection through the eye or injured skin. Identification, removal or disposal by conventional water filtration techniques is problematic due to small size and resistance of spores (there is also the possibility of their resistance to chlorine) and the treatment itself is often unsuccessful (Rinder 2004). The four most common microsporidian species, *Enterocytozoon bieneusi*, *Encephalitozoon hellem*, *E. cuniculi* and *E. intestinalis* have been diagnosed in various species of pets and wild birds. The most frequently detected species are *E. hellem* (Slodkowicz-Kowalska *et al*. 2006, Kasickova *et al*. 2009) and *E. bieneusi* (Slodkowicz-Kowalska *et al*. 2006, Graczyk *et al*. 2007, Bart *et al*. 2008, Kasickova *et al*. 2009), while in recent years, also species of *E. intestinalis* (Slodkowicz-Kowalska *et al*. 2006, Bart *et al*. 2008, Kasickova *et al*. 2009) and *E. cuniculi* (Reetz 1994; Kasickova *et al*. 2007, 2009; Malcekova *et al*. 2011) have been identified. Based on available informations, the presence of microsporidian in waterfowls has been described only in isolated cases in Poland, in wild and farmed species of birds, namely *E. hellem* (*Anas platyrhynchos*, *Cygnus olor*, *Anser anser*, *Cygnus melanocoryphus*, *Cygnus atratus*, *Coscoroba coscoroba*, *Balearic pavonina*; Slodkowicz-Kowalska *et al*. 2006) and in one case *E. intestinalis* (*Anser anser domestica*; Slodkowicz-Kowalska *et al*. 2006).

Paper presents detection of microsporidian *E. cuniculi* presence in several species of wild waterfowl (*Phalacrocorax*

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carbo, *Podiceps cristatus*, *Ciconia ciconia*) trapped in eastern Slovakia, using microscopic and molecular methods.

Materials and methods

Fecal samples

Fecal samples, provided by the Department of Infectious Disease and Epizootology and Avian and Exotic UVLF in Košice, originated from 40 great cormorants (*Phalacrocorax carbo*), 5 grebes (*Podiceps cristatus*) and two white storks (*Ciconia ciconia*). Cormorants, grebes and storks came from the same area of eastern Slovakia. The fecal samples were frozen and stored at –18°C prior examination.

Positive control

Spores of *E. cuniculi* isolated from kidney of domestic rabbit (*Oryctolagus cuniculus*; breed Nederland Dwarf) were used. Spores were cultured in RK 13 (rabbit kidney cells strain) applying RPMI 1640 medium supplemented with 5% BOFES (bovine fetal serum; Biocom Slovakia), antibiotics and antimycotics (penicillin G, Streptomycin, Amphotericin B; Biocom Slovakia).

Microscopic analysis

After processing the samples of faeces and staining them with optical brightener RYLUX D, the spores were observed under a fluorescence microscope (Vavra *et al*. 1993). Then they were examined in a fluorescent microscope ZEISS JENALUMAR with a 405–490 nm excitation filter, 510 nm colour rays, and a 550 nm barrier filter for green fluorescence.

Molecular analysis

DNA isolation: DNA from fecal samples was extracted following mechanical disruption: 200 µl of spores [100 µl spore suspension with 100 µl cell lysis buffer (NET 50: 4M NaCl; 0.5M EDTA pH 8.0; 1M Tris + 30% Sarcosine – Sigma/Aldrich)] boiled for 30 min, immersed into liquid nitrogen and de-frost by +100°C in turns. DNA extraction was performed using commercial isolation kit DNA Sorb B (AmpliSens, Ecoli) and procedure according to manufacturer's instructions.

Real-time SYBR Green PCR: The new *Encephalitozoon* – specific primer pair for real-time SYBR Green amplification was designed using program Primer Express® Software v. 2.0 (Applied Biosystems, Foster City, CA) and was chosen to amplify the ITS region, part of the small subunit (SSU) rRNA and part of the large subunit (LSU) rRNA genes of *E. cuniculi*, *E. intestinalis* and *E. hellem* at an annealing temperature of 60°C. The forward primer ecfITSf (5′-TGTACACACCGCCCGTCG-3′), complementary to positions 2920 to 2938 (*E. cuniculi*), 1187 to 1205 (*E. hellem*) and 2644 to 2627 (*E. intestinalis*), was designed by using a published GenBank sequences of *E. cuniculi* (accession number AJ005581.1), *E. hellem* (AF272836.1) and *E. intestinalis* (CP001951.1). The reverse primer ecfITSr (5′-TTTCACTCGCCGCTACTC-3′) was designed to be complementary to position 3252 to 3270 of GenBank sequences of *E. cuniculi* (AJ005581.1), 1528 to 1546 of *E. hellem* (AF272836.1) and 2327 to 2310 of *E. intestinalis* (CP001951.1). The species-specific primer pair (forward MSP3 5′-GGAATTCACACCGCCCGTC(A/G)(C/T)TAT-3′ and reverse MSP4B 5′- CCAAGCTTATGCTTAAGTCCAGGGAG-3′) was used for amplification of the ITS region, part of the small subunit (SSU) rRNA and part of the large subunit (LSU) rRNA genes of *E. bieneusi* at an annealing temperature of 54°C (Katzwinkel-Wladarsch *et al*. 1996). Amplifications were performed in 25 µl reactions containing 12.5 µl Fast Universal SYBR Green Master (Roche), 0.5 µl of each primer (30 pmol/µl) and 8 µl of template. PCR reactions were run in a BIOER Line-Gene thermocycler using a step cycle program. After initial step at 50°C for 2 min following initial denaturation of the DNA at 95°C for 10 min, and 40 amplification cycles were run: denaturation 95°C for 15 sec and hybridisation 54/60°C for 1 min. A sample was recorded as positive when the melting temperature was the same or ±0.5°C in comparison to a positive control. In each PCR the negative control was used (master mix + H_2O). PCR products were directly sequenced in both directions. Sequences were aligned and completed using Chromas Pro Programme, Bioedit and Custal X and compared to known sequences in the National Center for Biotechnology Information GenBank database.

Results

Samples of faeces obtained from 40 great cormorants (*Phalacrocorax carbo*), 5 grebes (*Podiceps cristatus*) and two white storks (*Ciconia ciconia*) were analyzed for the presence of microsporidia by microscopic and molecular methods.

Microscopic analysis showed presence of brightly fluorescing oval shapes of size 1.5 × 3 µm, characteristic of the strain Microsporidia in 32 samples (29 cormorants, 3 dippers). The real-time SYBR Green PCR method proved the presence of *E. cuniculi* spores in 19 avian samples which could be caused by low concentration of spores in the remaining samples of faeces (Notermans *et al*. 2005).

The real-time SYBR Green PCR using *Encephalitozoon*-specific (ecfITSf/ecfITSr) and species-specific (MSP3/MSP4B) primer pairs revealed the presence of microsporidian species *E. cuniculi* in 19 avian samples (40.4%), out of which was 17 cormorants (out of 40 in total), 1 grebe (out of 5 in total) and 1 stork (out of 2 in total; Table I). No *E. intestinalis*, *E. hellem* and *E. bieneusi* positive samples were identified. Positive PCR products were sent for sequenation. Sequences were compared with sequences in GenBank database and were identical with

Table I. Molecular detection of microsporidian species *E. cuniculi* genotype I in wild waterfowl of Slovakia

Species of examined birds	P	*E. cuniculi*
Ciconia ciconia	2	1
Phalacrocorax carbo	40	17
Podiceps cristatus	5	1
Total	47	19

P – number of examined samples.

the sequence of *E. cuniculi* genotype I (accession number AJ005581.1).

Discussion

Paper presents detection of microsporidian presence in several species of wild waterfowl in Slovakia. By molecular method of real-time SYBR Green PCR, there was first demonstrated presence of *E. cuniculi* genotype I species in waterfowls (total of 47 birds) *Phalacrocorax carbo*, *Podiceps cristatus* and *Ciconia ciconia*. No *E. intestinalis*, *E. hellem* and *E. bieneusi* positive samples were identified. Birds came from one water site in eastern Slovakia. Based on several studies addressing this topic, it can be assumed, that contamination of water source began through infected bird droppings and subsequently other individuals living in the same locality get infected because of environment (Kirschner *et al*. 2004, Rinder 2004). Host range of *E. cuniculi* is more diverse compared to other microsporidian species and there are constantly new host species discovered (Kasickova *et al*. 2007, Malcekova *et al*. 2011). Until recently, its presence was detected mostly in mammals, but in recent years its appearance increasing more often in birds population. Previous findings indicate the presence of *E. cuniculi* in different species of birds, but not in waterfowl (Reetz 1994; Kasickova *et al*. 2007, 2009; Malcekova *et al*. 2011). *E. cuniculi* represents the most frequently occurring microsporidian species in Slovakia, to be detected in several species of animals as well as in humans (Halanova *et al*. 1999; Malcekova *et al*. 2010, 2011; Valencakova *et al*. 2012).

In recent years, one is paying attention to problem of microsporidian presence in environment, their transmission, resistance, especially in water resources (surface, groundwater, and drinking water and waste resources). Microsporidian spores, due to presence of chitin cell wall, are highly resistant to environmental conditions in different environments (Didier *et al*. 2004). They can survive in aquatic environment for several months or even years, so immediate contact with animals or humans is not necessary to infect a new host (Didier *et al*. 2004). Some authors in their studies allow the possibility of contamination of water resources through water birds, whose faeces was proven to contain higher concentration of spores compared to other birds (Slodkowicz-Kowalska *et al*. 2006, Graczyk *et al*. 2008). Since microsporidia are able to infect a wide range of invertebrates and vertebrates species, that is why not only people are at the great risk, but also other animals living in water or in its surrounding.

The number of cases which prove the presence of microsporidian in water resources is rising not only in America, but also in Europe. The first demonstrated was *E. bieneusi* using molecular and microscopic techniques in a sample of water from Seine River, 15 km far from Paris in France (Sparfel *et al.* 1997). Subsequent analysis of other water samples also confirmed the presence of microsporidian spores. In America, namely in Arizona, after surface water examination, one out of four samples was proven to contain *E. bieneusi* (Dowd *et al*. 1998). A few years later, re-examination of Seina river water by microscopic and molecular analysis again confirmed the presence of *E. bieneusi* in 1 out of 25 samples. Another microsporidian species that was detected in water samples is *E. intestinalis*. It's presence was diagnosed in 2 out of 4 surface water samples, one sample of wastewater as well as in 1 out of 3 purified wastewater samples (Dowd *et al*. 1998). Report on its presence in irrigation waters used for crop and previous studies also contribute to the conclusion about the risk of infection from water sources (Thurston-Enriquez *et al*. 2002). This finding also supports the ability of microsporidia to survive and stay infectable in aquatic environment for a long time, namely for several weeks at temperatures from 10 to 30°C and are even able to survive at low temperatures (4°C) for as much as two years (Didier *et al*. 2004).

Microsporidian presence in water resources brings a question of, how could microsporidian get into the water sources. Some authors in their work tend to believe the contamination through waterfowl (Slodkowicz-Kowalska *et al*. 2006, Graczyk *et al.* 2008). Groundbreaking findings were published in the work of Slodkowicz-Kowalska (2006), which states the possibility of microsporidia water contamination and transmission of water sources, either surface water, groundwater as well as wastewater. Study results also indicate the presence of spores of *E. hellem* and *E. intestinalis* in birds, while the prevalence of manure excreted spores was statistically higher in waterfowl, than in other examined birds. Waterfowl droppings contained more spores (mean 3.6 x 105 spores/g) than ones of other birds living outside water sources (4.4 x 104 spores/g). One bird droppings can get into the water about 9.1 x 108 spores/g, while to infect an individual it is enough to be a low number of spores (Graczyk *et al.* 2008). Water birds live mainly in multiple flocks, where their numbers can amount to

hundreds or even thousands of what constitutes to an enormously large amount of droppings. These findings are epidemiologically important in several aspects. Most species of water birds are protected by low, have unrestricted access to surface waters (including waters used as drinking source), migrate long distances often between continents and thus may contribute to the transmission of spores from one area to another and contaminate new areas. Moreover, most of the daily activities of these birds involve grazing in shallow waters and subsequent discharge into the water. It has been also confirmed by several studies, that the presence of waterfowls decreases the water quality due to the high number of droppings. In the present work were investigated aquatic birds in one area of eastern Slovakia. Since cormorants, grebe and stork came from one water source location, it can be assumed that there was a transfer of fecal-oral route, but does not exclude the possibility of infection from the environment.

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