Isolation of *Encephalitozoon intestinalis* from crows living in urban parks of Tehran, Iran: an investigation with zoonotic aspect

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Abstract Microsporidia are eukaryotic, intracellular obligate parasites that widely involve many organisms including insects, fish, birds, and mammals. One of the genera of Microsporidia is *Encephalitozoon*, which contains several opportunistic pathogens. Since *Encephalitozoon* spp. are zoonotic and opportunistic pathogens, it is important to find their reservoir hosts; hence, the current study aimed at isolating and identifying *Encephalitozoon* spp. in the crows by the light microscopy observations and molecular methods. For this purpose, 36 samples were collected by the dropping method; however, due to the low volume of samples, the total samples were collected in a sterile stool container and the polymerase chain reaction (PCR) was performed to detect *Encephalitozoon* spp. Accordingly, 300-bp bands, specific to *Encephalitozoon* spp., were observed and by sequencing *E. intestinalis* was identified. Crows can be considered as the hosts of *E. intestinalis*.

Keywords Microsporidia · *Encephalitozoon intestinalis* · Crows · Molecular identification · Zoonotic aspect

Introduction

Microsporidia are obligate, intracellular parasites that widely infect many organisms including insects, fish, birds, and mammals (Didier et al. 2004). Previously,

Microsporidia were classified as protists, but currently they are classified as fungi (Didier and Weiss 2006); now there are more than 1500 species of Microsporidia among the 187 genera (Peyretailade et al. 2015). One of the genera of Microsporidia is *Encephalitozoon*, which has several species of which *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, and *Encephalitozoon intestinalis* are zoonotic with clinical protests (Mathis et al. 2005). *Encephalitozoon* spp. is mononuclear with merogoni and sporogoni life stages, produce small spores, possess a unique polar tube, and have intracellular life cycle inside the parasitophorous vacuoles (Keohanea et al. 1998).

Encephalitozoon cuniculi is generally isolated from three groups of patients as the ones with AIDS, transplant recipients, and patients with T-CD4⁺ deficiency due to idiopathic causes. *E. cuniculi* usually cause disseminated infection (Mathis et al. 2005). It has three main genotypes: rabbit, mouse, and dog, which are classified according to the repeatability of the 5'-GTTT-3' sequence of the internal transcribed spacer (ITS) ribosomal RNA gene (Kotler and Orenstein 1998). In animals, *E. cuniculi* was isolated from rabbits, laboratory mice, carnivores, monkeys (Snowden et al. 1999; Akerstedt 2002), and pigeons (Pirestani et al. 2013; Haro et al. 2005).

Encephalitozoon hellem is frequently isolated from patients with AIDS with ocular and disseminated infections (Connors et al. 2004; Scaglia et al. 1997; Deplazes et al. 1998). In animals, *E. hellem* is frequently isolated from birds, especially parrot species (Pulparampil et al. 1998; Snowden et al. 2000; Suter et al. 1998). Based on the ITS ribosomal RNA gene, *E. hellem* has three genotypes, which depend on the type of mutation (Xiao et al. 2001).

Encephalitozoon intestinalis is the second most common microsporidia reported from different parts of the world, and particularly is isolated from patients with AIDS and

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diarrhea; it is also associated with travelers' diarrhea. However, it is also isolated from immunocompetent individuals (Reynaud et al. 1998). In animals, it is isolated from goats, pigs, cows, dogs, monkeys, pigeons (Bornay et al. 1998), and cats (Nestor et al. 2012). *E. intestinalis* has the smallest genome with 2.3 Mbp among the microsporidia, whose genome is heavily compressed and many parts of it were deleted (Corradi et al. 2010). ITS fragments of rRNA gene of *E. intestinalis* isolated from humans and animals do not have polymorphic sites (Mathis et al. 2005); therefore, lack of genetic variation in the ITS fragment of *E. intestinalis* indicate no transmission barriers between the species recognized as hosts.

Due to the zoonotic and opportunistic nature of *Encephalitozoon* spp., it is very important to find its reservoir hosts. On the other hand, birds living in the vicinity of humans contaminate the environment by excretion of the spores of *Encephalitozoon* spp. (Haro et al. 2005) that in case of contact to water resources cause pollution of surface waters (Izquierdo 2011). So far, significant studies are conducted to isolated *Encephalitozoon* spp. from birds in the world (Haro et al. 2005; Pulparampil et al. 1998; Suter et al. 1998; Kasickova et al. 2009; Sak et al. 2010), but such studies are limited to two in Iran (Pirestani et al. 2013; Tavalla et al. 2017). Today, Iran is among the developing countries with increased number of patients with immunodeficiency states (Nabavi and Arshi 2016). Since opportunistic pathogens are a major threat to immunocompromised patients, for health measures and informing people, it is important to find the reservoirs of such pathogens. The current study was a sequel to the previous study conducted on pigeons in order to isolate *Encephalitozoon* spp. (Pirestani et al. 2013), and the current study was performed on the crows (*Corvus cornix*), mysterious birds that are the neighbors of humans in public parks and houses.

Material and method

Stool samples collection

Fecal samples were obtained from Pardisan Park, located in Northwest of Tehran, Iran. The trees in this park are the gathering place of crows. A large clean piece of plastic was placed under the trees and then, 120 sterile stool containers were placed on it. After eight hours, 36 containers containing feces were collected via dropping method. Due to the low volume of samples, and since there was no objective epidemiological work, all samples were placed in a container. The samples were immediately transferred to the laboratory and divided into two groups. One group was placed in 10% formalin fixative solution for staining and

the other group was mixed with twice the volume of 2.5% potassium dichromate and stored at 4 °C until DNA extraction.

Microscopic examination

The formalin group was placed in 10% KOH for 10 min, and then, centrifuged at 6000g for 10 min. Then, a thin sedimentary layer was provided and fixed with methanol. Finally, the smear was stained by the modified trichrome-blue method (Ryan et al. 1993).

DNA extraction

DNA extraction of stool samples was performed by CTAB/Chloroform-Isoamyl Alcohol method (Cullings 1992), however, modifications were made. Briefly, 400 µL of the collected stool sample (stored in potassium dichromate) was transferred into a 2-mL tube and washed three times with normal saline. To remove PCR inhibitors, the sample was placed in 1% KOH, it was washed again with saline and the supernatant was discarded; then, 200 µL guanidine hydrochloride buffer (8 M) and 200 µL TE buffer (0.05 M) were then added; the pellet was shaken and incubated at 60 °C for 20 min. Then, the stool sample was washed three times with normal saline and supernatant was discarded, 200 µL TE buffer and 200 µL sodium dodecyl sulfate (SDS) (10%) were added and shaken, then freezing–thawing action was performed five times using liquid nitrogen in 100 °C water bath. In next step, 400 µL TE buffer was added and shaken, 10 µL proteinase K was added and the sample was placed in a water bath at 60 °C for 16 h; again 10 µL proteinase K was added and the sample was placed in a water bath at 60 °C for 2 h, after incubation 200 µL NaCl (6 M) was added and shaken, then, 100 µL cetyltrimethylammonium bromide (CTAB) solution was added and the sample was placed for 10 min in a water bath at 60 °C; then, 600 µL chloroform-isoamyl alcohol solution was added and shaken vigorously for 20 s, the sample was centrifuged at 16,000g for 8 min. The supernatant was transferred into a 1.5-mL sterile tube and isopropanol was added as far as tube was filled, then incubated for 16 h at −20 °C; after incubation the sample was centrifuged at 19,000g for 15 min, supernatant was discarded and 1 mL of 70% ethanol was added to the pellet, centrifuged at 19,000g for 5 min. The tube was placed on a hot plate at 60 °C to evaporate the alcohol; finally, 50 µL distilled water was added to the pellet.

DNA concentration was measured using NanoDropOne system (Thermo Scientific Co). Then, DNA was stored at −20 °C until molecular identification.

Molecular identification

PCR for amplification of ITS

PCR was performed to amplify ITS fragment. For this purpose, the primer pair MSP3 and MSP4A (Katzwinkel-Wladarsch et al. 1996) was used. The primers amplified 300-bp fragments including the rDNA ITS specific to *Encephalitozoon* spp. PCR was conducted based on the Mathis et al. (1999) protocol with little modifications (Table 1). Since the specimens were poured into a container and there was only one sample container for DNA extraction, to avoid false negative responses, the PCR product was loaded into four wells of electrophoresis gel.

Sequence analysis

PCR product including ITS fragment was sequenced by the ABI3730XL sequence analyzer (Macrogen, Korea). The sequences were edited and aligned using ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and compared with the sequences formerly saved in the GenBank database.

Results

Microscopic examination

After staining by modified trichrome-blue method, the prepared smears were examined by an optical microscopy at 100× magnifications. Figure 1 shows the oval microsporidia spores observed in the stool of crows.

PCR and sequence analysis

Based on PCR amplification, 300-bp bands, specific to *Encephalitozoon* spp., were observed under transilluminator (Fig. 2). Sequence analysis results of PCR product were compared with those of GenBank database and

Fig. 1 Oval spores of *E. intestinalis* in feces of crows staining by modified trichrome blue method examined microscopically at a magnification of ×100

accordingly, *E. intestinalis* was detected (GenBank accession No. MG551530).

Discussion

Three factors are critical in the infection cycle of parasites, the causative agents, infection vectors, and reservoirs infection. Of these, finding the reservoirs of infection is very important to prevent and control of the disease (Didier et al. 2004). In terms of human microsporidiosis, the disease agents are identified, and it is obvious that the infection has no biological vectors, contrary to what is observed in the case of malaria. On the other hand, studies on finding the reservoirs of infection increased in the past two decades. These efforts demonstrated that human microsporidiosis is a zoonotic disease (Didier 2005).

Among microsporidia, *E. intestinalis* is known to cause diarrhea, particularly in patients with AIDS (Nestor et al. 2012). Also, *E. intestinalis* is isolated from patients receiving chemotherapy, patients with bone marrow transplant, and children with growth retardation (Cetinkaya et al. 2016). Since the first report of *E. intestinalis* in 1993 (Cali et al. 1993) to date, there is a significant point about *E. intestinalis*; it is frequently reported in a wide range of animals (Bornay et al. 1998), but rarely in birds. It is clear that if the birds living in the vicinity of humans are contaminated with *E. intestinalis*, with the disposal of spores of this microorganism, they can contaminate soil and

Table 1 Primers and conditions of PCR (35 cycles)

Forward primer (MSP3)	GGAATTCACACCGCCCGTC (A/G) (C/T) TAT
Reverse primer (MSP4A)	CCAAGCTTATGCTTAAGT(C/T) (A/C) AA (A/G) GGGT
Initial cycle	94 °C, 5 min
Denaturation	94 °C, 30 s
Annealing	64 °C, 60 s
Extension	72 °C, 60 s
Final extension	72 °C, 10 min

Fig. 2 **a** Gel analysis of PCR. 1, 2, 3, 4: *E. intestinalis*, 5: negative control. **b** Gel analysis of PCR. 1: *E. intestinalis*, 2: negative control

surface waters (Izquierdo 2011). For this reason, these birds are the potential reservoirs of the microorganism.

The current study examined crows, as one of the birds living near humans, in order to identify *Encephalitozoon* spp. by molecular methods and accordingly, *E. intestinalis* was detected in feces of the birds. Another birds living in the vicinity of human and examined similarly are pigeons. Haro et al., for the first time isolated microsporidia from pigeons in Spain (Haro et al. 2005). They isolated *Enterocytozoon bieneusi* (a pathogen of microsporidia from the *Enterocytozoonidae* family), *E. intestinalis*, and *E. hellem*

from pigeons, but no *E. cuniculi* was identified. In another study conducted in Iran by Pirestani et al., *E. bieneusi*, *E. hellem*, and *E. cuniculi* were isolated from pigeons, but *E. intestinalis* was not identified (Pirestani et al. 2013). Recently, a study was conducted on exotic birds in southwestern Iran, in which *E. bieneusi* and *Encephalitozoon* spp. isolated from these birds; the frequency of *E. intestinalis* was significant among *Encephalitozoon* spp. (Tavalla et al. 2017). In the current study, from genus *Encephalitozoon*, only *E. intestinalis* was isolated from the feces of crows and *E. bieneusi* was not detected (unpublished data).

Since diarrhea in patients with AIDS is a major problem and if not controlled cause death, it is necessary to identify the agents causing diarrhea for urgent treatment (Call et al. 2000); therefore, it is essential to identify *E. intestinalis*, which is a pathogen that causes chronic diarrhea. As mentioned above, due to lack of transmission barriers to this microorganism, finding its animal reservoirs is of great important. One of the important animal reservoirs for these pathogenic agents is the birds that live in the vicinity of humans, of which crows are one of the most important ones; although crows are always overlooked, in case of contamination with *E. intestinalis* they play a significant role in the zoonotic transmission with the excretion of *E. intestinalis* spores. It is clear that exposure of patients with AIDS to *E. intestinalis* spores, especially in public places such as the parks, can be a health threat to such patients.

Another important point about *E. intestinalis*, in addition to diarrhea in patients with AIDS, is its correlation with the travelers' diarrhea (Izquierdo 2011). The travelers' diarrhea is mainly related to drinking water and obviously the contamination of drinking water with infectious agents increases the risk of diarrhea for travelers. Identification of *E. intestinalis* in water resources indicates that health systems, especially in the developing countries, should apply best practices in drinking water disinfection in order to eliminate the spores of *E. intestinalis* and other pathogenic Microsporidia (Johnson et al. 2003). On the other hand, one of the main causes of water resources contamination, undoubtedly, is the spores of Microsporidia excreted by the feces of birds. Hence, alerts should be given to people who use surface drinking waters, especially river water.

The studied crows lived in a public park with many visitors; therefore, the presence of adults, children, and people with imperfect immune systems in this park and exposure to *E. intestinalis* spores, excreted by the feces of crows, can be a potential risk factor to cause infection, especially that *E. intestinalis* has no transition barrier. This is also valid for pigeons with Microsporidia that live in public parks, as Haro et al. (2005) explained. Finally, it is noteworthy that detection of *E. intestinalis* in crows and pigeons, the birds living close to humans, can help to

understand the route of transmission of this infectious agent, however, proof of the direct transmission of the spores from the feces of birds to humans requires extensive studies.

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Author contributions My: PhD student doing research work (PhD thesis), JS: Thesis supervisor and Corresponding authors, AD: First advisor, particularly in fieldwork, MP: Second advisor, especially in molecular work.

Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

Ethical approval The sampling was done in the form of dropping out and there was no ethical issue.

Appendix: Sequence data

MG551530.1 *Encephalitozoon intestinalis* isolate IR.TM1 small subunit ribosomal RNA gene and internal transcribed spacer 1, partial sequence TGG AATTCACACCGCCGTCGT TATCTAAGATGACGCAGTGGACGAAGATTGGAAGGTCTG AGTCCTTCG TGTTAGATAAGATATAAGTCGTAACACGGC TGCTGTTGGAGAACCAGCAGCAGGATCAGTATTTGAGAG A TTGGGGGGAATTTTTTTGATTGAGGATCCACGGGAT TGATAGGCATTAGCAGCAGGAAGGACGTGTAG GAGGAC GATATGCCTTTTTGAGGATGCTGAATGAAGCGGAAGCG CTTGGGGACCCCTTNNACTTAAGCA TAAGCTTGAACCC TTCCTTT

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