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Molecular characterization and genotyping of human related microsporidia in free-ranging and captive pigeons of Tehran, Iran

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ABSTRACT

Microsporidia are opportunistic pathogens in nature infecting all animal phyla. Samples of intestinal content from 147 pigeons from various regions of Tehran (Iran) were analyzed for occurred microsporidia by PCR and sequencing. The DNA isolated from 31 samples (21%) of microsporidia-positive was amplified with specific primers for the four most frequent human microsporidia. *Enterocytozoon bieneusi* was the most common species and was recognized in thirteen pigeons (42%). Four pigeons were positive for *Encephalitozoon intestinalis* (12.9%), six for *Encephalitozoon hellem* (19.3%) and two for *Encephalitozoon cuniculi* (6.4%). Mixed infections were detected in six another pigeons: *E. bieneusi* and *E. hellem* were detected in two cases (4.8%); *E. bieneusi* was associated with *E. intestinalis* in one case (0.8%); *E. hellem* and *E. intestinalis* coexisted in one; and *E. hellem* and *E. cuniculi* were identified in two pigeons. The genotypes of internal transcribed spacer (ITS) of the rRNA gene were determined for all isolates with single infections. Six isolates of *E. bieneusi* belonged to the genotype D (46.2%), three to the genotype M (23%), and four to the genotype J (30.8%). Sequences of four *E. hellem* isolates were same to genotype 1A and two were identical with genotype 3. In two cases of the pigeons, *E. cuniculi* genotype I, II were identified. This study implicates pigeons as potential sources of microsporidia infection for humans.

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1. Introduction

Microsporidia are a diverse group of obligate intracellular parasites infecting many invertebrates and vertebrates, including humans. At first, microsporidia assumed as primitive protozoa, but in the 1990s, based on molecular and phylogenetic analysis, proofs revealed relationship of these organisms and Fungi (Thomarat et al., 2004). Until now, more than 1200 microsporidia species identified which divided into about 150 genera (Keeling and Fast, 2002). Most infect invertebrates and fish, but 14 species in eight genera infect humans (Didier et al., 2004; Didier and Weiss, 2006). *Enterocytozoon bieneusi* and the *Encephalitozoon* species (*Encephalitozoon cuniculi*, *Encephalitozoon intestinalis*, and *Encephalitozoon hellem*) are the four major microsporidia species infecting humans and animals. It is established now that these four microsporidia species are zoonotic pathogens. *E. bieneusi* first found in enterocytes of a Haitian AIDS patient by Desportes et al. (1985). *E. bieneusi* found in a broad range of animals such as macaques, dogs, cats, cattle, llamas, racoons, muskrats, beavers, foxes, otters, chickens, pigeons, parrots, sparrows, aquatic birds, pigs, and falcons (Chalifoux et al., 1998; Fayer et al., 2003, 2007; Graczyk et al., 2007; Haro et al., 2006, 2005; Lobo et al., 2006; Mansfield et al., 1997; Mathis et al., 1999a; Muller et al., 2008; Reetz et al., 2002; Rinder et al., 2000; Santin et al., 2005; Slodkowicz-Kowalska et al., 2007; Stewart et al., 1979; Sulaiman et al., 2003b). This species often diagnosed in humans, particularly in immunocompromised patients with AIDS and chronic diarrhea. Among the *Encephalitozoon* species, *E. intestinalis* is the most identified species in humans and occurs also in birds as sporadic case (Bornay-Llinares et al., 1998; Valencakova et al., 2006). So far, also humans, *E. hellem* detected mainly in birds (Mathis et al., 2005) and in a European brown hare (De Bosschere et al., 2007). *E. cuniculi* especially discovered in rabbits, rodents and carnivores (Deplazes et al., 2000; Didier, 2005; Mathis et al., 2005) and swine (Reetz et al., 2009).

Based on ITS sequence of the rRNA, *E. bieneusi* has founded with over 90 genotypes in humans and animals. In the ITS sequence of *E. intestinalis* isolated from humans and animals have not detected any polymorphic sites, so far. Lack of genetic difference in ITS of *E. intestinalis* revealed there are no transmission barriers between the species identified as hosts. The existence of three different genotypes of *E. cuniculi* have confirmed based on the number of 5′-GTTT-3′ repeats present in the ITS of the rRNA. Genotype I or rabbit strain (three repeats), genotype II or mouse strain (two repeats) and genotype III or dog strain (four repeats) has isolated from mice and rabbits, mice and dogs, dogs and fox, respectively

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(Didier et al., 1995). Intraspecies genotype variability of *E. hellem* was initially based on the sequence of the ITS of the rRNA genes (Mathis et al., 1999b), but later also characterized in two other markers (IGS-TH and IGS-HZ) and in the PTP, preserving to describe new genotypes (Haro et al., 2003; Xiao et al., 2001). By ITS sequences, *E. hellem* has divided to four genotypes (1A, 1B, 1C/2A/2B/2C). Genotypes 1A, 1B, and 1C were identical with genotype 1, genotype 2A with genotype 2 and genotype 2C with genotype 3 that reported by Mathis et al. (1999b).

Our aim was to carry out experiments on 147 fecal samples of pigeons that we earn from the different region of Tehran. Take the view that these samples were infected with microsporidia therefore we have detected this pathogen with use specific primers in this study.

2. Materials and methods

2.1. Collection of stool samples

Fecal samples (dropping) taken from 147 pigeons from five public parks and five commercial aviaries located in different geographical areas of Tehran (Iran) (Table 1). Free-living pigeons seized by environmental officers and kept in individual cages until the end of the sampling and returned to the environment. The pigeons were without apparent clinical signs. After defecation, fecal samples directly collected, transported to the laboratory on ice, and mixed with two volumes of 2.5% potassium dichromate (K2Cr2O7), and stored at 4 °C until further analysis. All specimens characterized by multiplex/nested-PCR (hot start technique) based on rRNA gene to confirm the existence of microsporidian spores.

2.2. Molecular characterization of microsporidia species and genotypes

2.2.1. DNA extraction

For molecular characterization, DNA extraction carried out on all the samples. DNA extracted by alkaline digestion method as previously described (Pirestani et al., 2008; Xiao et al., 1999).

2.2.2. Molecular characterization

The PCR primers used were previously described by Katzwinkel-Wladarsch and nested PCR protocol used to amplify the ITS region of the rRNA of human pathogenic microsporidia including, *E. bieneusi*, *E. intestinalis*, *E. cuniculi*, *E. hellem* (Katzwinkel-Wladarsch et al., 1996). In the first PCR, three outer primers used including: MSP-1, TGAATG(G,T)GTCCCTGT; MSP-2A, TCACTCGCCGCTACT; MSP-2B, GTTCATTCGCACTACT. The second PCR run by taking 2 µl of the first PCR product to the mix containing three inner primers (MSP-3, GGAATTCACACCGCCCGTC(A,G)(C,T)TAT; MSP-4A, CCAAGC TTATGCTTAAGT(C,T)(A,C)AA(A,G)GGGT; MSP-4B, CCAAGCTTATGC TTAAGTCCAGGGAG).

For both PCR steps 45 cycles, each consisting of 95 °C for 45 s, 55 °C for 30 s and 72 °C for 45 s were performed. An initial incubation at 95 °C for 3 min, final extension at 72 °C for 10 was included. PCR products were visualized in a 2% agarose gel containing 0.2 mg ml^{-1} ethidium bromide.

2.2.3. Restriction fragment length polymorphism (RFLP) analysis

For determination of species of *Encephalitozoon* spp., RFLP performed according to described previously by Katzwinkel-Wladarsch et al. (1996) with change in a final volume of 30 µl consisting of 10 µl of secondary PCR product, 1 µl of the *Mnl*I enzyme (FastDigest, Fermentas, EU), 2 µl of buffer (10× FastDigest buffer supplemented with BSA, Fermentas, EU) and 17 µl of distilled water (nuclease-free). After incubation at 37 °C for 1 h, fragments visualized in a 3.5% agarose gel containing 0.25 mg ml^{-1} ethidium bromide.

The restriction enzyme *Mnl*I split the amplified sequence into several fragments with specific patterns for species of *Encephalitozoon* as follows: *E. hellem* was expected to produce fragments ~180, 90 and 50 bp; *E. intestinalis* ~160, 60 bp and *E. cuniculi* ~210 and 90 bp.

2.2.4. Sequence analysis

PCR product of each sample with single infection sequenced by the ABI3730XL sequence analyzer (Macrogen, Korea). The sequences edited and aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and compared with reference sequences from GenBank.

For *E. bieneusi* ITS sequences, the phylogenetic tree was built with the neighbor-joining (NJ) algorithm using molecular evolutionary genetics analysis (MEGA) software (version 5.0), including sequences of representative *E. bieneusi* isolates from the GenBank.

3. Results

3.1. Molecular characterization of microsporidia species and genotypes

Fecal samples of pigeons were analyzed for the presence of microsporidia by molecular methods. The multiplex/nested-PCR method with specific primers, proved the presence of

Table 1
Molecular characterization of human related microsporidia in pigeons of Tehran.

Region of Tehran		No. of pigeons	PCR positive									
			E. bieneusi		*E. intestinalis*		*E. hellem*		*E. cuniculi*		Mixed infection	
			No.	%	No.	%	No.	%	No.	%	No.	%
West	[PP]CHA	16	1	6.25	0	0	2	12.5	0	0	0	0
	[CA]SHA	14	1	7.1	1	7.1	0	0	0	0	0	0
East	[PP]BAS	15	1	6.7	0	0	2	13.3	0	0	2	13.3
	[CA]HRI	15	2	13.3	0	0	0	0	0	0	2	13.3
South	[PP]BEA	17	3	17.4	1	5.9	0	0	1	5.9	1	5.9
	[CA]RAH	14	2	14.3	0	0	0	0	1	7.1	0	0
North	[PP]JAM	14	0	0	0	0	0	0	0	0	0	0
	[CA]TAJ	15	1	6.7	1	6.7	1	6.7	0	0	0	0
Center	[PP]LAL	14	2	14.3	0	0	0	0	0	0	0	0
	[CA]ENG	13	0	0	1	7.7	1	7.7	0	0	1	7.7
PP		76	7	9.2	1	1.3	4	5.3	1	1.3	4	5.3
CA		71	6	8.45	3	4.2	2	2.8	1	1.4	2	2.8
total		147	13	8.8	4	2.7	6	4.1	2	1.4	6	4.1

Note: PP: public park; CA: commerical aviary. CHA: Chitgar; SHA: Shahriar; BAS: Basij; HRI: 17th Shahrivar; BEA: Beasat; RAH: Rah ahan; JAM: Jamshidieh; TAJ: Tajrish; LAL: Laleh; ENG: Enghelab.

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