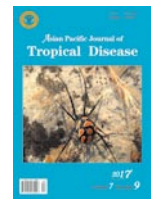


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Molecular identification of *Enterocytozoon bieneusi* and *Encephalitozoon* species in pigeons of southwest of IranMehdi Tavalla^{1,2*}, Masoumeh Mardani-Kateki^{2,3}, Forough Kazemi^{2,3}¹Health Research Institute, Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran²Department of Parasitology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Science, Ahvaz, Iran³Student Research Committee, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

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ABSTRACT

Objective: To evaluate microsporidia species in pigeons of Ahvaz city, southwest of Iran by the staining and multiplex/nested PCR methods.**Methods:** Initially, 131 stool specimens were randomly collected from pigeons. The specimens were stained by the modified trichrome (Weber) and were microscopically examined. The DNA was extracted by DNA stool extraction kit (Bioneer) and was examined by the multiplex/nested PCR method. For differentiating the species of *Encephalitozoon*, the products of PCR were explored by the restriction fragment length polymorphism (RFLP) using the Mnl1 restriction enzyme.**Results:** Of 131 specimens, 22 and 18 cases were positive by the staining and the multiplex/nested-PCR methods, respectively. Of 18, 7 and 11 samples were detected as *Enterocytozoon bieneusi* and *Encephalitozoon* spp., respectively. In addition, 2 *Encephalitozoon cuciculi* and 9 *Encephalitozoon intestinalis* were *Encephalitozoon* species. Of 7 *Enterocytozoon bieneusi* samples, 5 and 2 cases were detected as genotype M and L, respectively. Also, all *Encephalitozoon cuciculi* samples were identified as genotype II.**Conclusions:** Our findings showed a relatively high prevalence of microsporidia at pigeons and these birds can be a significant and important source of microsporidiosis. It is essential that the high-risk individuals including patients with immunodeficient diseases should be receiving the accurate and valid information about the risk of direct and indirect contact with the infected pigeons.

1. Introduction

Microsporidia are an obligate intracellular parasite. Although, the phylum Microsporidia consists of 150 genera and 1200 species, *Encephalitozoon* spp. [including *Encephalitozoon intestinalis* (*E. intestinalis*), *Encephalitozoon hellem* (*E. hellem*), and *Encephalitozoon cuciculi* (*E. cuciculi*)] and *Enterocytozoon bieneusi* (*E. bieneusi*) are the most frequent reasons of human microsporidiosis[1]. These microorganisms can cause the infection of wide range of animals and humans. The life cycle of the parasite

is including three stages: spore formation stage (sporogony), growing or replicating stage (schizogony) and infectious stage. Also, the symptoms of microsporidiosis are including myositis, keratoconjunctivitis, hepatitis, sinusitis, disseminated infections, chronic diarrhea, severe weight loss, nausea and confusion[2].

In recent years, *E. bieneusi* and *Encephalitozoon* spp. have been detected in birds[1,3,4], especially in pigeons[5,6]. *E. bieneusi* and *Encephalitozoon* spp. have been identified in animals that it is implying the zoonotic nature and potential of these microorganisms, but valid and accurate proofs for the transmission from these animals and birds to human are lacking[7]. It is very notable that exposure to pigeons may be a significant link in the epidemiology of zoonotic microsporidiosis, particularly in elderly people and children[8]. Also, it is reported that some patients with ocular microsporidiosis were exposed to pet birds[9]. These microorganisms have zoonotic source and are transferred by water contaminated with the stool of animals and birds[6]. On the other hand, the parasites can be life-

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threatening in immunodeficient individuals[2]. Hence, because of the zoonotic nature of microsporidia as well as the increasing prevalence of immunodeficiency diseases, the aim of this study was to evaluate microsporidia species in pigeons of Ahvaz city, southwest of Iran by the staining and multiplex/nested PCR methods.

2. Materials and methods

2.1. Sample collection

At first, 131 fecal samples were randomly collected from home pigeons of Ahvaz city, southwest of Iran. The collected specimens were transferred to Department of Parasitology, Ahvaz Jundishapur University of Medical Sciences. Then, a part of the specimens was used for smear preparation and staining of Weber. The rest of feces were mixed with twice the volume of potassium dichromate 2.5% and were kept at 4 °C[2].

2.2. Samples examination by staining

All stool samples of pigeons were stained by modified trichrome (Weber). The slides were fixed with methanol and were placed in trichrom color for 240 min. After decolorization with acid-alcohol, and washing with 95% ethanol, the slides were placed in absolute ethanol. At the next stage, to identify the spores of microsporidia, the slides were examined microscopically at magnification of 100× together with immersion oil. The positive samples were detected by dorsal vacuoles in microsporidia spore[2,10].

2.3. Extraction of DNA

The DNA was extracted by DNA extraction kits for stool (Bioneer) and extracted DNA was stored at -20 °C. This kit is including a spin column that the parasite DNA was absorbed by the column and after twice washing with special buffers, the purified DNA was obtained[2].

2.4. Molecular detection

The extracted DNA was examined by multiplex/nested PCR that both types of genders of *Enterocytozoon* and *Encephalitozoon* were identified by this method. At this stage, we used from specific primers that were designed by Katzwinkel-Wladarsch et al.[11]. These primers were designed based on small subunit ribosomal RNA (16S rRNA) gene that was used at the identification of all the different species of human microsporidia. The primers were purchased from Bioneer Company and were stored at -20 °C. Table 1 shows the primary and secondary primers used for multiplex/nested PCR. The amplified fragment length by the primers was 500 bp and 300 bp for gender of *Enterocytozoon* and *Encephalitozoon*, respectively. At first, the samples were examined with the primary and secondary primers by multiplex/nested PCR method. Then, for differentiating the species of *Encephalitozoon*, the products of multiplex/nested PCR were explored by restriction

fragment length polymorphism (RFLP) method using restriction enzyme of MnlI[2].

Table 1

The used primary and secondary primers for multiplex/nested PCR[11].

The primary primers	The secondary primers
MSP-1: TGAATGKGTCCCTGT	MSP-3: GGAATTCACACCGCCCGT C(A,G) (C,T) TAT
MSP-2A: TCACTCGCCGCTACT	MSP-4A: CCAAGCTTATGCTTAAGT (C,T) (A,C)AA(A,G)GGGT
MSP-2B: GTTTCATTCGCACTACT	MSP-4B: CCAAGCTTATGCTTAAGTCCAGGGAG

2.5. Sequencing

For genotyping, the positive samples of RFLP were sequenced by Bioneer Company (Daejeon, South Korea). Afterwards, the specified sequence was compared against the sequence of the registered isolates available in the GenBank library (NCBI) and homology between them was examined by software of BLAST[2].

3. Results

3.1. Staining

Figure 1 demonstrates the microsporidia spore in the stool samples obtained from pigeons. Of 131 specimens, 22 cases were suspected positive by the staining with modified trichrome (Weber), of which 18 cases were observed positive by the molecular method.

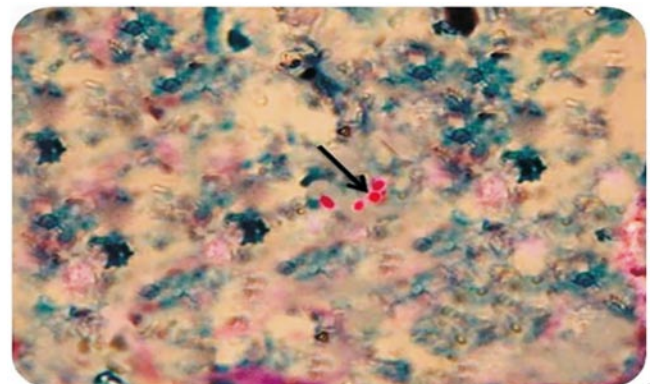


Figure 1. The microsporidia spore in the stool sample of pigeons that were stained by Weber staining and were microscopically examined at magnification of 100×.

3.2. Molecular analysis and genotyping

Table 2 demonstrates the results of molecular analysis of the stool samples obtained from pigeons. According to the finding, of 131, 18 samples were positive by the multiplex/nested-PCR test. Among them, 7 and 11 samples were detected as *E. bienersi* and *Encephalitozoon* spp., respectively. Furthermore, 2 *E. cuniculi* and 9 *E. intestinalis* were *Encephalitozoon* species. According to Table 2, of 7 *E. bienersi* samples, 5 and 2 cases were detected as genotype M and L, respectively. Also, all *E. cuniculi* samples were identified as genotype II.

Table 2

The results of molecular analysis of pigeon's fecal samples.

Samples	Number	Positive	<i>E. bienewsi</i> (Genotype)	<i>Encephalitozoon</i>	<i>E. intestinalis</i>	<i>E. hellem</i>	<i>E. cuniculi</i> (Genotype)
Pigeons	131	18	5 (M) 2 (L)	11	9	0	2 (II)
Total	131	18	7	11	9	0	2

4. Discussion

With improvement of diagnostic methods and molecular markers, epidemiological knowledge of microsporidia infections has been developed in particular in the last decade[5]. The different infectious diseases can be threatening the public health[12]. Microsporidia have the zoonotic potential and the parasites can be life-threatening in immunodeficient individuals[2]. Hence, because of the zoonotic nature of microsporidia as well as the increasing prevalence of immunodeficiency diseases, the aim of this study was to evaluate microsporidia species in pigeons of Ahvaz city, southwest of Iran by the staining and multiplex/nested PCR methods. In this study, we selected pigeons for examination; since these birds have indirect and direct contact with humans, and microsporidia are transferred by water contaminated with the birds stool[6].

The highest prevalence of microsporidiosis in humans is related to *E. bienewsi*, but in the current study, *E. intestinalis* had the highest prevalence in the birds. The results of the research showed that 9 fecal samples were infected with *E. intestinalis* (Table 2), but 7 cases were observed positive for only *E. bienewsi*. In contrast to our study, in 2013 in the center of Iran, Pirestani *et al.*[5] showed that *E. bienewsi* was the most common species and was recognized in 13 pigeons (42%) from 147 pigeons. Four samples were positive for *E. intestinalis* (12.9%), six for *E. hellem* (19.3%) and two for *E. cuniculi* (6.4%). Co-infections were identified in another six pigeons[5]. In addition, in 2005, Haro *et al.*[8] showed that of 124, 12 pigeon samples were positive for *E. bienewsi* (9.7%), five for *E. intestinalis* (4%), and one for *E. hellem* (0.8%). Co-infections were identified in eight additional pigeons: *E. bienewsi* and *E. hellem* were identified in six birds (4.8%); *E. bienewsi* was associated with *E. intestinalis* in one case (0.8%); and *E. hellem* and *E. intestinalis* coexisted in one pigeon. No positive cases were detected for *E. cuniculi*. Also, they identified genotype J of *E. bienewsi* in pigeons from urban parks for the first time[8]. Although, in our study, no positive samples for *E. hellem* were detected, two samples were positive for *E. cuniculi*. Consistent with our finding, *E. cuniculi* has been detected in chickens[13] and pigeons[6].

This research is important and significant in terms of the health public; because the opportunistic pathogens and parasites were isolated from pigeons of southwest of Iran. Because of the indirect and direct relationships of microsporidia with humans, these birds are an important source of the contamination. In conclusion, our findings showed a relatively high prevalence of microsporidia at pigeons and this bird can be a significant and important source of microsporidiosis. It is essential that the high-risk individuals including patients with immunodeficient diseases should be receiving the accurate and valid information about the risk of direct and indirect contact with the infected pigeons.

Conflict of interest statement

We declare that we have no conflict of interest.

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