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Molecular diagnosis of potentially human pathogenic *Enterocytozoon bieneusi* and *Encephalitozoon* species in exotic birds in Southwestern Iran

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ABSTRACT

Microsporidia are obligate intracellular parasites that produce spores. The infections caused by these parasites are mostly considered to be opportunistic in immunodeficient patients. Because of the zoonotic nature of microsporidia as well as the increasing prevalence of immunodeficiency diseases, the aim of this study was to evaluate the molecular diagnosis of *Enterocytozoon bieneusi* (*E. bieneusi*) and *Encephalitozoon* spp. in exotic birds in southwestern Iran. Initially, 816 stool specimens were collected and stained by modified trichrome (Weber) staining. The slides were explored using light microscopy. In the next stage, the extracted DNA was amplified using a multiplex/nested PCR method. RFLP with the MnlI restriction enzyme was used to differentiate the *Encephalitozoon* species in the products of the molecular analysis. Out of 816 samples, 138 and 181 cases were found to be positive by the staining and the multiplex/nested-PCR methods, respectively. Of the 181 samples, 103 and 78 samples were positive for *E. bieneusi* and *Encephalitozoon* spp., respectively. The *Encephalitozoon* species were 17 *E. cuniculi*, 52 *E. intestinalis* and 9 *E. hellem*. Of 103 *E. bieneusi* samples, 57, 39, 2 and 5 cases were detected as genotypes D, M, E and L, respectively. The results showed a relatively high prevalence of microsporidia in exotic birds, and according to the results of the genotyping, these birds can be an important source of microsporidiosis. It is essential that high-risk individuals, including patients with immunodeficiency diseases, receive accurate and valid information about the risk of direct and indirect contact with infected exotic birds.

Introduction

Microsporidia are obligate intracellular parasites. Although the phylum microsporidia consists of 150 genera and 1200 species, *Encephalitozoon* spp. (including *E. intestinalis, E. hellem,* and *E. cuniculi*) and *Enterocytozoon bieneusi* (*E. bieneusi*) are the most frequent causes of human microsporidiosis [1]. These microorganisms can cause infection in both a wide range of animals and humans [2,3]. The life cycle of the parasite consists of 3 stages: the infectious stage, the growing or replicating stage (schizogony) and the spore formation stage (sporogony) [4]. The symptoms of microsporidiosis include myositis, keratoconjunctivitis, hepatitis, sinusitis, disseminated infections [5], chronic diarrhea, severe weight loss, nausea and confusion [6]. It is possible that these parasites are transferred through water contaminated with animal stool [7].

In recent years, *Encephalitozoon* spp. and *E. bieneusi* have been detected in birds. Birds are considered as the primary hosts of *E. hellem* [8]. For example, the parasite was found in ducks, pigeons, geese, crows, puffins, hummingbirds, swans and cranes [1,8–11] and in captive birds from the order Psittaciformes, which includes lovebirds, budgerigars, Eclectus parrots, parrots, cockatoos and lories. *E. hellem* has also been identified in ostriches and Gouldian finches [1,12–16]. In addition, *E. cuniculi* has been detected in cockatiels, chickens, and pigeons [10,17,18], and *E. intestinalis* has been found in pigeons and geese [9–11]. Furthermore, *E. bieneusi* has been identified in chickens, grey parrots, pigeons, cockatiels, lovebirds, finches, falcons and other birds [10,19–23]. These examples

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Table 1
The exotic bird families and their species included in the current study.

Exotic bird families	Species
Fringillidae	*Carduelis spinus; Carduelis flavirostris; Linchura cantans*
Canary	*Serinus canarius canarius*
Psittacidae	*Psittacula eupatria or Psittacus erithacus*
African grey parrot	*Psittacula krameri*
Budgerigar	*Melopsittacus undulatus*
White-eared Bulbul	*Pycnonotus leucotis*
Passeriformes or Myna	*Acridotheres tristis*

imply the zoonotic potential of microsporidia [24]. Notably, exposure to pigeons may be the significant link in the epidemiology of human microsporidiosis [9], as well as exposure to pet birds of some patients with ocular microsporidiosis [25]. On the other hand, these parasites can be life-threatening in immunodeficient individuals [2,7]. 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 the molecular diagnosis of *E. bieneusi* and *Encephalitozoon* spp. in exotic birds in southwestern Iran.

Methods

Sample collection

Initially, 816 fecal specimens were collected from several pet shops and houses in Ahvaz city, Khuzestan province, southwestern Iran, during the period 2012–2014. Table 1 shows the exotic bird families and their species included in the current study. The collected samples were transferred to the Department of Parasitology, Ahvaz Jundishapur University of Medical Sciences. Part of the fecal sample was used for the smear preparation and staining. The rest of the feces was mixed with two volumes of potassium dichromate 2.5% and was stored at 4 °C [26].

Staining

First, 816 stool samples were stained using modified trichrome (Weber) staining. The slides were fixed with methanol and placed in the trichrome stain for 240 min. After decolorization with acid-alcohol and washing with 95% ethanol, the slides were placed in absolute ethanol. Next, to identify the spores, the slides were examined by optical microscopy at 100× magnification with immersion oil. The positive samples were identified by the dorsal vacuoles in the microsporidia spores [27,28].

Extraction of DNA

The DNA was extracted using DNA stool kits (Bioneer), and the extracted DNA was stored at −20 °C. This kit consisted of spin columns that absorbed the parasite DNA and eluted the purified DNA after washing twice with special buffers [28].

Molecular detection

The extracted DNA was examined using the multiplex/nested PCR method that was used to identify the microsporidial genera of *Enterocytozoon* and *Encephalitozoon*. For PCR, we used the specific primers that were designed by Katzwinkel-Wladarsch et al. [29]. These primers were designed based on the small subunit ribosomal RNA (16S rRNA) gene that was used for the identification of different species of microsporidia. The primers were purchased from Bioneer Company and stored at −20 °C. Table 2 shows the primary and secondary primers used for the multiplex/nested PCR method. The lengths of the fragments amplified by the primers were 500 bp and 300 bp for the microsporidial genera of *Enterocytozoon* and *Encephalitozoon*, respectively. First, the samples were examined with the primary and secondary primers using the multiplex/nested PCR method. Then, the RFLP method with Mnl1 was used to differentiate the species of *Encephalitozoon* in the multiplex/nested PCR products [28].

Table 2
The primary and secondary primers used for the multiplex/nested PCR [29].

Primary primers	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: GTTCATTCGCACTACT	MSP-4B: CCAAGCTTATGCTTAAGTCCAGGGAG

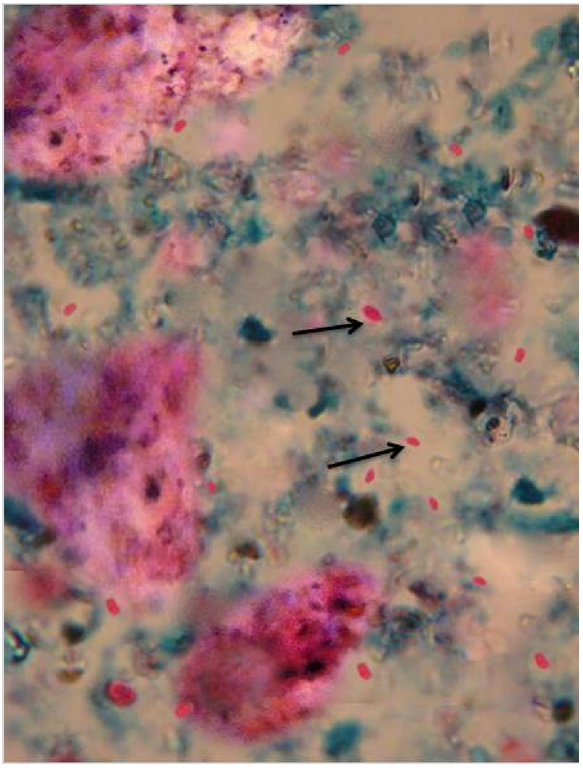

Fig. 1. The microsporidia spores in the stool sample of exotic birds that were stained by Weber staining and examined microscopically at a magnification of 100×.

Sequencing

For genotyping, the positive samples from the RFLP assay were sequenced by the Bioneer Company (Daejeon, South Korea). Afterwards, the specified sequence was compared with the sequences of the registered isolates available in the GenBank library (NCBI), and the homology between sequences was examined using BLAST software [28].

Results

Staining

Fig. 1 demonstrates the stained microsporidia spores in the stool samples of exotic birds. Of 816 samples, 138 cases were suspected to be positive for the parasite spore by the staining method, and of these 138 samples, 119 cases were verified as positive by the

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