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Molecular Survey of Microsporidia, *Blastocystis*, *Cryptosporidium* and *Giardia* in Pet Avian Species in Tehran, Iran

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Abstract

Background: Opportunistic microorganisms of the intestinal tract including *Cryptosporidium* spp., *Giardia* spp., *Blastocystis* sp., and microsporidia are increasingly being responsible for clinical disorders in various host species including human.

Objectives: This study was conducted with the aim of searching and tracking the above parasites in the feces of pet birds using parasitological and molecular methods in Tehran.

Methods: In the current study, fecal samples of avian birds were collected and investigated with modified Ziehl–Neelsen, modified trichrome and trichrome staining for the presence of microsporidia, *Cryptosporidium, Blastocystis* and *Giardia*. All the samples were examined molecularly with specific primers and PCR method as well.

Results: Three of the examined droppings contained *Encephalitozoon hellem* genotype 1B (2%) by PCR and sequencing. The microsporidian organisms were recovered from the droppings of clinically normal green-cheek parakeet, an African gray parrot, and a lovebird. Other examined parasites were not found in the examined samples.

Conclusion: The current study proved the captive pet birds as a possible source of microsporidian infection. The highly resistant nature of the microsporidia spores, besides the fact that

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encephalitozoonosis is predominantly subclinical in birds, can put the owners at increased risk of disease acquisition via spore inhalation or ingestion.

Keywords: *Blastocystis*, *Cryptosporidium, Giardia*, *Microsporidia, Zoonosi*

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1. Introduction

Microsporidia*, Blastocystis, Cryptosporidium,* and *Giardia* are eukaryotic zoonotic pathogens thriving in the intestinal tract of human, mammalian, and avian hosts. These opportunistic parasites are among the most common causes of gastrointestinal disorders in humans, domestic and wild mammals, and birds. Microsporidia are obligate, intracellular organisms infecting a wide host range including insects, fish, mammals, and birds (Laksemi *et al.,* 2020; Kašičková *et al.,* 2009; Feng & Xiao, 2010).

More than 1500 species of microsporidia from 200 different genera have been identified among which *Enterocytozoon bieneusi* and *Encephalitozoon* species including *E. cuniculi, E. intestinalis* and *E. hellem* are responsible for intestinal infections with the ability of crossing the host species barrier (Li *et al.,* 2020; Sak *et al*., 2010; Keeling & Fast, 2002; Li *et al*., 2009). *E. hellem* is the dominant species of microsporidia in birds and the third most reported species in human

microsporidiosis. Based on genomic markers, there are seven *E. hellem* genotypes which 1A, 1B, 1C and 2B where identified to be zoonotic (Robertson *et al*., 2019).

Blastocystis sp., is a frequent intestinal protist including various genetic subtypes. Several studies have shown that people with close contact with animals are at higher risk for *Blastocystis* sp., infection. While ST1-ST9 and ST12 were isolated from human samples, ST6 and ST7 are considered "avian STs", because of their relative predominance in birds (Dogruman-Al *et al*., 2009; Alfellani *et al*., 2013; Cian *et al*., 2017).

Cryptosporidiosis is considered a protozoan infection in humans, domestic and wild mammals, birds, and lower vertebrates (Quah *et al*., 2011; Ryan *et al*., 2016). Along with the bird specific species, *C. hominis*, *C. parvum*, and *C. muris* are the zoonotic species reported by birds, causing respiratory and digestive illness. *C. meleagridis* is the third agent of human cryptosporidiosis, which is a turkey (*Meleagris gallopavo*) specific species (Malik *et al*., 2021; Ibrahim *et al*., 2007).

There are bird-specific *Giardia* species, besides reports of *G. duodenalis* assemblages A and B infecting both humans and different species of birds (Ichikawa *et al*., 2019). Zoonotic giardiasis can be acquired through direct contact with infected asymptomatic carrier hosts including humans, domestic and wild animals, and birds and ingestion of infected water sources (Malik *et al*., 2021; Erlandsen and Bemrick, 1987).

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Zoonotic diseases of public health importance are studied considerably though wild, domestic, caged, ornamental, and companion avian hosts are recently being contemplated for their roles in the transmission and spread of important zoonotic pathogens (Malik *et al*., 2021). Some of isolates were shown to be possibly transmitted from these animals to their in-contact workers Epidemiological surveys have revealed that at intensive commercia

Considering the close contact of humans and companion avian birds, and given that zoonotic species and genotypes of the aforementioned parasites have been reported in humans and birds, avian hosts may be a risk factor for human infection. By virtue of the limited number of studies in the population of companion birds in the world and in the country, this study was designed to investigate the occurrence and to evaluate the zoonotic potential of these common parasitic protozoa in pet avian species referred to clinics in Tehran, Iran.

2. Materials and Methods

Sample collection: From April 2020 to July 2020 fresh droppings were collected from cages of pet birds referred to veterinary clinics in Tehran, the capital city of Iran located at 35.5501° N, 51.5150° E coordinates. The samples were collected on site upon admission to the clinic. A total of 150 fecal samples were collected in suitable sealed, labeled, and clean containers, and transported to the parasitology laboratory in the faculty of veterinary medicine in Tehran, Iran without preservative solutions. Prior to preservation in freeze condition, fecal smears were

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prepared and stained with the modified Ziehl–Neelsen method (MZN) for *Cryptosporidium*, Weber's chromotrope-based modified trichrome for microsporidia and trichrome for *Giardia* detection as described by Garcia (Garcia, 2006) . The smears were evaluated microscopically. In addition, a portion of samples was also transferred to sterile 1.5 mL tubes and stored at −20 °C for DNA extraction and further analyses.

DNA extraction and purification: In order to extract total DNA from samples, 250 mg of stool samples was suspended in one ml sterile PBS (pH =7–8). Fecal samples were homogenized by 0.5 mm glass bead disruption. Samples were centrifuged at 2500×*g* for 3 min, the supernatant was discarded, and DNA was extracted from the remaining pellet using stool DNA Extraction kit (MBST, Tehran, Iran). The purified DNA samples were stored at − 20 °C until assessment via PCR technique.

PCR amplification: Four specific primers pairs targeting ribosomal genes of *Cryptosporidium* spp., *Blastocystis* sp., microsporidia (*E. bieneusi* and *Encephalitozoon* spp), and *Giardia* were selected (Quiles *et al*., 2019; Scicluna *et al*., 2006; Hopkins *et al*., 1997; Jalas & Tavalla, 2018) (table 1). PCR amplification was performed in a volume of 25 µL containing 12.5 µL of ready to use master mix, 200 nM of each primer (1 µL each primer), 2 µL of the target DNA sample and 8.5 µL double distilled H_2O. Reactions were performed by Eppendorf thermocycler (Master cycler personal). Samples were denatured at 94 °C for 5 min, followed by 35 (PCR) cycles of

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denaturation for 30 s at 94 °C, annealing for 30 s at the appropriate respective annealing temperature, and extension for 30 s at 72 °C, with a final extension at 72 °C for 5 min. For each organism, positive identified samples (kindly provided by Dr. Mirjalali) were used in parallel with the clinical sample during the extraction, PCR reaction and electrophoresis as positive control. Amplified fragments were analyzed by 1.5% agarose gel electrophoresis stained with GelRed™ (Biotium, USA).

Table 1. Sequence of primers used to investigate microsporidia, *Blastocystis* sp., *Giardia* spp., and *Cryptosporidium* spp. in avian hosts.

Target organism	Primer name	Primers sequence (5′ to 3′)	Approximate size of amplified fragment (bp)	Annealing (°C)	Target Gene	Accession number
Microsporidia	v1f UNIr	CACCAGGTTGATTCTGCCTGAC TCAGGCTCCCTCTCCGGAAT	~300	60	ssUrRNA	MK719236
Blastocystis sp.	RD5 BhRDr	ATCTGGTTGATCCTGCCAGT GAGCTTTTTAACTGCAACAACG	~600	55	ssUrRNA	DQ232775
Giardia	RH11 RH4	CATCCGGTCGATCCTGCC AGTCGAACCCTGATTCTCCGCCAGG	~290	57	ssUrRNA	MK487707
Cryptosporidium	Cry F Cry R	CTGACCTATCAGCTTTAGA GCTGAAGGAGTAAGGAACA	~750	53	ssUrRNA	MW521259

SSU rRNA: small subunit ribosomal RNA

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Sequencing and genotyping: Samples yielding an amplified product of the expected size were considered positive even if they were not sequenced successfully. The positive samples were sequenced (Niagen Noor Company, Iran) in both directions using the amplifying PCR primers. DNA sequences were assembled by means of the BioEdit software (Schneider & Stephens, 1990) and aligned with homologous sequences published in the GenBank database using MEGAX software (Kumar *et al*., 2016). The obtained sequences were compared and blasted with the sequences available in the GenBank collection (Zhang *et al*., 2000). A phylogenetic tree was drawn using the MEGAX software and Neighbor-Joining method (Kumar *et al*., 2016). Bootstrapping with 1000 replicates was used to determine support for the generated clades. In case of identified organisms, an appropriate method was applied in order to characterize the genotype/subtype of the parasite to elucidate its zoonotic potential.

Determination of microsporida genotype by nested PCR: Because of the length polymorphism among *E. hellem* genotypes in the polar tube protein (PTP) gene, two sets of primers were used for the detection and differentiation of *E. hellem* by nested PCR analysis (Table 2). This primer set generate PCR products of known sizes for genotypes 1A, 1B, 1C, and 2B (Xiao *et al*., 2001) .

Table 2. The primers used for genotyping of *Encephalitozoon hellem* isolates based on Polar Tube Protein (PTP) PCR.

Primers sequence (5′ to 3′)	Target organism genotype	Target Size

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			(bp)
External primers	F_1-CTCATGCCAGTTGGTTCCT R_1TGGAGGCATTGCAATAGG	*E. hellem* 1A	461
		E. hellem 1B	521
Internal primers	F_2-CATGCTTGCCAACACAGG R_2-TGGAGGCATTGCAATAGG	*E. hellem* 1C	581
		E. hellem 2B	611

3. Results

Host species: In this study a total of 150 dropping samples derived from 17 bird species belonging to four bird orders from eight avian families were investigated for the presence of intestinal opportunistic pathogens including microsporidia, *Giardia* spp., *Blastocystis* sp., and *Cryptosporidium* spp. The studied host species are summarized in table 3.

Table 3 . The number and variety of species of pet birds have been investigated for the presence of *Cryptosporidium* spp., *Giardia* spp., *Blastocystis* sp., and microsporidia in fecal samples.

Order	Family	Common Name	Scientific Name	Frequency (%)
Psittaciformes	Psittaculidae	Budgerigar	*Melopsittacus ndulatus*	7 (4.66)

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		Lovebird	*Agapornis sp.*	14 (9.33)
		alexandrine Parakeet	*Psittacula eupatria*	6 (4)
		Ring necked parakeet	*Psittacula krameria*	3 (2)
		Lorikeet Parakeet	*Trichoglossus moluccanus*	1 (0.66)
	Psittacidae	African grey parrot	*Psittacus erithacus*	30 (20)
		Green cheeked parakeet	*Pyrrhura molinae*	8 (5.33)
		Amazon parrot	*Amazona*	1 (0.66)
		Sun Parakeet	*Aratinga Spix*	1 (0.66)
	Cacatuidae	Cockatiel	*Nymphicus hollandicus*	44 (29.33)
		Cockatoo	*Cacatua galerita*	4 (2.66)
Passeriformes	Passeridae	Bulbul	*Pycnonotus leucotis*	2 (0.75)
	Fringillidae	Canary	*Serinus canaria*	11 (7.33)
		Finch	*Taeniopygia guttata*	2 (1.33)

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	Sturnidae	Mynah	*Acridotheres tristis*	14 (9.33)
Columbiformes	Columbidae	Pigeon	*Columba livia*	1 (0.66)
Galliformes	Phasianidae	Quail	*Coturnix coturnix*	1 (0.66)

Microscopic and Molecular investigation: Microscopic observation of the fecal smears by modified Ziehl-Neelsen and trichrome staining for the detection of *Cryptosporidium* oocysts, *Giardia* or microsporidia revealed no parasite in the samples.

Among the total examined fecal samples, *Blastocystis* sp., *Cryptosporidium* spp., and *Giardia* spp., were not detected in the samples neither microscopically nor molecularly. A green cheeked parakeet (*Pyrrhura molinae*), an African gray parrot (AGP) (*Psittacus erithacus*) (Family: Psittacidae) and a lovebird (*Agapornis fischeri*) (Family: Psittaculidae) harbored microsporidia in the PCR method. The overall infection frequency of microsporidia was 2% (3/150) and the frequency among the Psittaciformes was 2.5% (3/119).

The expected ~300-bp PCR products were successfully sequenced for three positive samples. The resultant microsporidia sequences were submitted to the NCBI database under the accession numbers OM777676, OM777677, and OM777678. Pairwise alignment of the sequences from the present study revealed 99.59% identity between the green cheek and the lovebird isolate and 98.76% identity between the gray parrot and the green cheek and/or the lovebird isolates (figure 1).

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OM777676 1   ACCAGGTTGATTCTGCCTGACGTGGATGCTATTCTCTGGGGCTAAGCCATGCATGTTTAT  60
OM777678 3   ..........-.................................................  61
OM777677 3   ............................................................  62

OM777676 61  GAAGCCTTTATGGGGGATTGACGGACGGCTCAGTGATAG\TACGATGATTTGATTGGGAGC120
OM777678 62  .......................................\.................... 121
OM777677 63  .......................................T.................G.. 123

OM777676 121 CTGGATGTAACTGTGGGAAACTGCAGGTAAGTTCTGGGGGTGGTAGTTTGTAGCTACTGC 180
OM777678 122 ............................................................ 181
OM777677 124 ......................................T..................... 183

OM777676 181 GTACCGAGTAAGTTGTAGGCCTATCAGCTGGTAGTTAGGGTAATGGCCTAACTAGGCGGAG 241
OM777678 182 ............................................................. 242
OM777677 184 ............................................................. 243
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Figure 1- Pairwise alignment of small subunit ribosomal RNA sequences of microsporidia from the droppings of avian species. (OM777676: isolated from *Pyrrhura molinae*; OM777677: isolated from *Psittacus erithacus*; OM777678: isolated from *Agapornis* sp.)

Phylogenetic tree and genotyping: The isolates in the present study formed a well-supported clade with *Encephalitozoon hellem* sequences from different avian species and mammalian isolates. (Figure 2). The three isolates were further genotyped based on the sequences of PTP. The examined isolates were genotyped as 1B by yielding a 521 bp band after PTP PCR (Figure 3).

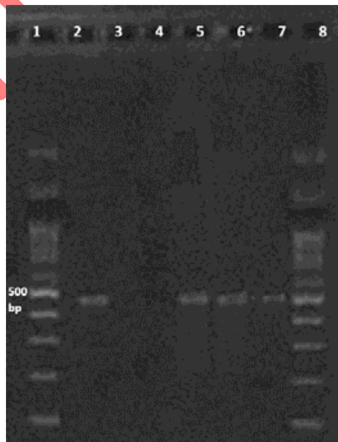

Figure 3- *E. hellem* isolates genotyping by PCR analysis of the PTP gene. Lanes 1 and 8, 100-bp ladders; lanes 2: positive control; lanes 3; lane 4: blank; and 5, genotype 1B; lanes 3 and 4, genotype 1A; lane 6, genotype 2B; and lanes 7 and 8, genotype 1C.

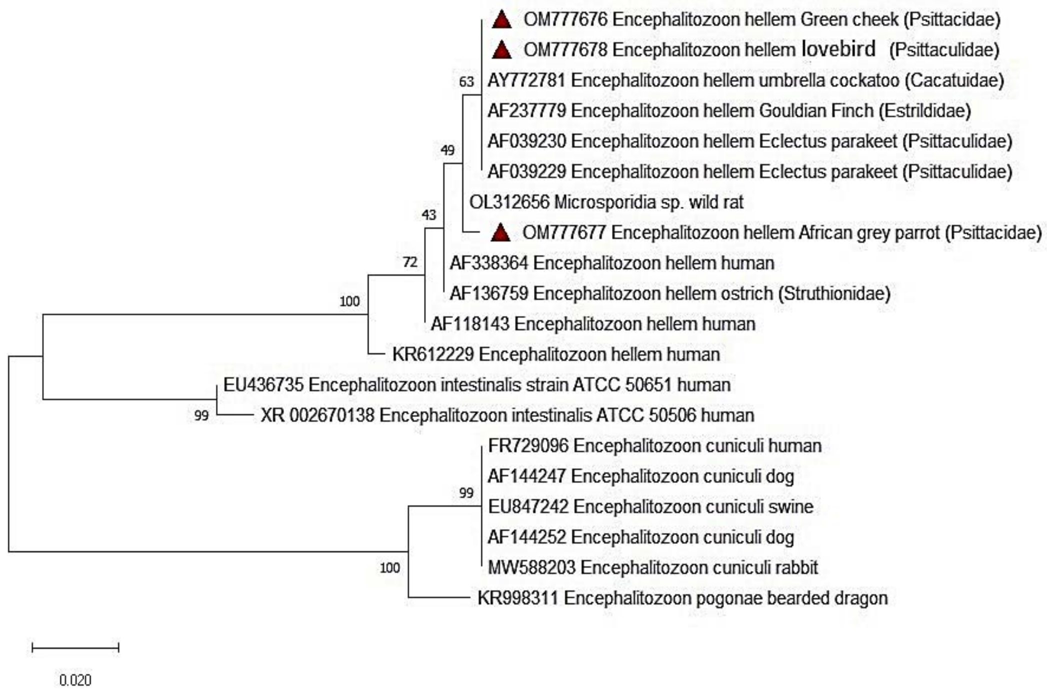

Figure 2- Phylogenetic tree of the small subunit ribosomal RNA sequence for *E. hellem* isolated from pet birds. The Phylogenetic tree was inferred by using the Maximum Likelihood method and Tamura 3-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X. Solid triangles indicate *Encephalitozoon* species identified in the present study.

4. Discussion

Pets including birds may act as a reservoir host for the transmission and/or propagation of pathogens between various animal species and humans. In the present study, pet avian species

were investigated for the occurrence of some of the most important zoonotic pathogens including *Cryptosporidium* spp., *Giardia, Blastocystis* sp., and microsporidia by means of PCR and special staining methods. Among 150 studied droppings from 8 families of pet birds, 3 samples were found to contain *E. hellem* (2 %) by PCR.

Microsporidia are known as opportunistic pathogens infecting a wide range of vertebrate hosts. The pathogen is spreading via contamination of food, water and air with the human and animal excretions (Ruan *et al*., 2021). Among the important zoonotic microsporidian species in humans, *E. hellem* is the dominant species in wild and captive birds (Jalas & Tavalla, 2018; Itoh *et al*., 2021). In the present study *E. hellem* infection, was determined in 3 bird species belonging to Psittaciformes. There are reports of the infection from other bird species including other parrots (Hopkins *et al*., 1997; Itoh *et al*., 2021). as well as hummingbirds, Gouldian finches, and ostrich. The prevalence of infection among companion birds in different studies ranged from 1.1-15.7% (Pllparampil *et al*., 1998; Snowden *et al*., 2000; Snowden and Logan, 1999; Suter *et al*., 1998). and it was 2% herein. According to SSU genotyping, genotypes 1A, 1C, 2B and according to PTP genotyping, genotypes 1A, 1B, 1C and 2B of *E. hellem* have zoonotic potential (Robertson *et al*., 2019). *E. hellem* has been identified in various bird families and Passeriformes, Apodiformes, and many Psittaciformes species were reported to be infected with genotype 1 (further divided to 1A,1B and 1C). In the present study all of the isolates were genotyped as potentially zoonotic genotype 1B. *E. hellem* genotypes 1A, 2B and 2C had been isolated from

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various wild and captive avian hosts. African gray parrot, green-cheek parakeet and lovebirds were reported to harbor genotypes 1A and 2 B (Kasicková *et al*., 2009; Barati *et al*., 2022; Pirestani *et al*., 2013; Rosell *et al*., 2016; Malcekova *et al*., 2010; Lee *et al*., 2011). The hosts in the present study were infected with genotype 1B and according to the best of the authors' knowledge it has been reported from an *Agapornis roseicollis* (Snowden *et al*., 2000) and human cases (Xiao *et al*., 2001). Studies on bird microsporidiosis in Iran include feral and captive avian species. Pigeons, crows, budgies and canraies were reported to be infected with *E. hellem*. The prevalence was from 1.1% in petshop and captive samples to 4.1% in fecal samples collected from public parks. The genotypes were identified in one of these studies which were rported as *E. hellem* genotypes 1A and 3 based on ITS sequence analysis (Pirestani *et al*., 2013; Tavalla et al., 2018; Yazdanjooie *et al.,* 2018). Although it been speculated that birds may act as a mechanical vector for microsporidia, passing and disseminating it through their digestive tract, recently it has been proven that *E. hellem* is proliferating in various tissues of the infected companion birds (Kicia *et al*., 2022). Since *E. hellem* infection in birds is not always associated with clinical disorder (Lee *et al*., 2011; Hinney *et al*., 2016; Mathis *et al*., 2005), pet shop staff and bird owners may be unaware that their environment is contaminated with feces and aerosols from infected pet birds.

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There are reports of bird infection with different species of *Cryptosporidium* with a worldwide prevalence of 0.8-44.4% (Quah *et al*., 2011; Gharagozlou *et al*., 2014; Nakamura & Meireles, 2015; Zaheer *et al*., 2021; Al-Abedi *et al.,* 2022). Aside from *C. meleagridis* which is prevalent in birds and a proven cause of zoonotic cryptosporidiosis in humans, other zoonotic species are rarely reported from birds (Ibrahim *et al*., 2007; Goodwin & Krabill, 1989; Meamar *et al*., 2007). In the present study *Cryptosporidium* was not detected neither microscopically nor molecularly. The mammalian *Cryptosporidium* species identified from pet birds seem to be rare and mechanically spreading to humans (Hopkins *et al*., 1997; Li *et al*., 2016). Giardiasis in avian hosts has been reported with varying prevalence in different bird populations (Ichikawa *et al*., 2019). Despite the reports of *G. psittaci* and different *G. duodenalis* assemblages from pet birds, in the present study *Giardia* was not detected in any of the samples. Despite the low numbers of *Giardia* cysts in fecal samples, the subclinical nature of infection in birds make avian species a source for human infections via direct or indirect contact (Ichikawa *et al*., 2019; Hopkins *et al*., 1997; Heyworth, 2016; Saleh Mohammed Al-Samarrai *et al.,* 2022). *Blastocystis* sp., was not identified in the examined samples in the current study. There are reports of zoonotic *Blastocystis sp.,* subtypes in pet avian species (Barati *et al*., 2022; Asghari *et al*., 2019; Maloney *et al*., 2020; Mohammad Rahimi *et al*., 2021; Hublin *et al*., 2021). There should be more epidemiological investigations to explore the factors associated with *Blastocystis sp.* and public health importance (Wang *et al*., 2018).

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To elaborate the role of pet animals in dissemination of zoonotic pathogens, molecular and genotype data have to be interpreted in association with the supporting epidemiologic and clinical information (Robertson *et al*., 2019). This comprehensively includes the pathogens such as *E. hellem* with its broad avian and mammalian hosts, which apparently complicates the significance of avian pets as a source of human infection. Due to the small size of the spore and the intermittent spore excretion, conventional microscopy is usually insufficient for parasite detection in routine stool examination. Thus, further diagnostic methods such as special stains by light or fluorescence microscopy, transmission electron microscopy, serological tests, flow cytometry, histological analysis, cell culture and molecular-based tests in addition to extensive samplings may strengthen the results of the epidemiological studies.

5. Conclusion

The current study proved the captive pet birds as a source of microsporidian infection. The highly resistant nature of the microsporidia spores, besides the fact that *Encephalitozoonosis* is predominantly subclinical in birds, can put the owners especially children and elderly with impaired immune systems, at increased risk of disease acquisition via spore inhalation or

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ingestion. Further, studies designed with a broader sampling population using repeated sampling to overcome the intermittent spore shedding and multi-loci molecular diagnostics are recommended to truly evaluate the role of pet birds in the epidemiology of zoonotic opportunistic pathogens.

Ethical Considerations

Compliance with ethical guidelines

All procedures were conducted according to the animal care guideline of the Research Committee of the Faculty of Veterinary Medicine, University of Tehran.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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بررسی آلودگی تک یاخته های میکروسپوریدیا، *بلاستوسیستیس*، *ژیاردیا* و *کریپتوسپوریدیوم* در پرندگان خانگی ارجاع شده به درمانگاه‌های دامپزشکی شهر تهران به روش انگل شناسی و مولکولی

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زمینه مطالعه: میکروارگانیسم‌های فرصت طلب دستگاه گوارش از جمله.spp *Cryptosporidium*، .spp *Giardia*، .sp *Blastocystis* و microsporidia به طور چشمگیری مسئول اختلالات بالینی در گونه‌های مختلف میزبان از جمله انسان هستند.

هدف: در مطالعه حاضر حضور ارگانیسم‌های فوق در میزبان پرندگان خانگی بررسی شد.

روش کار: نمونه‌های دفع شده از 150 پرنده خانگی از کلینیک‌های دامپزشکی شهر تهران جمع‌آوری و به صورت میکروسکوپی و مولکولی بررسی شد.

نتایج: سه مورد از مدفوع‌های مورد بررسی حاوی ژنوتیپ (B1) (2٪) *انسفالیتوزوون هلم* با روش PCR و تعیین توالی بود. ارگانیسم‌های میکروسپوریدیایی از مدفوع یک طوطی گرین چیک، یک طوطی خاکستری آفریقایی و یک طوطی برزیلی جدا شدند. سایر انگل‌ها در نمونه های بررسی شده یافت نشد.

نتیجه‌گیری نهایی: مطالعه حاضر ثابت کرد که پرندگان خانگی نگهداری شده در قفس منبع احتمالی عفونت میکروسپوریدیای هستند. ماهیت بسیار مقاوم اسپورهای میکروسپوریدیا، علاوه بر این واقعیت که انسفالیتوزونوزیس عمدتاً در پرندگان تحت بالینی است، می‌تواند صاحبان آن‌ها را در معرض افزایش خطر ابتلا به بیماری از طریق استنشاق یا بلعیدن اسپور قرار دهد. اگرچه منبع و ماهیت عفونت ناشناخته باقی مانده است، ممکن است مطالعات بیشتری برای روشن کردن نقش پرندگان خانگی در اپیدمیولوژی پاتوژن های فرصت‌طلب مشترک بین انسان و دام توصیه شود.

کلمات کلیدی: *بلاستوسیستیس*، بیماریهای مشترک، *ژیاردیا*، *کریپتوسپوریدیوم*، میکروسپوریدیا

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