Original Article

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, such as *Cryptosporidium* spp. *Giardia* spp. *Blastocystis* sp. and microsporidia, are increasingly responsible for clinical disorders in various host species, including humans.

Objectives: This study was conducted to search for the above parasites in the feces of pet birds using parasitological and molecular methods in Tehran City, Iran.

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 methods.

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 a clinically normal green-cheek parakeet, an African gray parrot, and a lovebird. Other parasites that were examined were not found in the analyzed samples.

Conclusion: The current study proved that captive pet birds are a possible source of microsporidian infection. Besides the fact that *encephalitozoonosis* is predominantly subclinical in birds, the highly resistant nature of the microsporidia spores can put the owners at increased risk of disease acquisition via spore inhalation or ingestion.

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

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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 broad 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 *Enterocytozoon cuniculi*, *Enterocytozoon intestinalis* and *Enterocytozoon hellem* are responsible for intestinal infections with the ability to cross the host species barrier (Li et al., 2020; Sak et al., 2010; Keeling & Fast, 2002; Li et al., 2019). *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 were identified as zoonotic (Robertson et al., 2019).

Blastocystis sp. is a frequent intestinal protist that includes 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 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, *Cryptosporidium hominis*, *Cryptosporidium parvum* and *Cryptosporidium muris* are the zoonotic species reported by birds that cause respiratory and digestive illnesses. *Cryptosporidium meleagridis* is the third agent of human cryptosporidiosis, a turkey (*Meleagris gallopavo*) specific species (Malik et al., 2022; Ibrahim et al., 2007).

There are bird-specific *Giardia* species, besides reports of *Giardia 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., 2022; Erlandsen & Bemrick, 1987).

Zoonotic diseases of public health importance are studied considerably though wild, domestic, caged and ornamental; companion avian hosts have recently contemplated their roles in transmitting and spreading important zoonotic pathogens (Malik et al., 2022). Some of the isolates were shown to be possibly transmitted from these animals to their in-contact workers.

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

Materials and Methods

Sample collection

From April 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, Tehran University, in Tehran, Iran, without preservative solutions. Before preservation in freeze condition, fecal smears were prepared and stained with the modified Ziehl-Neelsen method for *Cryptosporidium*, Weber's chromotrope-based modified trichrome for microsporidia and trichrome for *Giardia* detection as described by Garcia (2006). The smears were evaluated microscopically. In addition, a portion of the samples was transferred to sterile 1.5 mL tubes and stored at -20 °C for DNA extraction and further analyses. 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 1.

Table 1. Pet bird species of investigated for the presence of zoonotic parasites in dropping samples

Order	Family	Common Name	Scientific Name	No. (%)
Psittaciformes	Psittaculidae	Budgerigar	*Melopsittacus undulates*	7(4.66)
		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	*P. erithacus*	30(20)
		Green cheeked parakeet	*P. 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)
	Sturnidae	Mynah	*Acridotheres tristis*	14(9.33)
Columbiformes	Columbidae	Pigeon	*Columba livia*	1(0.66)
Galliformes	Phasianidae	Quail	*Coturnix coturnix*	1(0.66)

DNA extraction and purification

To extract total DNA from samples, 250 mg of stool samples was suspended in 1 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 a 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 2). 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 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, positively identified samples (kindly provided by Mirjalali) were used in parallel with the clinical sample during the extraction. PCR reaction and electrophoresis were used as positive controls. Amplified fragments were analyzed by 1.5% agarose gel electrophoresis stained with GelRed™ (Biotium, USA).

Sequencing and genotyping

Samples yielding an amplified product of the expected size were considered positive even if not sequenced successfully. The positive samples were sequenced (Niagen Noor Company, Iran) in both directions using the amplifying PCR primers. DNA sequences were assembled using BioEdit software, version 7.2.5 (Schneider & Stephens, 1990) and aligned with homologous sequences

Table 2. 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′)	The Approximate Size of Amplified Fragment (bp)	Annealing (°C)	Target Gene	Accession Number
Microsporidia	v1f UNIr	F: CACCAGGTTGATTCTGCCTGAC R: TCAGGCTCCCTCTCCGGAAT	~300	60	ssUr-RNA	MK719236
Blastocystis sp.	RD5 BhRDr	F: ATCTGGTTGATCCTGCCAGT R: GAGCTTTTTAACTGCAACAACG	~600	55	ssUr-RNA	DQ232775
Giardia	RH11 RH4	F: CATCCGGTCGATCCTGCC R: AGTCGAACCCTGATTCTCCGC-CAGG	~290	57	ssUr-RNA	MK487707
Cryptosporidium	Cry F Cry R	F: CTGACCTATCAGCTTTAGA R: GCTGAAGGAGTAAGGAACA	~750	53	ssUr-RNA	MW521259

SSU rRNA: Small subunit ribosomal RNA.

published in the GenBank database using MEGAX software (Kumar et al., 2018). 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, version 10.1.8 and the Neighbor-Joining method (Kumar et al., 2018). Bootstrapping with 1000 replicates was used to determine support for the generated clades. In the case of identified organisms, an appropriate method was applied to characterize the genotype/subtype of the parasite to elucidate its zoonotic potential.

Determination of microsporidia 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 to detect and differentiate *E. hellem* by nested PCR analysis (Table 3). This primer set generates PCR products of known sizes for genotypes 1A, 1B, 1C and 2B (Xiao et al., 2001).

Results

Microscopic and molecular investigation

Microscopic observation of the fecal smears by modified Ziehl-Neelsen and trichrome staining for detecting *Cryptosporidium* oocysts, *Giardia* or microsporidia revealed no parasites in the samples.

Among the total examined fecal samples, *Blastocystis* sp. *Cryptosporidium* spp. and *Giardia* spp. were not detected in the samples microscopically or 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 3.119(2.5%).

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

Table 3. The primers used for genotyping of *E. hellem* isolates based on *PTP* PCR

Primers Sequence (5′ to 3′)		Target Organism Genotype	Target Size (bp)
External primers	F-CTCATGCCAGTTGGTTCCT	*E. hellem* 1A	461
	R-TGGAGGCATTGCAATAGG	*E. hellem* 1B	521
Internal primers	F-CATGCTTGCCAACACAGG	*E. hellem* 1C	581
	R-TGGAGGCATTGCAATAGG	*E. hellem* 2B	611

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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

Note: OM777676: Isolated from *P. molinae*; OM777677: Isolated from *P. erithacus*; OM777678: Isolated from *Agapornis* sp.

98.76% identity between the gray parrot and the green cheek and or the lovebird isolates (Figure 1).

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).

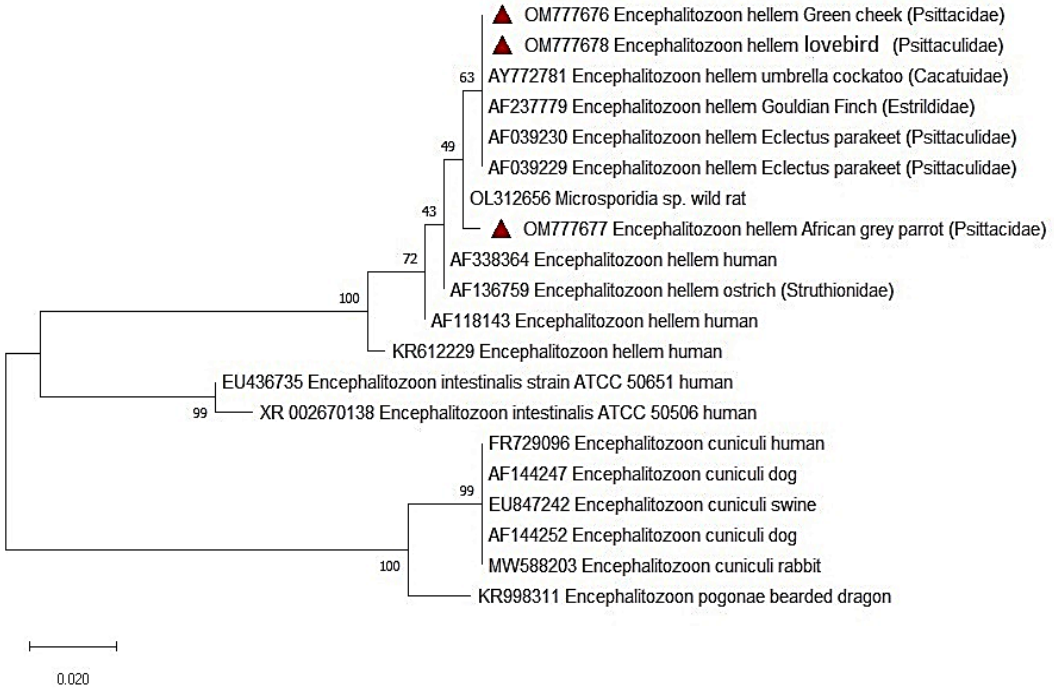

Figure 2. Phylogenetic tree of the small subunit ribosomal RNA sequence for *E. hellem* isolated from pet birds

Note: The phylogenetic tree was inferred using the maximum likelihood method and the 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.

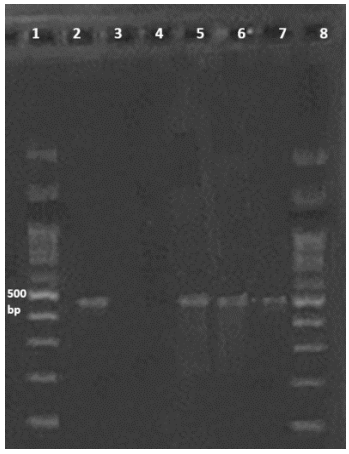

Figure 3. *E. hellem* isolates genotyping by PCR analysis of the *PTP* gene

Note: Lanes 1 and 8: 100-bp ladders; lanes 2: Positive control; lanes 3 and 4: blank; lane 5: Genotype 1B; lanes 3 and 4: Genotype 1A; lane 6: Genotype 2B; lanes 7 and 8: Genotype 1C.

Discussion

Pets, including birds, may act as reservoir hosts for the transmission and or propagation of pathogens between animals 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 utilizing 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 food, water and air contamination with human and animal excretions (Ruan et al., 2021). Among humans' important zoonotic microsporidian species, *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 ostriches. The prevalence of infection among companion birds in different studies ranged from 1.1% to 15.7% (Pulparampil et al., 1998; Snowden et al., 2000; Snowden & Logan, 1999; Suter et al., 1998) and it was 2% herein. According to SSU genotyping, genotypes 1A, 1C and 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 into 1A,1B and 1C). All isolates were genotyped as potentially zoonotic genotype 1B in the present study. *E. hellem* genotypes 1A, 2B and 2C had been isolated from various wild and captive avian hosts. African gray parrots, green-cheek parakeets 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. According to the authors' knowledge, it has been reported from *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 canaries were reported to be infected with *E. hellem*. The prevalence was from 1.1% in pet shops and captive samples to 4.1% in fecal samples collected from public parks. The genotypes were identified in one of these studies, which were reported as *E. hellem* genotypes 1A and three based on ITS sequence analysis (Pirestani et al., 2013; Tavalla et al., 2018; Yazdanjooie et al., 2018). Although it has 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.

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 either microscopically or molecularly. The mammalian *Cryptosporidium* species identified from pet birds seem rare and mechanically spreading to humans (Hopkins et al., 1997; Li et al., 2019). Giardiasis in avian hosts has been reported to have varying prevalence in different bird populations (Ichikawa et al., 2019). Despite the reports of *G. psittaci* and different *G. duodenalis* assemblages from pet birds, *Giardia* was not detected in any of the samples in the present study. Despite the low number of *Giardia* cysts in fecal samples, the subclinical nature of infection in birds makes avian species a source of 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).

To elaborate on the role of pet animals in disseminating zoonotic pathogens, molecular and genotype data must be interpreted in association with the supporting epidemiologic and clinical information (Robertson et al., 2019). This search comprehensively includes pathogens such as *E. hellem* with its broad avian and mammalian hosts, which 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, molecular-based tests, and extensive samplings may strengthen the results of the epidemiological studies.

Conclusion

The current study proved that captive pet birds are a source of microsporidian infection. Besides the fact that Encephalitozoonosis is predominantly subclinical in birds, the highly resistant nature of the microsporidia spores can put the owners, especially children and elderly with impaired immune systems, at increased risk of disease acquisition via spore inhalation or 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 Guidelines of the Research Committee of the Faculty of Veterinary Medicine, Tehran University (Code: (28864/6/6).

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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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مقاله پژوهشی

بررسی آلودگی تک‌یاخته‌های میکروسپوریدیا، بلاستوسیستیس، ژیاردیا و کریپتوسپوریدیوم در پرندگان خانگی ارجاع‌شده به درمانگاه‌های دامپزشکی شهر تهران به روش انگل‌شناسی و مولکولی

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چکیده

زمینه مطالعه: میکروارگانیسم‌های فرصت‌طلب دستگاه گوارش ازجمله Blastocystis sp.، Giardia spp.، Cryptosporidium spp. و microsporidia به‌طور چشمگیری مسئول اختلالات بالینی در گونه‌های مختلف میزبان ازجمله انسان هستند.

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

روش کار: نمونه‌های دفع‌شده از ۱۵۰ پرنده خانگی از کلینیک‌های دامپزشکی شهر تهران جمع‌آوری و به‌صورت میکروسکوپی و مولکولی بررسی شدند.

نتایج: سه مورد از مدفوع‌های موردبررسی حاوی ژنوتیپ (2%) (B1) 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 نمونه‌های بررسی‌شده یافت نشد.

نتیجه‌گیری نهایی: مطالعه حاضر ثابت کرد پرندگان خانگی نگهداری‌شده در قفس منبع احتمالی عفونت میکروسپوریدیای هستند. ماهیت بسیار مقاوم اسپورهای میکروسپوریدیا، علاوه‌براین 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، مطالعات بیشتری برای روشن کردن نقش پرندگان خانگی در اپیدمیولوژی پاتوژن‌های فرصت‌طلب مشترک بین انسان و دام توصیه می‌شود.

کلیدواژه‌ها: بلاستوسیستیس، بیماری‌های مشترک، ژیاردیا، کریپتوسپوریدیوم، میکروسپوریدیا

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