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Original Article

Microsporidial Spores in Fecal Samples of Some Domesticated Animals Living in Giza, Egypt

***Ahmad Z. AL-HERRAWY, Mahmoud A. GAD**

Dept. of Water Pollution Research, National Research Centre, 12622 Dokki, Giza, Egypt

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***Correspondence**
Email:
alherrawy@gmail.com

Abstract

Background: The aim of the present work was to investigate the prevalence and species of intestinal microsporidiosis among animals in Giza, Egypt.

Methods: A total of 869 animal fecal samples were collected from domesticated animals (dogs, cats, rabbits, cattle, buffaloes, sheep, goats, donkeys and pigs) living in Giza, Egypt. Spores of microsporidia were concentrated from collected samples by centrifugation and finally stained with modified trichrome (MT) stain to detect microsporidial spores. Microsporidial spores in microscopically-positive samples were molecularly confirmed and identified using species-specific primers.

Results: Spores of microsporidia were microscopically detected in 17.0% of the examined animal fecal samples. The highest and lowest rates of infection with intestinal microsporidia were recorded in dogs (33.3%) and buffaloes (6.9%), respectively. Molecularly, the obtained microsporidial spores were classified as *Enterocytozoon bieneusi* and *E. intestinalis*. Dual infection with both identified species was observed in fecal samples from buffalo, rabbit, goat, cat, pig and dog.

Conclusion: Domestic animals may play a role in dissemination of intestinal microsporidiosis in the environment. Examined animals were infected with *E. bieneusi* in a higher percentage than *E. intestinalis*.

Introduction

Microsporidia are single-celled microorganisms defined as obligate intracellular eukaryotic parasites capable for infection of protozoa, other invertebrates, and vertebrates (1). Morphologically, spores of microsporidia may be spherical, ovoid, rod-shaped, or crescent-shaped, although most are ovoid (2). These organisms are microscopically defined by the presence of a nucleated sporoplasm, a coiled polar tube

and an anchoring disk. They lack several eukaryotic organelles such as mitochondria, Golgi membranes, and eukaryotic ribosomes (3).

Historically, microsporidial spores were first recognized as the causative agent of pe´brine (pepper) disease, severely affecting the silkworm industry in France and Italy during the mid-17th century (4). Thenceforth microsporidiosis have affected honeybee, fish, and mink industries have been compromised by microsporidiosis (5). Since 1985, microsporidia have been incriminated as a causative agent of opportunistic infections associated with persistent diarrhea and weight loss in persons with AIDS (6-8). At first, microsporidia were microscopically considered as primitive protozoa, but in the 1990s, molecular and phylogenetic evidences revealed relationship of these organisms and fungi (9).

More than 1300 species of microsporidia were identified and divided into about 150 genera (10, 11). About 14 microsporidia species infect humans (11). *Enterocytozoon bieneusi* is considered the most prevalent enteric species infecting humans worldwide, followed by *E. intestinalis* (12). Vertical transmission of microsporidiosis from mother to offspring has been represented in animals (rodents, rabbits, carnivores, and non-human primates) (13, 14). The presence of microsporidia in the respiratory and intestinal tracts of infected individuals and the excretion of spores in urine and feces illustrate that horizontal transmission is possible through fecal–oral transmission, oral–oral transmission, inhalation of contaminated aerosols, and ingestion of contaminated food and water (8, 15).

Risk factors associated with microsporidiosis that support horizontal transmission include homosexual practices, intravenous drug use, and exposure to water in swimming pools and hot tubs as well as occupational contact with water contaminated with microsporidial spores (16, 17). Those organisms are involved in Drinking Water Contaminant Candidate List of the US Environmental Protection Agency (USEPA) (18). *E. bieneusi* is found in a variety of mammals including macaques, dogs, cats, cattle, llamas, raccoons, muskrats, beavers, foxes, otters and pigs (19-24).

To our knowledge scarce data is available concerning the occurrence of intestinal microsporidiosis in domestic animals in Egypt. Therefore, the aim of the present work was to investigate the prevalence and species of intestinal microsporidiosis among animals in Giza, Egypt.

Materials and Methods

Animal fecal samples

A total of 869 fecal samples were separately collected from animals in Giza, Egypt in 2012-2013. Fecal samples were collected from 108 dogs "*Canis lupus familiaris* ", 104 cats "*Felis catus*", 98 cattle "*Bos taurus*", 116 buffaloes, "*Bubalus bubalis* ", 83 goats "*Capra hircus*", 89 sheep "*Ovis aries*" 96 pigs "*Sus scrofa*", 88 donkeys "*Equus asinus* " and 87 rabbits "*Oryctolagus cuniculus*". Fecal samples were separately collected in clean plastic containers. Animal fecal samples were labeled with species of animal. The collected samples were carried out to the laboratory at the same day of collection.

Concentration of microsporidial spores

Fecal samples were separately homogenized with clean spatula and then divided into two equal parts. One part of each fecal specimen was preserved in 10% formalin solution (Merck) and used for microscopic detection of microsporidial spores. The second part of each fecal sample was kept at -20 ^{o}C until used for DNA extraction. Ethyl acetate concentration method was used to concentrate the spores of microsporidia in fecal samples (25).

Staining and light microscopy

The obtained concentrated pellet was fixed on a clean glass slide using absolute methyl alcohol (Merck) and stained with modified trichrome (MT) stain (26).

Stained smears were microscopically examined with oil immersion lens for the presence of microsporidia spores. Stained microsporidia spores appeared reddish with clearly defined edges and a vacuole against a green background.

DNA extraction

The preserved part of each fecal sample was washed with phosphate buffer saline (PBS) and centrifuged at 2500 g for 5 min. The supernatant was decanted and the remaining pellet was washed again two times as mentioned before. The final washed pellet was re-suspended in 1 ml of PBS. Two hundred microliters of fecal suspension were extracted using the Qiagen QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) with a modified protocol. The temperature of the initial lysis step was increased to 95 °C for 5 min, followed by incubation with Proteinase K for 4 h in a water bath at 55 °C, and then 100 µl of AE buffer was used for elution. The elution step was repeated to increase the amount of DNA yield by reapplying the original 100 µl to the spin column. DNA eluate was stored at −20 °C until PCR analysis.

PCR amplification and electrophoresis

PCR was performed using three different diagnostic primer pairs: i) generic microsporidia primer pair (PMP1 and PMP2) to confirm the presence of microsporidia (27); ii) species specific primer pair (EBIEF1/EBIER1) for amplification of microsporidial small subunit rRNA (SSU-rRNA) coding regions of *E. bieneusi* (28); and iii) species specific primer pair (SINTF/SINTR) for *E. intestinalis* (29) (Table 1).

Amplification of DNA was performed using Maxima Hot Start Green PCR master mix (Thermo Scientific). A hot-start procedure for microsporidia and *E. bieneusi* was used with an initial denaturation at 95 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30s, primer annealing at 60 °C for 30 s and extension at 72 °C for 30s. A final extension step was performed at 72 °C for 10 min (27, 28). The optimal PCR conditions for the SINTF/SINTR primers began with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30s, annealing at 55 °C for 30s, and extension at 72 °C for 90 s. A final extension step was performed at 72 °C for 10 min (29). Agarose gel electrophoresis was consequently used for the detection of PCR products.

Table 1: Primers of microsporidia, *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*

Organism	Primer name	Sequence	Fragment length (bp)	Reference
Microsporidia	PMP1 PMP2	CACCAGGTTGATTCTGCCTGAC CCTCTCCGGAACCAAACCCTG	*250 :E. bieneusi* *268 : E. cuniculi* 270:*E. intestinalis* *279 : E. hellem.*	27
Enterocytozoon bieneusi	EBIEF1 EBIER1	GAAACTTGTCCACTCCTTACG CCATGCACCACTCCTGCCATT	607	28
Encephalitozoon intestinalis	SINTF SINTR	TATGAGAAGTGAGTTTTTTTC CCGTCCTCGTTCTCCTGCCCG	545	29

Results

Microscopic examination of 869 fecal samples from different animals revealed the presence of intestinal microsporidia in 17.0% of them by using modified trichrome stain. The highest rate of infection with intestinal microsporidia was recorded in dogs (33.3%), followed by 23.1, 20.5, 18.8, 14.9, 14.3, 11.2, 9.1 and 6.9% in cats, goats, pigs, rabbits, cattle, sheep, donkeys and buffaloes, respectively (Table 2 and Fig. 1).

Table 2: Prevalence of intestinal microsporidia in animal fecal samples by modified trichrome stain

Source of fecal samples	Fecal samples		
	Total no. examined	Microsporidia +Positive by MT stain	
		No.	%
Cattle	98	14	14.3
Buffalo	116	8	6.9
Sheep	89	10	11.2
Goat	83	17	20.5
Rabbit	87	13	14.9
Cat	104	24	23.1
Dog	108	36	33.3
Donkey	88	8	9.1
Pig	96	18	18.8
Total	869	148	17.0

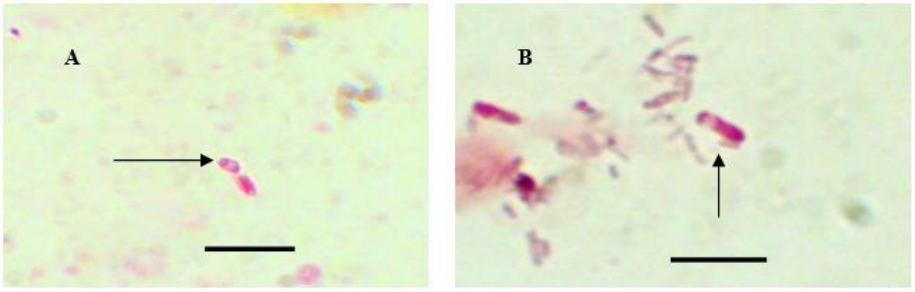

Fig. 1: Original picture of different microsporidial spores stained with MT stain. Bar = 5µm

Molecularly, intestinal microsporidial spores were recorded in 95 (64.2%) out of the 148 microscopically positive samples. 76.8% and 42.1 % of the PCR positive animal fecal samples had *E. bieneusi* and *E. intestinalis,* respectively. The highest rate of infection with *E. bieneusi* reached 100% in cattle, buffaloes, sheep and goats. No infection with *E. bieneusi* was recorded in donkey fecal samples. The highest rate of infection with *E. intestinalis* was recorded in donkeys (100%), followed by 82.6, 36.4, 31.3, 26.7, 25.0 and 20.0%, in dogs, goats, cats, pigs, buffaloes and rabbits, respectively. There was no infection with *E. intestinalis* in cattle and sheep. Dual infection with both *E. bieneusi* and *E. intestinalis* was recorded in examined fecal samples from buffalo, rabbit, goat, cat, dog and pig (Table 3 and Fig 2-4).

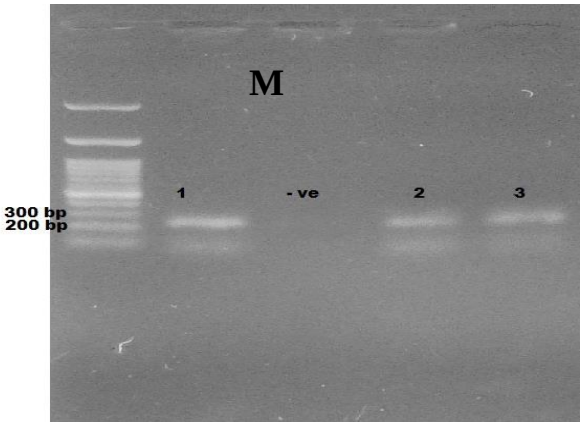

Fig. 2: Ethidium bromide stained 2% agrose showing PCR products of microsporidia. M: Marker (100 plus bp), -ve: negative control, samples 1,2,3: positive samples at 250-279bp.

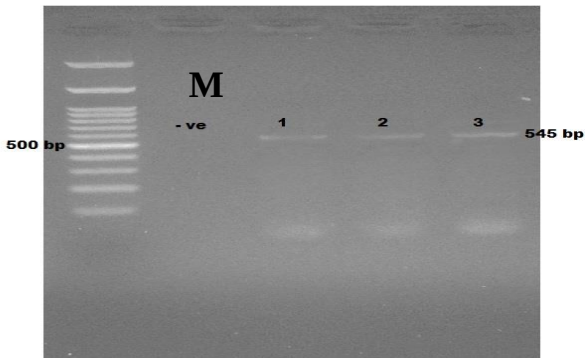

Fig. 3: Ethidium bromide stained 2% agrose showing PCR products of *Encephalitozoon intestinalis*. M: Marker (100 plus bp); -ve: negative control; lanes 1-3: positive samples

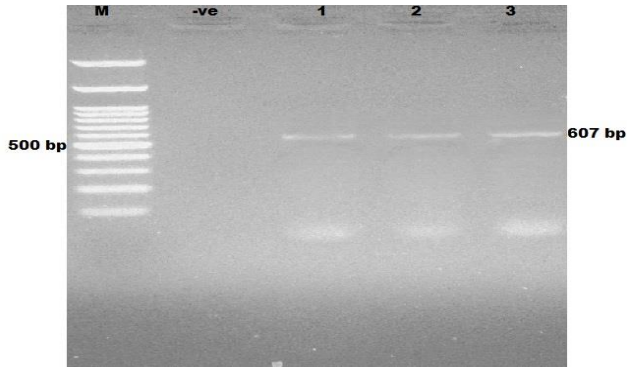

Fig. 4: Ethidium bromide stained 2% agrose showing PCR products of *Enterocytozoon bieneusi*. M: Marker (100 plus bp); -ve: negative control; lanes 1-3: positive samples

Table 3: Species identification of intestinal microsporidia in examined animals by PCR

Sources of fecal samples	PCR positive fecal samples for intestinal microsporidia				
	Total + positive samples by PCR	*E. bieneusi* positive samples		*E. intestinals* positive samples	
		NO.	%	NO.	%
Cattle	9	9	100	0	0.0
Buffaloes	4	4	100	1	25.0
Rabbits	5	4	80	1	20.0
Sheep	6	6	100	0	0.0
Goat	11	11	100	4	36.4
Cats	16	13	81.3	5	31.3
Dogs	23	14	60.9	19	82.6
Donkeys	6	0	0.0	6	100
Pigs	15	12	80	4	26.7
Total	95	73	76.8	40	42.1

Discussion

Overall, 148 out of 869 examined animal fecal samples were positive for intestinal microsporidia by MT stain. Only 95 out of 148 microscopically positive fecal samples were molecularly positive for intestinal microsporidia. This result agreed with another study (30) where the detection of microsporidia by PCR showed lower correlation with their detection by light microscopy. Furthermore, in Portugal the inhibitors could interfere with PCR results and therefore PCR was postulated to have a limited value for identification of the species of microsporidia present in fecal samples (31).

In the present study, a total of 14 (14.3%) cattle fecal samples were positive for intestinal microsporidia by modified trichrome stain. Other workers in Spain found no infection with intestinal microsporidia in fecal samples from three caws by Weber's chromotrope stain and this might be attributed to the small number of samples (32). In Mexico, *E. intestinalis* was detected in one caw fecal sample

stained with quick-hot Gram chromotrope stain (33).

The present result showed that nine out of 14 microscopically-positive cattle fecal samples were molecularly-positive and identified as *E. bieneusi*. In other molecular studies, *E. bieneusi* was reported in cattle feces in Germany (11.7%) (34), USA (9.5%) (35), Korea (14.9%) (36) and Argentina (14.3%) (37).

In our study, a total of 8/88 (9.1%) donkeys had intestinal microsporidia by MT stain. Our result was greatly lower than that of Lores et al. (32) where one of two examined fecal samples of donkeys had intestinal microsporidia and Bornay-linares et al. (33) who examined only two donkeys and found that one of them had intestinal microsporidia. The greater difference in the number of examined samples could not be dependent due the very low number of examined animals.

The present results showed that 6 out of 8 donkey fecal samples (positive for microsporidia by MT stain) had intestinal microsporidia by PCR. Molecularly, the identified species in these 6 donkeys were all *E. intestinalis*. In USA, molecular examination of only 2 donkeys revealed that one of them had intestinal microsporidia identified as *E. intestinalis* (33), while in Spain no microsporidia was detected by PCR in donkey fecal samples that were microscopically positive for microsporidia (32).

Microscopic examination of pig fecal samples stained with MT stain in the current investigation revealed the presence of microporidial spores in 18.8% of them. A higher occurrence (82%) of intestinal microsporidia in pigs was recorded in Peru using calcoflour M2R stain (38). This variation between our result and that of Sak et al. (38) might be attributed to difference in diagnostic tools. In Spain, no intestinal microsporidia was observed in 4 pigs by using Weber's chromotrope stain and this might be attributed to the low number of the examined fecal samples (32).

The current work revealed that 15 out of 18 pig fecal samples (that were microscopically positive for microsporidia) were positive by PCR. The identified species of microsporidia in these samples were: 12 pigs had *E. bieneusi*, four pigs had *E. intestinalis*. Other workers recorded higher incidences (94% and 92.6%) of *E. bieneusi* by PCR in the examined pig fecal samples from Czech Republic and Slovakia (38, 39), while lower incidences were reported in Switzerland, Massachusetts (USA) and Japan (40-43).

To our knowledge there were no available data concerning the detection of intestinal microsporidia in sheep by using staining techniques. Using PCR technique in the present work, it was found that 60% of microscopically positive sheep fecal samples for intestinal microsporidia were PCR positive and all samples had only a single infection with *E. bieneusi*. Other workers agreed with our result in that *E. bieneusi* was the only species identified in feces of sheep (43).

In the present work, 20.5% of the examined goat fecal samples (n=83) by MT stain were microscopically positive for intestinal microsporidia. Other workers in Spain observed clusters of microsporidia-like spores within a vacuole inside epithelial cells in fecal smears of one goat using Weber's chromotrope-based stain (32).

In the present study, 64.7% of microscopically positive goat fecal samples (n=17) were positive by PCR. In addition, 41.2% of PCR positive goat fecal samples had a single infection with *E. bieneusi*. Other workers in Spain (32) and Peru (43) found that 14.2% and 2% of the examined goat fecal samples had *E. bieneusi* only, while Bornay-linares et al. (33) in USA examined one goat fecal sample by PCR and identified *E. intestinalis* in it.

14.9% of the examined rabbit fecal samples had intestinal microsporidia by MT stain which was higher than the result of Lores et al. (32) in Spain who found that 2/22 (9.1%) of rabbits had intestinal microsporidia by Weber's chromotrope stain.

Molecularly, single as well as mixed infections with *E. bieneusi* and *E. intestinalis* were observed in pellets of rabbits in the present

work. *E. bieneusi* was microscopically identified and by PCR for the first time in rabbit fecal samples in Spain, a single infection of rabbits with either *E. bieneusi* (44) or *E. cuniculi* (32) was recorded, while no infection with intestinal microsporidia was reported in Germany (34).

Intestinal microsporidia were microscopically detected in cat feces in our study in Egypt (23.1%) and in Portugal (29.4%) (31), but not in Spain (32) might be attributed to difference in geographic criteria of these different countries.

The present study showed that both *E. bieneusi* and *E. intestinalis* were present in feces of cats. In Germany (34), Japan (42) and USA (45), *E. bieneusi* was the only identified species in 5, 17 and 14.3% of cat fecal samples by PCR, respectively. In Portugal, both *E. bieneusi* and *E. cuniculi* were detected in feces of cats (31), while no intestinal microsporidia were found in ten examined cat fecal samples in Spain (32).

In the present study, 36/108 (33.3%) of dogs had intestinal microsoporidia by MT stain. Other workers in Spain found a lower incidence (5.9%) of intestinal microsporidia in dogs by Weber's chromotrope stain (32). In Portugal, a lower incidence [13.8% (5/36)] of intestinal microsporidia was detected in dogs by MT stain (31). The variation between our result and the previous results (31, 32) might be attributed to difference in the number of the examined samples.

The present work declared that dogs could have *E. bieneusi*, *E. intestinalis* or both in their feces. Other workers in Colombia (46), Spain ((32), Portugal (31) and Japan (42) found molecularly that 15, 11.8, 60 and 2.5% of examined dogs had intestinal microsporidia belonging to only *E. bieneusi*, while in no microsporidial spores were found in 60 dog fecal samples examined by PCR in Germany (34).

Conclusion

E. bieneusi was the most prevalent intestinal microsporidia in domesticated animals that may play a role in dissemination of intestinal microsporidiosis in the environment.

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