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Original Article

Molecular Detection and Identification of Zoonotic Microsporidia Spore in Fecal Samples of Some Animals with Close-Contact to Human

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Abstract

Background: Microsporidia species are obligatory intracellular agents that can infect all major animal groups including mammals, birds, fishes and insects. Whereas worldwide human infection reports are increasing, the cognition of sources of infection particularly zoonotic transmission could be helpful. We aimed to detect zoonotic microsporidia spore in fecal samples from some animals with close – contact to human.

Methods: Overall, 142 fecal samples were collected from animals with closed-contact to human, during 2012-2013. Trichrome – blue staining were performed and DNA was then extracted from samples, identified positive, microscopically. Nested PCR was also carried out with primers targeting SSU rRNA gene and PCR products were sequenced.

Results: From 142 stool samples, microsporidia spores have been observed microscopically in 15 (10.56%) samples. *En. cuniculi* was found in the faces of 3 (15%) small white mice and 1 (10%) laboratory rabbits (totally 2.81%). Moreover, *E. bienersi* was detected in 3 (10%) samples of sheep, 2 (5.12%) cattle, 1 (10%) rabbit, 3 (11.53%) cats and 2 (11.76%) ownership dogs (totally 7.74%). Phylogenetic analysis showed interesting data. This is the first study in Iran, which identified *E. bienersi* and *En. Cuniculi* in fecal samples of laboratory animals with close – contact to human as well as domesticated animal and analyzed them in phylogenetic tree.

Conclusion: *E. bienersi* is the most prevalent microsporidia species in animals. Our results can also alert us about potentially zoonotic transmission of microsporidiosis.

Introduction

Microsporidia group is an obligatory intracellular agent, which can infect all major animal groups including mammalian, birds, fishes and insects (1-3). At first, *Microsporidia* spp., were only known as causative agent of economic losses in silkworm, honeybee and fishery industries (3). With identification of microsporidiosis in human in 1959 (4) and increasing the reports of the infection in immunocompromised patients particularly in recent years, the worth of identification of infection in animals as potentially sources of zoonotic transmission, has been illuminated more than before (3).

Although there are vast information in epidemiology and infection of microsporidia, but the manner of transmission and source of human infection have not been clearly known (5, 6). The zoonotic transmission of microsporidia has not been demonstrated yet, but this way could be concerned as a considerable reason of *Microsporidia* infection in Human populations. The species that infect human, particularly *E. bienersi* and *Encephalitozoon* spp., have been reported frequently from wide range of mammalian hosts (7-10). Several studies have been carried out on laboratory, household and wild animals as well as birds, which introduced them as potentially zoonotic sources of human intestinal microsporidiosis (8, 9, 11-18).

However, only few studies have been carried out around intestinal *Microsporidia* species in animals related to human in Iran. To date, some studies were performed on pigeon (19), cat and dog (14), but other human closed-contiguity animals have not been considered as potentially zoonotic sources. This study aimed to determine intestinal *Microsporida* species in laboratory rabbits and small white mice, ownership dogs, cats, dairy cattle and sheep.

Materials and Methods

Animal stool samples

A total of 142 fecal samples were collected from animals with closed-contact to human,

containing laboratory small white mice (n=20), rabbits (n=10), ownership dogs (n=17), cats (n=26), sheep (n=30) and dairy cattle (n=39) during 2012-2013. Samples of ownership dog and cats were provided from Meshkin shahr district, Ardebil, Iran. Sheep and dairy cattle were provided from same farm in Tehran Province and finally laboratory animals sample was obtained from Animal House of School of Public Health, Tehran University of Medical Sciences.

Samples were washed according to method that mentioned elsewhere (20). Briefly, the samples were suspended in sterile PBS (pH: 7.5) and filtrated with sterile gases for debris exclusion. Remained suspension washed three times by sterile PBS (pH: 7.5). After final centrifuging at 2500 rpm for 10 min and removing the supernatant, remained pellet of each isolate was re-suspended in sterile PBS (pH: 7.5), formalin – PBS 5% for molecular and parasitological survey, respectively.

Parasitology

Slim slides of each sample, stained with trichrome - blue staining, were performed (21). All the slides were examined under light microscope with high magnifications (1000 X). Ovoid, pinkish spore that were about 1-2.5 μm were considered as *Microsporidia* spp.

DNA extraction

DNA extraction was carried out for all microscopically positive and negative samples (20). Briefly, 250 μl of PBS suspended stool were transferred to 1.5 ml tube. After centrifuging in 10000 rpm for 5 min and removing the supernatant, 400 μl of lysis buffer (Tris 100mM, EDTA 10 mM, SDS 2% and 20 μg per μl of Proteinase K) and 300 μl volume of acid washed Glass beads (size 425-600 μm) were added to pellet. Every tube was shaken vigorously for 2 min and was placed in 60 °C for 4 hours. Samples were shaken every 30 min intensely. Subsequently, after centrifuging at 3000 rpm for 5 min and supernatant was

collected and transferred to Bioneer stool DNA extraction kit (Bioneer Corporation, Daejeon, Korea). Purified DNA stored at -20 °C until use.

Nested - Polymerase Chain Reaction

Nested- PCR was carried out in final volume 25 µl, containing 2.5 µl of 10X PCR buffer, 2 mM MgCl₂, 200µM dNTP, 1.5 unit of Taq DNA polymerase (Fermentase, Thermo Fisher Scientific, Lithuania) and 10 µM of each primers. Amplifications were carried out in PeqLab thermocycler (PEQLAB Biotechnologie GmbH, Germany) using Primers that mentioned by Mirjalali et al. (20), previously. Outer primers PMicF (5'- GGTTGATTCTGCCTGACG - 3') and PMicR (5' - CTGCGAGC (G/A)TACTATCC - 3') amplify 779bp fragment of *Encephalitozoon* spp. and *E. bienersi* under condition: 95 °C for 5 min as initial denaturation, following 35 cycles of 94 °C for 40 sec, 55 °C for 45 sec and 72 °C for 45 sec and finally 72 °C for 5 min as final extension. Second PCR was carried out by two pairs of genus – specific internal primers including EnbF (5'- GGTAATTTGGTCTCTGTGTG - 3') and EnbR (5'- CTACACTCCCTATCCGTTTC -3') and also

EncepF (5'- AGTACGATGATTGGTTG-3') and EncepR (5'- ACAACACTATA-TAGTCCCGTC- 3') which amplify 440 bp and 629 bp fragments of *E. bienersi* and *Encephalitozoon* spp., respectively. The second PCR program conditions were: initial denaturing at 95 °C for 5 min and then 25 cycles of 94 °C for 35 sec, 57 °C for 35 sec, 72 °C for 40 sec and as a final extension, 72 °C for 3 min. Sterile distilled water and a sequenced microsporida isolate which were obtained previously (20), were used as negative and positive control, respectively and run beside all the samples. Subsequently, 5µl of PCR products were electrophoresed on 1.5% agarose gel and then were visualized by ethidium bromide staining. For more confirmation of molecular results, 20 µl of each PCR product was sequenced using by ABI 3130 (California, USA) sequencer.

Phylogenetic analysis

Phylogenetic tree was drawn based on our isolates and some non- human isolates retrieved from GenBank database. All of isolates information and their accession numbers were summarized in Table 1.

Table 1: All of information about isolates, which submitted to phylogenetic tree

Isolate	Host	Source	Accession Number
	Cat	Our study	KJ414446
	Cat	Our study	KJ414447
	Cattle	Our study	KJ414443
	Dog	Our study	KJ414448
<i>E. bienersi</i>	Sheep	Our study	KJ414444
	Rabbit	Our study	KJ414449
	Monkey	GenBank database	AF023245
	Cattle	GenBank database	AY257180
	Falcon	GenBank database	DQ793212
<i>En. cuniculi</i>	Laboratory Mouse	Our study	KJ414452
	Laboratory Mouse	Our study	KJ414445
	Laboratory Mouse	Our study	KJ414451
	Laboratory Rabbit	Our study	KJ414449
	Rabbit	GenBank database	L17072
	Canis	GenBank database	X97469
	<i>Mouse musculus</i>	GenBank database	X98467

Molecular alignment was performed using by ClustalW in Bioedit software and then phylogenetic tree was constructed using Molecular & Evolution Genetic Analysis software version 6 (MEGA 6) in Neighbor- Joining test and Tamura 3 parameter model. For calculating the reliability of the tree, Bootstrap value with 1000 replication was considered.

Ethical approval

All experiments on the animals, which were included in our study, were performed based on the guidelines of the Ethical Board of Tehran University of Medical Sciences, Iran, where the study was approved.

Results

From 142 stool samples, collected from the animals, *Microsporidia* spores were observed microscopically in 15 (10.56%). Size of spores in *E. bienersi* was 1.2 – 2µm and in *En. cucinuli* was about 2-2.5 µm. All of spores were pinkish, ovoid and ellipse in form. They were also differentiable from each other in microscopic field based on spore sizes. In most of samples, parasite rate was low to moderate. Nested-PCR using new primers, which mentioned above, could confirm all of the microscopically positive samples (Fig. 1).

E. bienersi and *En. cucinuli* were identified in 11 (7.74%) and 4 (2.81%) samples, respectively. *En. cucinuli* was found in the feces of 3 (15%) small white mice and 1 (10%) laboratory rabbits. Moreover, *E. bienersi* was detected in 3 (10%) samples of sheep, 2 (5.12%) cattle, 1 (10%) rabbit, 3 (11.53%) cats and 2 (11.76%) ownership dogs. Results are summarized in Table 2.

Sequencing results and BLAST analysis confirmed the Nested-PCR results. All of samples, which were amplified with *Encephalitozoon* spp. specific primers, were characterized as *En. cucinuli*. The results of those, which were amplified with *E. bienersi* specific primers, were

also confirmed by sequencing. Accession numbers of submitted sequences are KJ414443 to KJ414452.

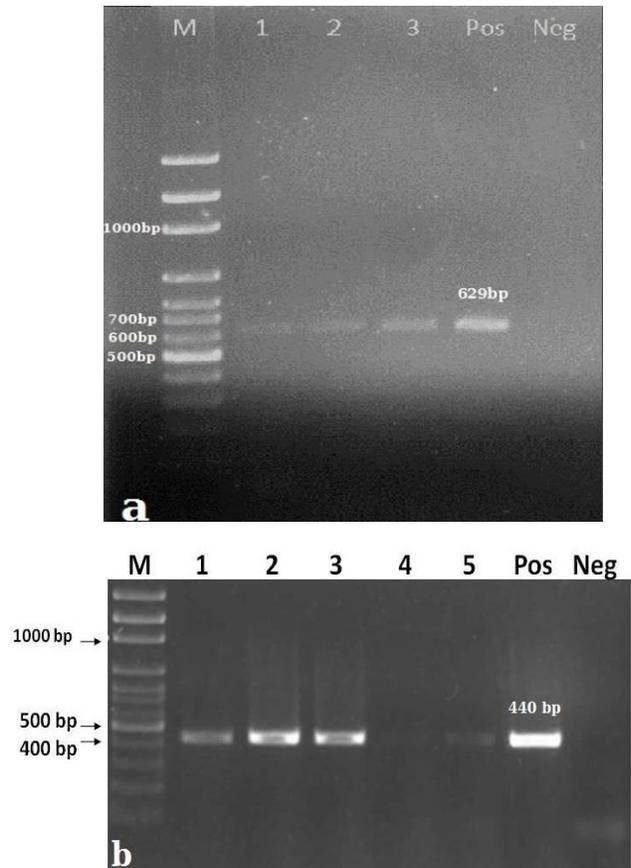


Fig. 1: Gel electrophoresis of Nested- PCR products using by genus- specific primers; a: A 629bp fragment of *Encephalitozoon* spp., b: A 440 bp fragment of *E. bienersi*. Abbreviations are M: 100bp marker (Fermentase, Thermo Fisher Scientific, Lithuania), Pos: Positive control and Neg: Negative control

According to phylogenetic tree analysis, all of isolates placed in two clades (*Encephalitozoon cucinuli* & *Enterocytozoon bienersi*). All of *En. cucinuli*, obtained in this study, were grouped in one clade with highest Bootstrap value. *E. bienersi* isolates which were obtained from dog, cats and rabbit were clustered together and those, which were isolated from sheep and cattle, were also placed in separated but close together (Fig. 2).

Table 2: Prevalence of *Microsporidia* species according to under investigation hosts and parasite species

	Host (percent)					
	Cat	Dog	Sheep	Cattle	Laboratory mice	Laboratory rabbit
<i>E. bienersi</i>	11.53	11.76	10	5.12	-	10
<i>En. cuniculi</i>	-	-	-	-	15	10

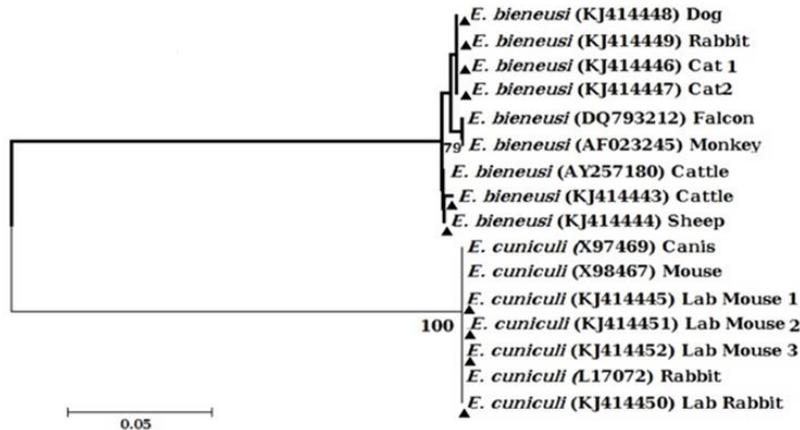


Fig. 2: Phylogenetic tree of the SSU rRNA gene region nucleotide sequences of *E. bienersi* and *E. cuniculi* isolates recovered from cat, ownership dog, laboratory rabbit and mouse, cattle and sheep and reference accession numbers, which retrieved from Gene Bank database. All of our isolates were marked with black filled triangle. The tree was constructed by using the Tamura3-parameter model in MEGA software version 6. The numbers above the branches indicate the percentage of bootstrap percentages. Branches without numbers have frequencies of less than 70%

Discussion

Microsporidia infections are reported in immunocompromised patients, frequently. *E. bienersi* and *Encephalitozoon* spp., which known as most prevalent species, could be main agents of variety range of involvements in immunocompromised individuals (22-24). Different sources have been concerned as potential agents of spreading the infection, so far (5, 25-27). In spite of the fact that zoonotic source probably play important role in human microsporidia infections, but the main transmission route of infection have not been clearly proved yet. Microsporidia spores released from stool, urine and collapsed carcasses can disperse via water and food sources. As discussed above, animals that have close – contact to human can also spread infected microsporida spores in human populations. Ad-

ditionally, it is important to consider that there was no diarrhea or other characteristic sign of microsporidia infection in these animals and the parasite rate was moderate to low as well. Indeed, this subject can lead to more shedding and distributing of the spores in human society, because they were not considered as sick animals and were abandoned without receiving the treatment, subsequently. To date, study of the microsporidia infection in animals in Iran, have been carried out in two-limited study: cat, dog (14) and more recently on pigeon (19). Accessible data shows that, this study is the first assessment of intestinal microsporidiosis in vaster spectrum of domesticated and laboratory mammalian animals with close - contact to humans in Iran.

In this study, *E. bienersi* and *En. cuniculi* spores were detected in fifteen cases. In addition, the percentage of infection among all of

under assessment groups was approximately equal and prevalence of *E. bienersi* was significantly more than *En. cuniculi*.

Our finding demonstrates that *E. bienersi* more likely is the most prevalent microsporidia species among animals with close - contact to humans in Iran. This subject is in agreement with Pirestani and colleagues study that showed more incidence of *E. bienersi* in pigeons (19). It is interest to mention that *E. bienersi* in human population in Iran, particularly individuals with immune system deficiency, is also predominant species (20, 28, 29). Reports of widespread infection of *E. bienersi* in mammalian animals as well as human reveal this fact that, even though zoonotic transmission has not been clearly proved yet, but more likely, infection via contiguity with farm, pet and laboratory animals could be one of the main routes in transmission cycle of *E. bienersi*.

The numbers of animals infected with *En. cuniculi*, were significantly less than *E. bienersi*. These findings can represent this fact that *Encephalitozoon* spp., has probably less effluence in domesticated animals as well as human in Iran (20, 28, 29). Additionally, all *En. cuniculi* infected cases were determined in both laboratory mice and rabbits. This finding is in agreement with other studies that emphasis on high prevalent of *En. cuniculi* in those animals (30, 31).

Small Subunit ribosomal RNA (SSU rRNA) is one of conserved gene and can be useful in phylogenetic research of Microsporidia spp. (32, 33), According to molecular finding of SSU rRNA gene, phylogenetic analysis showed near relationship between all of *En. cuniculi* which obtained in this study. As mentioned above, all of laboratory rabbits and mice samples were collected from same laboratory, so more likely the source of all *En. cuniculi* is same. On the other hand, *E. bienersi*, which isolated from cats, ownership dog and laboratory rabbits, showed complete proximity in this fragment of SSU rRNA gene. Although sampling place of cat and ownership dog was equal and probably they were infected from

same source, but the interesting point is similarity of laboratory rabbit isolates with those, which were isolated from cat and ownership dog. Furthermore, it is interest to mention that although cattle and sheep isolates have trivial differences with each other in sequence analysis at the positions but they were grouped more near together than those isolated from cats, laboratory rabbit and dog and this fact could be related to place of sampling. As mentioned former, cattle and sheep isolates, obtained from same dairy farm and different from other isolates, geographically.

Conclusion

E. bienersi probably is the most prevalent microsporidia species in animal hosts as well as human in Iran and more likely infected animals with close- contact to human play an irrefutable role in transmission cycle of Microsporidia spp., particularly *E. bienersi*. Our results also show that animals especially domesticated ones, without clinical sign could be importance in zoonotic transmission of microsporidia species.

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