Comparative Immunology, Microbiology and Infectious Diseases 65 (2019) 37–40

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

First insight into *Encephalitozoon cuniculi* infection in laboratory and pet rabbits in Iran

Zainab Sadeghi-Dehkordi[a,*], Ebrahim Norouzi[b], Hidokht Rezaeian[a], Alireza Nourian[a], Vahid Noaman[c], Alireza Sazmand[a]

[a] Department of Pathobiology, Faculty of Veterinary Science, Bu-Ali Sina University, Hamedan, Iran
[b] Department of Laboratory Animal Breeding, Agricultural Research, Education and Extension Organization (AREEO), Razi Vaccine and Serum Research Institute, Hessarak, Karaj, Alborz, Iran
[c] Group of Veterinary Medicine, Animal Sciences Research Department, Isfahan Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization (AREEO), Isfahan, Iran

ARTICLE INFO

Keywords:
Encephalitozoonosis
Zoonosis
Oryctolagus cuniculus
Hamedan
Karaj

ABSTRACT

Encephalitozoon cuniculi infects a wide variety of domestic and wild mammalian species including humans. Although the infection status has been studied in laboratory and pet rabbits worldwide, there is shortage of information regarding the disease in Iran. In the present study, the occurrence of infection in brains of 117 asymptomatic rabbits from six breeding and experimental units with highest population of rabbit colonies in the country ($n = 60$) as well as pet rabbits of pet stores in two cities ($n = 57$) were examined by nested-PCR. Histological sections of brains and kidneys were also studied by light microscopy. PCR results revealed that 3.3% of laboratory rabbits (2/60) and 59.6% of pet rabbits (34/57) harboured *E. cuniculi* in their brains. Histopathology on the other hand showed spores of the parasite in kidney and brain of one and kidney of another pet rabbit. As encephalitozoonosis may interfere with results of experiments performed on laboratory rabbits, routine screenings for identification and culling of infected animals is recommended. Furthermore, infected companion rabbits can transmit *E. cuniculi* to people in close contact with them, therefore, improving public knowledge of this zoonotic infection is suggested.

1. Introduction

Encephalitozoon cuniculi Levaditi, Nicolau & Schoen, 1923 (Synonym: *Nosema cuniculi*) is an obligate intracellular microsporidian parasite belonging to the fungi kingdom [1]. the organism has a wide host range among mammals including humans and birds. To date, four genotypes of *E. cuniculi* have been identified in animal and human hosts namely I, II, III and IV [2]. These genotypes have no strict host preference and human infections with all of them have been documented hence *E. cuniculi* is recognized as emerging pathogen with increasing zoonotic concerns [3]. Although *E. cuniculi* can infect several domestic and wild mammalian species, it has mainly been investigated in rabbits. Various aspects of the disease such as etiology, epidemiology, clinical signs, pathophysiology, immunology, diagnosis, treatment and control have been discussed in detail [4,5].

Encephalitozoonosis has a worldwide distribution in both pet and laboratory rabbits with reports of infection in up to 73% of conventional rabbit colonies [6]. Transmission can occur via both horizontal (ingestion or inhalation of spores) and vertical (transplacental) routes [7]. *Encephalitozoon cuniculi* is occasionally associated with clinical disease in rabbits most commonly with neurological signs, renal insufficiency and ocular disorders, however, neurological signs have predominantly been reported [5]. Neurologic manifestations including behavioural changes, depression, head tilting, ataxia, circling, rotation, seizures and paralysis [8]. At present, no effective treatment exists for encephalitozoonosis in rabbits however, benzimidazoles have shown to be partially effective in prevention and treatment of the disease [9]. No vaccine is currently available for immunization of susceptible hosts.

As the naturally infected rabbits usually do not display clinical symptoms [10], and on the other hand, clinical signs except for phacoclastic uveitis, are considered not pathognomonic enough to exclude differential diagnoses including bacterial infection or injury [11], diagnosis continues to be problematic. Several serological and molecular techniques have been used for the diagnosis of infection however, nested polymerase chain reaction (nested PCR) has been proven to be an advantageous method for detection of the pathogen in various organ

* Corresponding author at: Department of Pathobiology, Faculty of Veterinary Science, Bu-Ali Sina University, Felestin Sq., 6517658978, Hamedan, Iran.
E-mail address: z.sadeghidehkordi@basu.ac.ir (Z. Sadeghi-Dehkordi).

https://doi.org/10.1016/j.cimid.2019.04.005
Received 26 October 2018; Received in revised form 8 April 2019; Accepted 8 April 2019

tissues, urine and CSF [12].

In Iran three *Encephalitozoon* species of *E. cuniculi* Levaditi, Nicolau & Schoen, 1923, *E. hellem* Didier et al., 1991 and *E. intestinalis* Cali, Kotler & Orenstein, 1993 have been identified in feces of pigeons, exotic birds, mice, rats, dogs and immunodeficient human patients by PCR [13–17]. Although *E. cuniculi* is among infectious agents recommended by the Federation of European Laboratory Animal Science Associations (FELASA) to be monitored every three months in colonies in breeding and experimental units [18], to the best of the authors' knowledge, those recommendations are not currently being practiced in the country. Except for a single study on fecal samples of laboratory rabbits from a University animal house [19], no information is available regarding the infection status in Iran. The present study aimed to investigate the infection of *E. cuniculi* in laboratory reared rabbits in six breeding units as well as pet rabbits using molecular and histopathological examinations.

2. Materials and methods

2.1. Study population

During the year 2015, totally 117 laboratory rabbits of New Zealand white breed ($n = 60$) and pet rabbits (*Oryctolagus cuniculus*) ($n = 57$) were examined. Six breeding and experimental units with highest population of rabbit colonies were chosen: i. Razi Vaccine and Serum Research Institute, ii. Pasteur Institute of Iran, iii. Royan Institute, iv. Toliddaru Pharmaceutical Company, v. School of Pharmacy of Tehran University of Medical Sciences and vi. Imam Khomeini Hospital, and 60 New Zealand White rabbits (10 rabbits per unit) were collected from them. Moreover, 57 pet rabbits of both sexes were bought from pet stores in Hamedan ($n = 48$) and Karaj ($n = 9$). The animals were between one to two years old, weighing 800–3100 g with no clinical signs of vestibular disorder or other neurological diseases.

Pet rabbits were put in a carbon dioxide (CO_2) chamber and euthanized with increasing the gas concentration from 70% to 100%.

2.2. Sampling

Cerebra of all rabbits were dissected aseptically, half of which, was kept in sterile tubes at –20 °C until further use, and the other half was fixed in 10% neutral buffered formalin. In the case of pet rabbits, kidneys were also dissected and processed for histopathology.

2.3. DNA isolation and PCR assay

Extraction of DNA from cerebral tissue was performed on a 200 mg homogenate using phenol/chloroform extraction and ethanol precipitation as described elsewhere [20]. A species-specific nested-PCR targeting fragments of *E. cuniculi* ribosomal RNA was performed with the obtained product from external PCR as template for nested PCR [12]. Primer sequences and PCR conditions are listed in Table 1. All PCRs were performed using Taq DNA Polymerase (Yekta Tajhiz Azma, Tehran, Iran) in SimpliAmp® thermal cycler (Applied Biosystems, California, United States). The amplified products were gel electrophoresed on 2% w/v agarose gel, and viewed under UV transilluminator. Positive and negative controls were included in each run of PCR reactions and positive samples were rechecked twice.

2.4. Histopathology

The fixed tissue samples were dehydrated, cleared, paraffin embedded, sectioned at 5 µm thick, stained with haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS), examined independently by a pathologist under a light microscope (Olympus CX41) equipped with a digital camera (Olympus DP25).

3. Ethical statement

This study was carried out in accordance with the guidelines and under supervision of the animal ethics and welfare committee of Bu-Ali Sina University.

4. Results

4.1. Nested-PCR

Species-specific nested-PCR from homogenate of cerebra of laboratory and pet rabbits revealed an overall prevalence of 30.7% in all sampled regions of the country. Two out of sixty laboratory rabbits (3.3%) were positive for *E. cuniculi*. In the case of pet rabbits however, 34 out of 57 (59.6%) rabbits were found positive for *E. cuniculi*. The rate of infection was higher in Karaj (77.8%) than Hamedan (56.2%).

4.2. Histopathological findings

No pathologic changes indicating the infection were found in cerebra of examined lab rabbits. However, spores of *E. cuniculi* were observed in two pet rabbits (1.7%). One rabbit had spores in its kidney, and the other in both kidney and brain (Fig. 1).

5. Discussion

In the present study, molecular techniques and microscopic examination were used for detection of *E. cuniculi* in laboratory and pet rabbits in Iran.

Common diagnostic tools for detection of *E. cuniculi* in organs, body fluids and excreta of rabbits include i. molecular biology techniques (e.g., conventional, nested and real-time PCR) [11,12,21,22], and iii. serological analysis (e.g., indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), and carbon immunoassay (CIA), Western blot analysis and C-reactive protein (CRP) measurement). Although each test has advantages and disadvantages, the PCR method is generally accepted as the standard molecular technique for the detection of microsporidia in animals and humans [5]. On the other hand, histopathology has the ability to objectively pinpoint the spores in place. Serological methods are widely used worldwide however, seronegativity was observed in considerable rates of 22.9%, 16% and 28.6% in rabbits with neurological symptoms, phacoclastic uveitis and renal failure respectively [8]. As excretion period of the spores into urine and feces is short and intermittent [23] therefore, in the present study, nested-PCR and histopathological techniques were chosen for detection of *E. cuniculi* spores in cerebrum and kidneys of

Table 1
PCR conditions for detection of *E. cuniculi* in rabbit brain.

Target	Sequence (5′→3′)	No. of cycles	Annealing temperature (°C)	Primer concentration (pmol)	Product size (bp)
ssrRNA	Ence_549_forward ATGAGAAGTGATGTGTGTGCG Ence_549_reverse GCCATGCACTCACAGGCATC	35	61	5	549
ssrRNA	P2 TTGCGGGATGAGCAGTAGCTGCG M2 TGCTGCCACAAACACAACCCG	35	58	5	1460

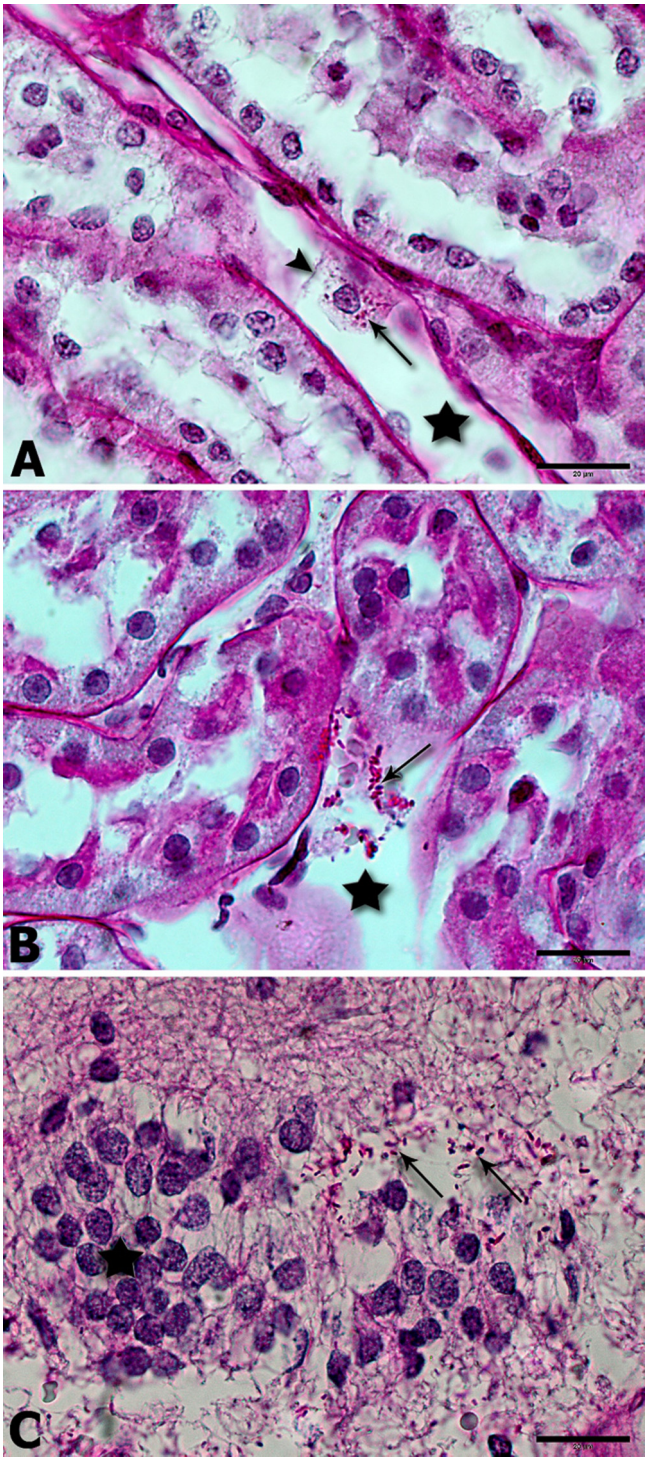

Fig. 1. Light microscopic view of infected organs of pet rabbits. A) renal tubule (star) of kidney showing degeneration of epithelial cells. One tubular cell (arrowhead) with intracellular spores of *E. cuniculi* (arrow) in cytoplasm around the cell nucleus. B) Tens of extracellular spores (arrow) in a renal tubule (star). C) Extracellular *E. cuniculi* spores (arrows) in a cerebral section of a rabbit along with moderate glial reaction in the form of focal glial cell aggregation (star). Masson's Trichrome staining, ×400, scale bar = 20 µm.

rabbits, as the most frequently affected areas by the parasite.

Species-specific nested-PCR on brain samples revealed an overall prevalence of 3.3% of laboratory rabbits of six breeding and experimental units in Iran. As PCR has not been routinely implemented for diagnosis of encephalitozoonosis in rabbits, the molecular epidemiological data are scarce. Several research groups from different countries have studied *E. cuniculi* in laboratory/breeding rabbits using different methods with reported seroprevalences of 0–95% [4]. *Encephalitozoon cuniculi* is among the infectious agents that is recommended by the Federation of European Laboratory Animal Science Associations (FELASA) to be monitored regularly in laboratory animals, as results obtained from experiments on those animals could be interfered by the infection [23]. Encephalitozoonosis should be investigated every three months and annually in laboratory rabbits (*Oryctolagus cuniculus*) and guinea pigs (*Cavia porcellus*), respectively. Testing of laboratory rats (*Rattus norvegicus*) and hamsters (*Mesocricetus auratus*) is optional, and in accordance with specific needs, with frequencies depending on local circumstances [18]. In the only documented study on rabbit encephalitozoonosis in Iran, the fecal material of 1 out of 10 laboratory rabbits from Animal House of School of Public Health, Tehran University of Medical Sciences was found positive by PCR [19]. Although the prevalence of infection in laboratory rabbits of major breeding and experimental units in this study and ours was low, screening for identification and culling of infected animals is recommended.

In the present study, 34 out of 57 (59.6%) examined pet rabbits were infected with the parasite. Prevalence of infection with *E. cuniculi* in healthy/asymptomatic pet rabbits in UK, Austria, Italy and Japan have been reported 36.8% to 68.1% [4]. People exposed to infected rabbits may be at risk of this zoonotic infection. Disseminated infection can occur particularly in hosts with impaired or supressed immune system such as those suffering from conditions like HIV-AIDS and recipients of transplanted organs [24]. However, apparently healthy immune-competent individuals with asymptomatic microsporidiosis can also intermittently shed organisms in feces and urine [25]. In a study in China, 9.76% of 300 tested persons from three regions in major rabbit producing and consuming provinces were found positive for *E. cuniculi* by ELISA [26]. Although a direct correlation between close contact of test-positive individuals with rabbits was not stated in that study, reporting a confirmed case of infection with *E. cuniculi* in an immunocompetent animal care worker in Turkey [27] urges routine examination of rabbit colonies as well as people close to them.

Histopathology sections showed the intra- and extracellular spores of the parasite in 1.7% of rabbits, a much lower figure compared to the results of nested-PCR. The molecular technique of PCR is able to detect and multiply limited amount of target sequence, virtually a single molecule of DNA, therefore increasing the possibility of finding the parasite in a tissue homogenate. However, histopathology is a robust method for in-place visualization of the parasite. Members of genus *Encephalitozoon* infect several cell types in mammalian hosts, including epithelial and endothelial cells, fibroblasts, macrophages, and astrocytes with a wide range of symptoms such as hepatitis, encephalitis, peritonitis, urethritis, prostatitis, nephritis, sinusitis, keratoconjunctivitis, cystitis and diarrhoea [1]. *Encephalitozoon cuniculi* initially infects organs with high blood flow such as the lung, liver, and kidney, with infection of nervous tissue occurring later in the course of the disease, so predilection organs for the organism in rabbits would be central nervous system particularly brain, kidney and eye [5]. In this study, one rabbit had spores in kidney, and the other in both kidney and brain. Similar to our finding, in previous studies more rabbits showed lesions in kidney in comparison with brain that led to hypothesis that the kidney is infected prior to the brain [10,12].

6. Conclusion

In our study, we report considerable infection of *E. cuniculi* in breeding and experimental units in Iran. Due to possible interferences with research as well as impacts on health and production of rabbits in colonies application of FELASA guidelines for monitoring of laboratory animals' health status is suggested. Moreover, as most of rabbits develop this zoonotic infection in chronic and subclinical forms, periodic

clinical examinations of pet rabbits are highly recommended.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Acknowledgments

The authors wish to acknowledge Bu-Ali Sina University of Hamedan for supporting the research. We would like to thank Sakineh Azami for her excellent support in the laboratory.

References

[1] B. Han, L.M. Weiss, Microsporidia: obligate intracellular pathogens within the fungal kingdom, Microbiol. Spectr. 5 (2) (2017).
[2] H. Talabani, C. Sarfati, E. Pillebout, T. van Gool, F. Derouin, J. Menotti, Disseminated infection with a new genovar of *Encephalitozoon cuniculi* in a renal transplant recipient, J. Clin. Microbiol. 48 (7) (2010) 2651–2653.
[3] B. Hinney, B. Sak, A. Joachim, M. Kváč, More than a rabbit's tale–*Encephalitozoon* spp. in wild mammals and birds, Int. J. Parasitol. Parasites Wildl. 5 (1) (2016) 76–87.
[4] F. Künzel, A. Joachim, Encephalitozoonosis in rabbits, Parasitol. Res. 106 (2) (2010) 299–309.
[5] F. Künzel, P.G. Fisher, Clinical signs, diagnosis, and treatment of *Encephalitozoon cuniculi* infection in rabbits, Vet. Clin. North Am. Exot. Anim. Pract. 21 (1) (2018) 69–82.
[6] A. Lyngset, A survey of serum antibodies to *Encephalitozoon cuniculi* in breeding rabbits and their young, Lab. Anim. Sci. 30 (3) (1980) 558–561.
[7] P. Baneux, F. Pognan, In utero transmission of *Encephalitozoon cuniculi* strain type I in rabbits, Lab. Anim. 37 (2) (2003) 132–138.
[8] F. Künzel, A. Gruber, A. Tichy, R. Edelhofer, B. Nell, J. Hassan, M. Leschnik, J.G. Thalhammer, A. Joachim, Clinical symptoms and diagnosis of encephalitozoonosis in pet rabbits, Vet. Parasitol. 151 (2–4) (2008) 115–124.
[9] S. Abu-Akkada, S. Oda, Prevention and treatment of *Encephalitozoon cuniculi* infection in immunosuppressed rabbits with fenbendazole, Iran. J. Vet. Res. 17 (2) (2016) 98–105.
[10] J. Csokai, A. Gruber, F. Künzel, A. Tichy, A. Joachim, Encephalitozoonosis in pet rabbits (*Oryctolagus cuniculus*): pathohistological findings in animals with latent infection versus clinical manifestation, Parasitol. Res. 104 (3) (2009) 629–635.
[11] G. Desoubeaux, A. Pantin, R. Peschke, A. Joachim, C. Cray, Application of Western blot analysis for the diagnosis of *Encephalitozoon cuniculi* infection in rabbits: example of a quantitative approach, Parasitol. Res. 116 (2) (2017) 743–750.
[12] J. Csokai, A. Joachim, A. Gruber, A. Tichy, A. Pakozdy, F. Künzel, Diagnostic markers for encephalitozoonosis in pet rabbits, Vet. Parasitol. 163 (1–2) (2009) 18–26.
[13] R. Ghoyounchi, E. Ahmadpour, A. Spotin, M. Mahami-Oskouei, A. Rezamand, N. Aminisani, M. Ghojazadeh, R. Berahmat, T. Mikaeili-Galeh, Microsporidiosis in Iran: a systematic review and meta-analysis, Asian Pac. J. Trop. Med. 10 (4) (2017) 341–350.
[14] E. Kazemi, M. Tavalla, S. Maraghi, M.J. Yad, M. Latifi, Frequency of microsporidial infection in immunocompromised patients with staining and molecular methods based on internal transcribed spacer region gene in two cities of southwest Iran during 2013–2014, Asian J. Pharm. Res. Health Care 9 (1) (2016) 7–16.
[15] M. Tavalla, M. Mardani-Kateki, R. Abdizadeh, R. Nashibi, A. Rafie, S. Khademvatan, Molecular identification of *Enterocytozoon bieneusi* and *Encephalitozoon* spp. in immunodeficient patients in Ahvaz, Southwest of Iran, Acta Trop. 172 (2017) 107–112.
[16] M. Tavalla, F. Kazemi, M. Mardani-Kateki, R. Abdizadeh, Molecular diagnosis of Enterocytozoon bieneusi and *Encephalitozoon*spp. in wild rats of southwest of Iran, Jundishapur J. Microbiol. 11 (3) (2018) e55961.
[17] M. Tavalla, M. Mardani-Kateki, R. Abdizadeh, S. Soltani, J. Saki, Molecular diagnosis of potentially human pathogenic *Enterocytozoon bieneusi* and *Encephalitozoon* species in exotic birds in Southwestern Iran, J. Infect. Public Health 11 (2) (2018) 192–196.
[18] M. Mähler, M. Berard, R. Feinstein, A. Gallagher, B. Illgen-Wilcke, K. Pritchett-Corning, M. Raspa, FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units, Lab. Anim. 48 (3) (2014) 178–192.
[19] Z. Askari, H. Mirjalali, M. Mohebali, Z. Zarei, S. Shojaei, T. Rezaeian, M. Rezaeian, Molecular detection and identification of zoonotic microsporidia spore in fecal samples of some animals with close-contact to human, Iran. J. Parasitol. 10 (3) (2015) 381–388.
[20] S. Jamshidi, A.S. Tabrizi, M. Bahrami, H. Momtaz, Microsporidia in household dogs and cats in Iran; a zoonotic concern, Vet. Parasitol. 185 (2–4) (2012) 121–123.
[21] M. Leipig, K. Matiasek, H. Rinder, D. Janik, D. Emrich, K. Baiker, W. Hermanns, Value of histopathology, immunohistochemistry, and real-time polymerase chain reaction in the confirmatory diagnosis of *Encephalitozoon cuniculi* infection in rabbits, J. Vet. Diagn. Invest. 25 (1) (2013) 16–26.
[22] L.E. Rodríguez-Tovar, A. Villarreal-Marroquín, A.M. Nevárez-Garza, U. Castillo-Velázquez, H.G. Rodríguez-Ramírez, M.C. Navarro-Soto, J.J. Zárate-Ramos, G. Hernández-Vidal, A. Trejo-Chávez, Histochemical study of *Encephalitozoon cuniculi* spores in the kidneys of naturally infected New Zealand rabbits, J. Vet. Diagn. Invest. 29 (3) (2017) 269–277.
[23] G. Maestrini, E. Ricci, C. Cantile, R. Mannella, F. Mancianti, G. Paci, C. D'Ascenzi, S. Perrucci, *Encephalitozoon cuniculi* in rabbits: serological screening and histopathological findings, Comp. Immunol. Microbiol. Infect. Dis. 50 (2017) 54–57.
[24] M. Kicia, M. Wesolowska, Z. Kopacz, M. Kváč, B. Sak, M. Sokulska, K. Cebulski, A.B. Hendrich, A. Pozowski, Disseminated infection of *Encephalitozoon cuniculi* associated with osteolysis of hip periprosthetic tissue, Clin. Infect. Dis. 67 (8) (2018) 1228–1234.
[25] E.S. Didier, L.M. Weiss, Microsporidiosis: not just in AIDS patients, Curr. Opin. Infect. Dis. 24 (5) (2011) 490–495.
[26] P. Yaoqian, W. Shuai, L. Xingyou, L. Ruizhen, S. Yuqian, Seroprevalence of *Encephalitozoon cuniculi* in humans and rabbits in China, Iran. J. Parasitol. 10 (2) (2015) 290–295.
[27] A. Carhan, O. Ozkan, E. Ozkaya, The first identification of *Encephalitozoon cuniculi* infection in an animal care worker in Turkey, Iran. J. Parasitol. 10 (2) (2015) 280–285.