

RESEARCH ARTICLE

Molecular characterization and multi-locus genotypes of *Enterocytozoon bieneusi* from captive red kangaroos (*Macropus Rfus*) in Jiangsu province, China

Zhijun Zhong¹✉, Yanan Tian¹✉, Yuan Song¹, Lei Deng¹, Junxian Li², Zhihua Ren¹, Xiaoping Ma¹, Xiaobin Gu¹, Changliang He¹, Yi Geng¹, Guangneng Peng¹*

1 Key Laboratory of Animal Disease and Human Health of Sichuan Province, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan, P.R. China, **2** Nanjing Hongshan Forest Zoo, Nanjing, Jiangsu, P.R. China

✉ These authors contributed equally to this work.

* pgn.sicau@163.com



OPEN ACCESS

Citation: Zhong Z, Tian Y, Song Y, Deng L, Li J, Ren Z, et al. (2017) Molecular characterization and multi-locus genotypes of *Enterocytozoon bieneusi* from captive red kangaroos (*Macropus Rfus*) in Jiangsu province, China. PLoS ONE 12(8): e0183249. <https://doi.org/10.1371/journal.pone.0183249>

Editor: Ulrike Gertrud Munderloh, University of Minnesota, UNITED STATES

Received: April 23, 2017

Accepted: August 1, 2017

Published: August 14, 2017

Copyright: © 2017 Zhong et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: Funding provided by National Natural Science Foundation of China-31272620, Prof. Guangneng Peng; National Key Research and Development Program of China-2016YFD0501009, Prof. Guangneng Peng; and the Chengdu Giant Panda Breeding Research Foundation-CPF2015-4, Zhijun Zhong.

Abstract

Enterocytozoon bieneusi is the most common pathogen of microsporidian species infecting humans worldwide. Although *E. bieneusi* has been found in a variety of animal hosts, information on the presence of *E. bieneusi* in captive kangaroos in China is limited. The present study was aimed at determining the occurrence and genetic diversity of *E. bieneusi* in captive kangaroos. A total of 61 fecal specimens (38 from red kangaroos and 23 from grey kangaroos) were collected from Nanjing Hongshan Forest Zoo and Hongshan Kangaroo Breeding Research Base, Jiangsu province, China. Using the nested PCR amplification ITS gene of rRNA of *E. bieneusi*, totally 23.0% (14/61) of tested samples were PCR-positive with three genotypes (i.e. one known genotype, CHK1, and two novel genotypes, CSK1 and CSK2). Multi-locus sequence typing using three microsatellites (MS1, MS3, and MS7) and one minisatellite (MS4) revealed one, five, two, and one types at these four loci, respectively. In phylogenetic analysis, the two genotypes, CHK1 and CSK1, were clustered into a new group of unknown zoonotic potential, and the novel genotype CSK2 was clustered into a separate clade with PtEb and PtEbIX. To date, this is the first report on the presence of *E. bieneusi* in captive red kangaroos in Jiangsu province, China. Furthermore, a high degree of genetic diversity was observed in the *E. bieneusi* genotype and seven MLGs (MLG1-7) were found in red kangaroos. Our findings suggest that infected kangaroo may act as potential reservoirs of *E. bieneusi* and be source to transmit infections to other animal.

Introduction

Enterocytozoon bieneusi is a unicellular enteric microsporidian that causes diarrhea in a variety of domestic and wild animals worldwide as well as in humans, especially in children and immunosuppressed individuals [1]. Since *E. bieneusi* was first found in enterocytes of Haitian

Competing interests: The authors have declared that no competing interests exist.

patients with AIDS and then identified in pig feces, many studies were concentrated on the role of other animals as reservoirs in the epidemiology of this pathogen [2, 3]. Animals infected with *E. bieneusi* can shed spores through feces into the environment, which can contaminate food or water, leading to microsporidiosis outbreaks [4]. To date, more than 240 genotypes of *E. bieneusi* have been identified based on the analysis of the sequences of the single internal transcribed spacer (ITS) region of the rRNA gene [5–8]. These genotypes of *E. bieneusi* have been divided into nine different groups based on phylogenetic analysis [9, 10]. Group 1, which is usually considered as a human-pathogen group, contains 94% of all the identified ITS genotypes of *E. bieneusi*, and the remaining eight major clusters (groups 2 to 9) are found mostly in specific hosts and wastewater [7, 11].

The genetic identity of *E. bieneusi* has been widely investigated in several domestic and wild animals in China, but reports on *E. bieneusi* in captive kangaroos are limited [12, 13]. Only one study so far has reported *E. bieneusi* infection in white kangaroos and grey kangaroos in the Zhengzhou zoo in China [12], and there are no reports on *E. bieneusi* infection in red kangaroos in China. The Nanjing Hongshan Forest Zoo and the Hongshan Kangaroo Breeding Research Base are the dominating kangaroo breeding bases in China, from where the kangaroos are mainly taken to other zoos as ornamental animals. Because the kangaroos are mainly maintained in zoos and their feces directly defecate to the environment, infectious spores of *E. bieneusi* from these kangaroos can be transmitted to other animals and even pose a threat to public health. Therefore, this study was aimed at examining the infection rate and genetic diversity of *E. bieneusi* in captive kangaroos from Jiangsu province in China using ITS sequencing and multilocus sequence typing (MLST) analysis.

Methods

Ethic statement

This study was reviewed and approved by the Research Ethics Committee and the Animal Ethical Committee of Sichuan Agricultural University. Appropriate permission was obtained from zoo managers before the collection of fecal specimens. During the collection of fecal specimens, the animals were not subjected to any kind of injury.

Specimen collection

A total of 61 fecal specimens (38 from red kangaroos and 23 from grey kangaroos) were collected between November 2016 and January 2017 from captive kangaroos at the Nanjing Hongshan Forest Zoo and Hongshan Kangaroo Breeding Research Base in Jiangsu province, China (Table 1). Fresh fecal specimens (approximately 10 g) from each kangaroo were collected immediately by feeders after defecation on the ground and then quickly transferred into individual 50-mL plastic containers. All the kangaroos showed no obvious clinical symptoms at the time of sampling. All the fecal specimens were stored at 4°C in 2.5% (w/v) potassium dichromate until use.

DNA extraction

Genomic DNA was extracted from 200 mg of each fecal sample using the EZNA® Stool DNA kit (Omega Biotek, Norcross, GA, USA) according to the manufacturer's protocol. The DNA was then eluted in 200 µL of absolute ethanol and stored at -20°C prior to PCR analysis.

Table 1. Prevalence and distribution of *E. bieneusi* genotypes in Nanjing Hongshan Forest Zoo and Hongshan Kangaroo Breeding Research Base.

Characteristics	No. examined	No. positive (%)	Genotype
Zoo			
Nanjing Hongshan Forest Zoo	34	0 (0%)	
Hongshan Kangaroo Breeding Research Base	27	14 (51.9%)	CHK1, CSK1, CSK2
Species			
Red kangaroo	38	14 (36.8%)	CHK1, CSK1, CSK2
Grey kangaroo	23	0 (0%)	
Gender			
Male	19	3 (15.7%)	CHK1
Female	42	11 (26.2%)	CHK1, CSK1, CSK2

<https://doi.org/10.1371/journal.pone.0183249.t001>

PCR amplification

All the DNA preparations were analyzed for the presence of *E. bieneusi* using nested PCR amplification of a 389-bp nucleotide fragment of the rRNA gene. Positive specimens were further characterized by MLST analyses using the MS1, MS3, MS4, and MS7 loci. The primers and cycling parameters employed for these reactions were as previously described [14, 15]. TaKaRa Taq DNA Polymerase (TaKaRa Bio Inc., Tokyo, Japan) was used for all the PCR amplifications. A negative control with no DNA added was included in all the PCR tests. All the secondary PCR products were subjected to electrophoresis on a 1.5% agarose gel and visualized by staining the gel with Goldenview.

Nucleotide sequencing and analysis

All the secondary PCR products of the expected size were directly sequenced with a set of primers used for the secondary PCR by Life Technologies (Guangzhou, China) using a Big-Dye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA).

The nucleotide sequences obtained in this study were aligned with each other and with reference sequences downloaded from GenBank using the Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST/>) and ClustalX 1.83 (<http://www.clustal.org/>) to determine the *E. bieneusi* genotypes. The genotypes were assigned previously published names if found to be identical to known genotypes. Genotypes with single nucleotide substitutions, deletions, or insertions in 243 bp of the ITS gene region of *E. bieneusi* relative to the those of the known genotypes were considered novel genotypes and named according to the established nomenclature system [16].

Phylogenetic analysis

To better assess the diversity of the *E. bieneusi* genotypes in the present study and to determine the genetic relationship between the novel ones isolated from the kangaroos and the reference sequences previously published in GenBank, phylogenetic analysis was performed by constructing a neighboring-joining tree using Mega 6 software (<http://www.megasoftware.net/>), which is based on evolutionary distances calculated using a Kimura 2-parameter model. The reliability of these trees was assessed using bootstrap analysis with 1,000 replicates. The topology tree was constructed by a maximum parsimony analysis of the same sequence alignment, with all alignment sites taken into consideration [17].

Nucleotide sequence accession numbers

Representative nucleotide sequences were deposited into the GenBank database under the following accession numbers: KY706126 to KY706128 for the rRNA gene ITS sequences of *E. bieneusi*, and KY706117 to KY706125 for the microsatellite loci (MS1, MS3, and MS7) and minisatellite (MS4).

Results and discussion

In this study, 14 (23.0%) out of 61 captive kangaroos were found to be infected with *E. bieneusi*, and all the positive specimens belonged to red kangaroos from the Hongshan Kangaroo Breeding Research Base, which acts as the largest kangaroo breeding zoo in China. The male and female kangaroos showed an infection rate of 15.7% and 26.2%, respectively, with no significant difference ($P > 0.05$) (Table 1). The overall infection rate was lower than that observed in captive Pere David's deer (34.0%, 16/47) in the Henan province of China and in captive black bear (27.4%, 29/106) in the Sichuan province of China [18, 19], but higher than the rates reported for captive wildlife in the Zhengzhou Zoo (15.8%) and for pet chinchillas (3.6%) in China [12, 20].

Sequence analysis of the amplified PCR products revealed three ITS genotypes: a known genotype, CHK1, and two novel genotypes, CSK1 and CSK2. The known genotype, CHK1, has been identified previously in white kangaroos at the Zhengzhou zoo [12], and the novel genotype, CSK1, has 20 bases different from AF267144; the other novel genotype, CSK2, has higher genetic variability than the genotype KIN-3 (JQ437575). Phylogenetic analysis also showed that the genotypes CHK1 and CSK1 were clustered into a new group, which was first reported by our laboratory [6], and this group contains genotypes almost from bears and kangaroos [12]. The novel CSK2 genotype was grouped in a separate cluster together with genotypes PtEb IX and PtEb from dogs [21] (Fig 1). Genotypes of the new group have been detected in different animals in the past; however, whether these genotypes have the potential for transmission across species needs to be investigated in the future. Furthermore, the origin of *E. bieneusi* in the kangaroo population is not clear. All kangaroos in Hongshan Kangaroo Breeding Research Base are imported from Australia through Kangaroos Exchanging Program between China and Australia. However, no report for *E. bieneusi* infection in kangaroos in Australia, indicating the *E. bieneusi* infection in kangaroos is likely introduced in China. Thus further work should focus on the occurrence of these genotypes in other kangaroos population as well as other animals in China.

MLST using MS1, MS3, MS4, and MS7 has been developed for studying the taxonomy and population genetics of *E. bieneusi* [22]. Recently, high multilocus genotype (MLG) diversity has been observed for genotypes such as I-like, I, J, CHB1, SC01, BEB6, D, and horse1 that have the same ITS gene sequences [6, 10, 23–25]. In this study, all the ITS-positive samples were amplified and sequenced at the four loci. A total of 11, 13, 11, and 12 fecal samples were successfully amplified at the MS1, MS3, MS4, and MS7 loci, respectively, but only 10 samples were simultaneously positive at all four loci (S1 Table). Sequence analysis revealed one, five, two, and one genotypes at the MS1, MS3, MS4, and MS7 loci, respectively, and seven distinct MLGs (MLG1–7) were observed in genotype CHK1. Thus, our findings revealed the high genetic diversity of genotype CHK1 of *E. bieneusi* in kangaroos.

In conclusion, this is the first report on *E. bieneusi* infection in captive red kangaroos in Jiangsu province, China. Two novel genotypes (CSK1 and CSK2) were identified by analysis of the ITS gene. Genetic diversity was observed in genotype CHK1 using the MLST tool, and seven MLGs were found in red kangaroos. Because of the high-density feeding environment in zoos and the lack of systematic fecal collection method from animals, proper advice should be given

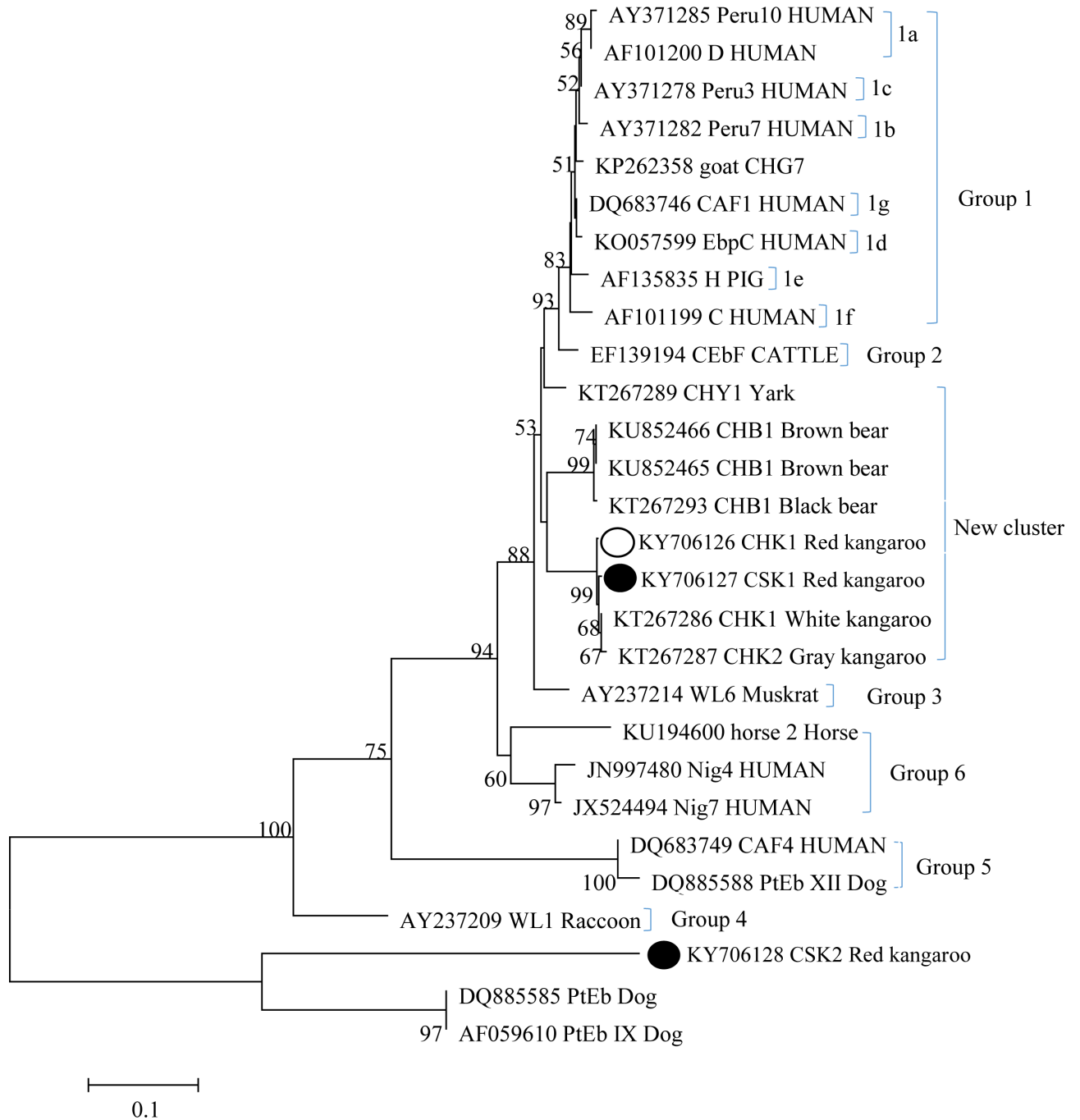


Fig 1. Phylogenetic relationship among the ITS loci of *E. bieneusi* isolates. Phylogenetic relationship of the genotypes of *E. bieneusi* identified in this study and known genotypes previously published in GenBank as inferred by a neighbor-joining analysis of ITS sequences based on genetic distances calculated by the Kimura 2-parameter model. A similar topology tree was also performed by maximum parsimony analysis, with the exception that the CSK2 genotype grouped together with genotypes PtEb and PtEbIX, with 99% bootstrap value. The numbers on the branches are percent bootstrapping values from 1000 replicates, with more than 50% shown in the tree. Each sequence is identified by its accession number, genotype designation, and host origin. Genotypes marked with black circles and open circle are novel and known genotypes identified in this study, respectively.

<https://doi.org/10.1371/journal.pone.0183249.g001>

to the managers of the Kangaroo Breeding Research Base to take steps to avoid interspecies transmission of *E. bieneusi*.

Supporting information

S1 Table. Multi-locus sequence typing of *Enterocytozoon bienewsi* in red kangaroos in Hongshan Kangaroo Breeding Research Base, Jiangsu province, China.
(DOCX)

Acknowledgments

We thank Junxian Li for giving advice on sample collection. We sincerely thank Tao Wang from University of Melbourne for comment on the draft manuscript.

Author Contributions

Conceptualization: Zhijun Zhong, Lei Deng, Guangneng Peng.

Data curation: Zhijun Zhong, Yinan Tian, Yuan Song.

Formal analysis: Zhijun Zhong, Zhihua Ren, Xiaoping Ma.

Investigation: Zhijun Zhong, Junxian Li, Xiaobin Gu, Changliang He, Yi Geng.

Methodology: Zhijun Zhong, Yinan Tian, Lei Deng.

Software: Zhijun Zhong, Yinan Tian, Changliang He.

Writing – original draft: Zhijun Zhong, Lei Deng.

Writing – review & editing: Zhijun Zhong.

References

1. Matos O, Lobo ML, Xiao L. Epidemiology of *Enterocytozoon bienewsi* Infection in Humans. *J Parasitol Res.* 2012; 2012(4):981424–981424. <https://doi.org/10.1155/2012/981424> PMID: 23091702.
2. Desportes I, Le CY, Galian A, Bernard F, Coch-Priollet B, Lavergne A, et al. Occurrence of a new microsporidian: *Enterocytozoon bienewsi* n.g., n. sp., in the enterocytes of a human patient with AIDS. *J Protozool.* 1985; 32(2):250–254. PMID: 4009510.
3. Deplazes P, Mathis A, Müller C, Weber R. Molecular epidemiology of *Encephalitozoon cuniculi* and first detection of *Enterocytozoon bienewsi* in faecal samples of pigs. *J Eukaryot Microbiol.* 1996; 43(5):93S. PMID: 8822883.
4. Zhao W, Zhang W, Yang Z, Liu A, Zhang L, Yang F, et al. Genotyping of *Enterocytozoon bienewsi* in Farmed Blue Foxes (*Alopex lagopus*) and Raccoon Dogs (*Nyctereutes procyonoides*) in China. *PLoS One* 2015; 10(11):e0142611. <https://doi.org/10.1371/journal.pone.0142611> PMID: 26544711.
5. Zhang XX, Cong W, Lou ZL, Ma JG, Zheng WB, Yao QX, et al. Prevalence, risk factors and multilocus genotyping of *Enterocytozoon bienewsi* in farmed foxes (*Vulpes lagopus*), Northern China. *Parasit Vectors*, 2016; 9(1):1–7. <https://doi.org/10.1186/s13071-016-1356-1> PMID: 26847241.
6. Wei L, Lei D, Yu X, Zhong Z, Qiang W, Liu X, et al. Multilocus genotypes and broad host-range of *Enterocytozoon bienewsi* in captive wildlife at zoological gardens in China. *Parasit Vectors*. 2016; 9(1):1–9. <https://doi.org/10.1186/s13071-016-1356-1> PMID: 26847241.
7. Zhao W, Yu S, Yang Z, Zhang Y, Zhang L, Wang R, et al. Genotyping of *Enterocytozoon bienewsi* (Microsporidia) isolated from various birds in China. *Infect Genet Evol.* 2016; 40:151–154. <https://doi.org/10.1016/j.meegid.2016.02.037> PMID: 26944443.
8. Qi M, Wang R, Wang H, Jian F, Li J, Zhao J, et al. *Enterocytozoon bienewsi* Genotypes in Grazing Horses in China and Their Zoonotic Transmission Potential. *J Eukaryot Microbiol.* 2016; 63(5):591–597. <https://doi.org/10.1111/jeu.12308> PMID: 26909747.
9. Zhao W, Zhang W, Yang D, Zhang L, Wang R, Liu A. Prevalence of *Enterocytozoon bienewsi* and genetic diversity of ITS genotypes in sheep and goats in China. *Infect Genet Evol.* 2015; 32:265–270. <https://doi.org/10.1016/j.meegid.2015.03.026> PMID: 25818401.
10. Tian GR, Zhao GH, Du SZ, Hu XF, Wang HB, Zhang LX, et al. First report of *Enterocytozoon bienewsi* from giant pandas (*Ailuropoda melanoleuca*) and red pandas (*Ailurus fulgens*) in China. *Infect Genet Evol.* 2015; 34:32–35. <https://doi.org/10.1016/j.meegid.2015.06.015> PMID: 26079276.

11. Santin M, Fayer R. Enterocytozoon bienewsi, giardia, and Cryptosporidium infecting white-tailed deer. J Eukaryot Microbiol. 2015; 62(1):34–43. <https://doi.org/10.1111/jeu.12155> PMID: 25066778.
12. Li J, Qi M, Chang Y, Wang R, Li T, Dong H, et al. Molecular Characterization of Cryptosporidium spp., Giardia duodenalis, and Enterocytozoon bienewsi in Captive Wildlife at Zhengzhou Zoo, China. J Eukaryot Microbiol. 2015; 62(6):833–839. <https://doi.org/10.1111/jeu.12269> PMID: 26384582.
13. Li J, Luo N, Wang C, Qi M, Cao J, Cui Z, et al. Occurrence, molecular characterization and predominant genotypes of Enterocytozoon bienewsi in dairy cattle in Henan and Ningxia, China. Parasit Vectors. 2016; 9(1):1–5. <https://doi.org/10.1186/s13071-016-1425-5> PMID: 26968376.
14. Sulaiman IM, Fayer R, Lal AA, Trout JM, Schaefer FW, Xiao L. Molecular Characterization of Microsporidia Indicates that Wild Mammals Harbor Host-Adapted Enterocytozoon spp. as well as Human-Pathogenic Enterocytozoon bienewsi. Appl Environ Microbiol. 2003; 69(8):4495–4501. <https://doi.org/10.1128/AEM.69.8.4495-4501.2003> PMID: 12902234.
15. Feng Y, Li N, Dearen T, Lobo ML, Matos O, Cama V, et al. Development of a multilocus sequence typing tool for high-resolution genotyping of Enterocytozoon bienewsi. Appl Environ Microbiol. 2011; 77(14):4822–4828. <https://doi.org/10.1128/AEM.02803-10> PMID: 21622791.
16. Santin M, Fayer R. Enterocytozoon bienewsi Genotype Nomenclature Based on the Internal Transcribed Spacer Sequence: A Consensus. J Eukaryot Microbiol. 2009; 56(1):34–38. <https://doi.org/10.1111/j.1550-7408.2008.00380.x> PMID: 19335772.
17. Guo Y, Alderisio KA, Yang W, Cama V, Feng Y, Xiao L. Host specificity and source of Enterocytozoon bienewsi genotypes in a drinking source watershed. Appl Environ Microbiol. 2014; 80(1):218–225. <https://doi.org/10.1128/AEM.02997-13> PMID: 24141128.
18. Zhang Z, Huang J, Karim MR, Zhao J, Dong H, Ai W, et al. Zoonotic Enterocytozoon bienewsi genotypes in Pere David's deer (Elaphurus davidianus) in Henan, China. Exp Parasitol. 2015; 155:46–48. <https://doi.org/10.1016/j.exppara.2015.05.008> PMID: 25982030.
19. Deng L, Li W, Zhong Z, Gong C, Cao X, Song Y, et al. Multi-locus genotypes of Enterocytozoon bienewsi in captive Asiatic black bears in southwestern China: High genetic diversity, broad host range, and zoonotic potential. Plos One. 2017; 12(2):e0171772. <https://doi.org/10.1371/journal.pone.0171772> PMID: 28182656.
20. Qi M, Luo N, Wang H, Yu F, Wang R, Huang J, et al. Zoonotic Cryptosporidium spp. and Enterocytozoon bienewsi in pet chinchillas (Chinchilla lanigera) in China. Parasitol Int. 2015; 64(5):339–341. <https://doi.org/10.1016/j.parint.2015.05.007> PMID: 25988830.
21. Li W, Li Y, Song M, Lu Y, Yang J, Tao W, et al. Prevalence and genetic characteristics of Cryptosporidium, Enterocytozoon bienewsi and Giardia duodenalis in cats and dogs in Heilongjiang province, China. Vet Parasitol. 2015; 208(3–4):125–134. <https://doi.org/10.1016/j.vetpar.2015.01.014> PMID: 25665462.
22. Feng Y, Yang W, Ryan U, Zhang L, Kvač M, Koudela B, et al. Development of a Multilocus Sequence Tool for Typing Cryptosporidium muris and Cryptosporidium andersoni. J Clin Microbiol. 2011; 49(1):34–41. <https://doi.org/10.1128/JCM.01329-10> PMID: 20980577.
23. Wang XT, Wang RJ, Ren GJ, Yu ZQ, Zhang LX, Zhang SY, et al. Multilocus genotyping of Giardia duodenalis and Enterocytozoon bienewsi in dairy and native beef (Qinchuan) calves in Shaanxi province, northwestern China. Parasitol Res. 2016; 115(3):1–7. <https://doi.org/10.1007/s00436-016-4908-6> PMID: 26782809.
24. Deng L, Li W, Zhong Z, Gong C, Liu X, Huang X, et al. Molecular characterization and multilocus genotypes of Enterocytozoon bienewsi among horses in southwestern China. Parasit Vectors. 2016; 9(1):561. <https://doi.org/10.1186/s13071-016-1844-3> PMID: 27776551.
25. Deng L, Li W, Yu X, Gong C, Liu X, Zhong Z, et al. First Report of the Human-Pathogenic Enterocytozoon bienewsi from Red-Bellied Tree Squirrels (Callosciurus erythraeus) in Sichuan, China. PloS one 2016, 11(12):e0168631. <https://doi.org/10.1371/journal.pone.0168631> PMID: 27992597.