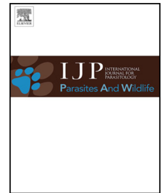
IJP: Parasites and Wildlife 11 (2020) 103–107

Contents lists available at ScienceDirect

IJP: Parasites and Wildlife

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



Genotypes and zoonotic potential of *Enterocytozoon bieneusi* in edible bullfrogs (*Lithobates catesbeiana*) in China

Hao Ding[a], Aiyun Zhao[a], Lingyun Wang[a], Na Gao[a], Yangang Sun[b], Junqiang Li[b,*], Meng Qi[a,**]

[a] College of Animal Science, Tarim University, Alar, Xinjiang, 843300, China
[b] Academy of Chinese Medical Sciences, Henan University of Chinese Medicine, Zhengzhou, 450046, China

ARTICLE INFO

Keywords:
Enterocytozoon bieneusi
Bullfrogs
Genotype
Zoonotic

ABSTRACT

Enterocytozoon bieneusi, an obligate intracellular pathogen of the intestinal epithelium, is commonly identified in humans and many other animals and is ubiquitous in water sources and the environment generally. To determine the molecular prevalence of *E. bieneusi* in edible bullfrogs (*Lithobates catesbeiana*) and evaluate the possibility of its potential zoonotic transmission to humans via food or water, the intestinal contents of 295 bullfrogs were intermittently collected from two open markets in Aksu, China. The samples were screened for the internal transcribed spacer by polymerase chain reaction amplifications, revealing that 20.7% (61/295) of them were infected with *E. bieneusi*, with no significant differences found between the two sampling locations ($p > 0.05$). Twenty-two different *E. bieneusi* genotypes were identified, including one known genotype (EbpC) and 19 novel ones (named BLC1 to BLC19). The zoonotic genotype EbpC was identified in most of the *E. bieneusi*-positive samples (65.6%, 40/61). The remaining genotypes were identified in either one or three samples each. Our phylogenetic analysis showed that 20 of the *E. bieneusi* genotypes belonged to Group 1. As far as we are aware, this is the first report of *E. bieneusi* infections in edible bullfrogs. Our findings suggest that *E. bieneusi* can be maintained in edible bullfrogs and potentially transmitted via food or water. It is possible that these amphibians are unsuspected zoonotic reservoirs of *E. bieneusi*.

1. Introduction

Enterocytozoon bieneusi, an obligate intracellular pathogen of the intestinal epithelial, is frequently identified in humans and a variety of other animals (Mori et al., 2013; Wang et al., 2018a; Udonsom et al., 2019). Infection with it can lead to chronic or acute enteric diarrhea and extra-intestinal infections (such as cholangitis), especially in patients with acquired immune deficiency syndrome (Santín and Fayer, 2011; Liu H et al., 2017). The infective spores of this pathogen are ubiquitous in water sources and the environment, and they are potentially transmitted through the fecal-oral route, either directly or indirectly by ingesting food or water contaminated with them (Santín and Fayer, 2011). The National Institute of Allergy and Infectious Diseases (NIAID) has classified *E. bieneusi* as a Category B Priority Pathogen (Karim et al., 2014).

Considerable genetic diversity exists among and within *E. bieneusi* isolates according to the sequence polymorphisms found in the internal transcribed spacer (ITS) region (Li et al., 2019a). To date, more than 500 *E. bieneusi* genotypes have been reported, placing them into 11 distinct groups (Groups 1–11) by phylogenetic analysis (Li et al., 2019a, 2019b). Some of these genotypes (e.g., EbpA, EbpC, and Type IV) are found in different species of animals as well as in humans, indicating their public health importance and zoonotic potential.

The *Lithobates catesbeiana* bullfrog, a native North American species first introduced into China in the 1950s, is mainly farmed as food in Mid-Southern China and then sold throughout China. In 2014, the yield of these farmed edible bullfrogs reached 25 million kilograms in China (Gao, 2017). Although there have been some reports of *E. bieneusi* infections in pigs, cows, goats, and other animals (Li et al., 2014; Fiuza et al., 2015; da Silva Fiuza et al., 2016; Shi et al., 2016; Udonsom et al., 2019), there are no such reports for edible bullfrogs. Therefore, the aim of this study was to determine the molecular prevalence of *E. bieneusi* in edible bullfrogs, and assess the possibility of its potential zoonotic transmission to humans via food or water.

* Corresponding author.
** Corresponding author. College of Animal Science, Tarim University, Tarim Road 1487, Alar, Xinjiang, 843300, China.
E-mail addresses: lijunqiangcool@126.com (J. Li), qimengdz@163.com (M. Qi).

https://doi.org/10.1016/j.ijppaw.2020.01.004
Received 12 December 2019; Received in revised form 9 January 2020; Accepted 10 January 2020
2213-2244/ © 2020 Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).

2. Materials and methods

2.1. Ethics statement

This study was conducted in accordance with the Chinese Laboratory Animal Administration Act (1988) after review, and its protocol was approved by the Research Ethics Committee of Henan University of Chinese Medicine (accession no.: DWLL201806009). The edible bullfrog sellers were not engaged in any form of discussion about this study.

2.2. Sample collections

Aksu city (N 41°09′, E 80°19′) is an urban area in the Xinjiang Uygur Autonomous Region of China where the climate is dry and precipitation is scarce. In 2018, the total population of Aksu was approximately 710,000. Two open markets in Aksu city selling edible bullfrogs were involved in the present study.

Bullfrogs were butchered by the shopkeepers in the markets, the edible bodies and limbs were sold to the consumers, and the entrails were discarded. The number of collected samples accounted for approximately 20% of the total number of sold bullfrogs each time. To avoid cross contamination during the sample collection, the whole rectum from each bullfrog was collected by our technicians when the shopkeepers cut open the belly of each bullfrog. Each rectum was collected, placed separately into a clean plastic zipper bag, and shipped under cool conditions to the laboratory where approximately 5 g of the rectal contents from each rectum sample was collected using a laminar flow cabinet and stored there at 4 °C. DNA was extracted from these samples within 72 h of collection.

According to the shopkeepers, the edible bullfrogs were raised and farmed in Southern China and imported into the Aksu city area batch by batch. To avoid cross contamination from the same batch of bullfrogs, a total of 295 intestinal contents from the bullfrogs were collected 17 times during December 2018 and from May to June 2019 from the two open markets (Table 1).

2.3. DNA extraction and polymerase chain reaction (PCR) amplification

Approximately 200 mg of each sample was used for direct DNA extraction. Total DNA was extracted with the E.Z.N.A.R® Stool DNA Kit (Omega Biotek Inc., Norcross, GA, USA), according to the manufacturer's instructions. The extracted DNA was stored at −20 °C until PCR amplification.

Enterocytozoon bieneusi DNA was screened by nested PCR amplification of the ITS region. The primers and thermal cycling parameters used for the two PCR amplifications have been described previously (Mirjalali et al., 2015). The 2 × EasyTaq PCR SuperMix (TransGene Biotech Co., Beijing, China) kit was used for the PCR amplifications. A positive control (cattle-derived *E. bieneusi* genotype J DNA) and negative control (2 μL of distilled water without DNA) were included in all the PCR runs. All secondary PCR products were subjected to electrophoresis on 1.5% agarose gels, and then visualized by ultraviolet transillumination after staining with GelRed (Biotium Inc., Hayward, CA, USA).

2.4. *E. bieneusi* sequences and phylogenetic analyses

Positive secondary PCR amplicons were sequenced by a commercial sequencing company (GENEWIZ, Suzhou, China). The sequence accuracy was confirmed via bidirectional sequencing, and the sequences obtained were aligned using ClustalX 2.1 (http://www.clustal.org/) with the reference sequences downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) to determine the species and genotypes. Representative genotypes of the nucleotide sequences we obtained were submitted to GenBank at the National Center for Biotechnology Information under accession numbers MN758739–MN758760.

Bayesian inference (BI) and Monte Carlo Markov chain methods were used to construct phylogenetic trees in MrBayes (version 3.2.6) (http://nbisweden.github.io/MrBayes/). The posterior probability values were calculated by running 1,000,000 generations. A 50% majority-rule consensus tree was constructed from the final 75% of the trees generated via BI. Analyses were run three times to ensure convergence and insensitivity to priors.

2.5. Statistical analyses

All statistical analyses in this study were performed using SPSS 22.0. The chi-square test was used for the significance analysis. Differences were considered significant only when $p < 0.05$.

Table 1
Occurrence and genotype distributions of *E. bieneusi* pathogens in edible bullfrogs (*L. catesbeiana*) in Aksu, China.

Collection sites	Collection time	No. positive/No. examined (%)	Genotypes (n)
Market 1	Dec 3, 2018	1/18 (5.6%)	BLC1 (1)
	Dec 9, 2018	0/11 (0)	
	Dec 15, 2018	0/16 (0)	
	Dec 20, 2018	5/15 (33.3%)	EbpC (4), BLC3 (1)
	Dec 23, 2018	3/19 (15.8%)	EbpC (2), BLC4 (1)
	Dec 28, 2018	5/13 (38.5%)	EbpC (5)
Subtotal		14/92 (15.2%)	EbpC (11), BLC1 (1), BLC3 (1), BLC4 (1)
Market 2	May 20, 2019	2/15 (13.3%)	EbpC (1), BLC5 (1)
	May 26, 2019	2/17 (11.8%)	EbpC (1), BLC6 (1)
	May 31, 2019	5/22 (22.7%)	EbpC (4), BLC7 (1)
	Jun 2, 2019	4/19 (21.1%)	EbpC (3), BLC8 (1)
	Jun 5, 2019	1/24 (4.2%)	BLC9 (1)
	Jun 9, 2019	2/19 (10.5%)	EbpC (2)
	Jun 13, 2019	2/19 (10.5%)	EbpC (2)
	Jun 17, 2019	4/14 (28.6%)	EbpC (3), BLC10 (1)
	Jun 20, 2019	7/21 (33.3%)	EbpC (2), BLC11 (2), BLC12 (1), BLC14 (1), BLC15 (1)
	Jun 25, 2019	9/18 (50.0%)	EbpC (6), BLC11 (1), BLC16 (1), BLC17 (1)
	Jun 28, 2019	9/15 (60.0%)	EbpC (5), BLC2 (1), BLC13 (1), BLC18 (1), BLC19 (1)
Subtotal		47/203 (23.2%)	EbpC (29), BLC2 (1), BLC5 (1), BLC6 (1), BLC7 (1), BLC8 (1), BLC9 (1), BLC10 (1), BLC11 (3), BLC12 (1), BLC13 (1), BLC14 (1), BLC15 (1), BLC16 (1), BLC17 (1), BLC18 (1), BLC19 (1)
Total		295	EbpC (40), BLC11 (3), BLC1 (1), BLC2 (1), BLC3 (1), BLC4 (1), BLC5 (1), BLC6 (1), BLC7 (1), BLC8 (1), BLC9 (1), BLC10 (1), BLC12 (1), BLC13 (1), BLC14 (1), BLC15 (1), BLC16 (1), BLC17 (1), BLC18 (1), BLC19 (1)

3. Results and discussion

Enterocytozoon bieneusi infections have been reported in humans and a broad range of other animals, highlighting its public health importance and potential for zoonotic transmission (Li et al., 2019a). Reports of *E. bieneusi* infections in various food-related animals (e.g., pigs, goats, sheep, beef cattle, and chickens) have been largely documented in China (da Cunha et al., 2016; Wang et al., 2018b; Li et al., 2019c; Udonsom et al., 2019). High infection rates (range, 17.2%–78.9%) for *E. bieneusi* have been observed in pig samples in China (Li et al., 2014, 2019c; Wang et al., 2018b; Zou et al., 2018). While the *E. bieneusi* infection rates in goats and sheep range from 10.6% to 42.8% (Zhao et al., 2015; Shi et al., 2016; Chen et al., 2018; Zhou et al., 2019), those in beef cattle range from 19.7% to 26.5% (Ma et al., 2015; Wang et al., 2016). However, the infection rate in chickens was reported to be lower in China at 11.8% (Li et al., 2014). In the present study, both positive controls and negative controls were included with the samples from the present study in the PCR runs to ensure the absence of sample contamination. Among the 295 intestinal contents samples from the edible bullfrogs, the *E. bieneusi* infection rate was 20.7% (61/295) (Table 1). The *E. bieneusi* infection rate in the edible bullfrogs from the two different collection sites was 15.2% (14/92) and 23.2% (47/203), with no significant difference found between the sampling locations ($p > 0.05$) (Table 1). To the best of our knowledge, this is the first report of *E. bieneusi* infection in bullfrogs.

Based on the ITS gene, 20 *E. bieneusi* genotypes were identified among the 61 successfully sequenced samples from our study. The genotypes included one known genotype (EbpC) and 19 novel genotypes (named BLC1 to BLC19). The genotype EbpC was identified in the most samples (65.6%, 40/61), making it the predominant genotype in the edible bullfrogs. Novel genotypes (BLC1 to BLC10, and BLC12 to BLC19) were one sample each, while BLC11 was found in three samples. The novel genotypes (BLC1 to BLC19) had 1 to 10 nucleotide substitutions or deletions, compared with the genotype EbpC (AF135832) (Fig. 1). Genotype EbpC has already been frequently found in humans (Mori et al., 2013; Liu et al., 2017; Li et al., 2019b) and in various other animals (Wang et al., 2018a; Udonsom et al., 2019), highlighting its public health importance and zoonotic potential (Li et al., 2019a). It has been suggested that bullfrogs are a source of human infections from microsporidial spores, especially as amphibian feces can contaminate water sources.

In the phylogenetic analyses conducted to date, more than 90% of the *E. bieneusi* genotypes were found to belong to Groups 1 or 2 (Li et al., 2019a, 2019b). Group 1 genotypes are considered of public health importance based on the widespread occurrence of D, EbpC, and type IV genotypes in a very large variety of hosts (Li et al., 2019a). The remaining genotypes correspond to host-adapted groups (Groups 2–5) and are associated with specific animals and probably have no public health significance (Thellier and Breton, 2008). Recently, Groups 6–11 have also been described in some animal groups and environmental samples (Karim et al., 2014). All 20 *E. bieneusi* ITS genotypes identified in the edible bullfrogs belonged to Group 1 in the present study (Fig. 2), suggesting that the bullfrogs were naturally infected with host-specific and/or zoonotic genotypes of *E. bieneusi*.

The infective spores of *E. bieneusi* are potentially transmitted through the fecal-oral route by ingesting food or water contaminated with them (Santín and Fayer, 2011). The results of the present study indicate that bullfrogs can serve as important reservoir hosts for *E. bieneusi*. There is also the attendant risk that *E. bieneusi* spores from infected bullfrogs can contaminate water sources, leading to the transmission of zoonotic infections. Therefore, monitoring for *E. bieneusi* spores in the water sources associated with farmed bullfrogs is warranted.

In conclusion, to the best of our knowledge, this is the first report of *E. bieneusi* infections in edible bullfrogs, and the zoonotic genotype EbpC was the predominant genotype. That all 20 genotypes belonged to Group 1 suggests that bullfrogs may be a potential source of human infections with this pathogen. Further studies on *E. bieneusi* contamination of the water sources and environments around farmed bullfrogs should be conducted to investigate the transmission

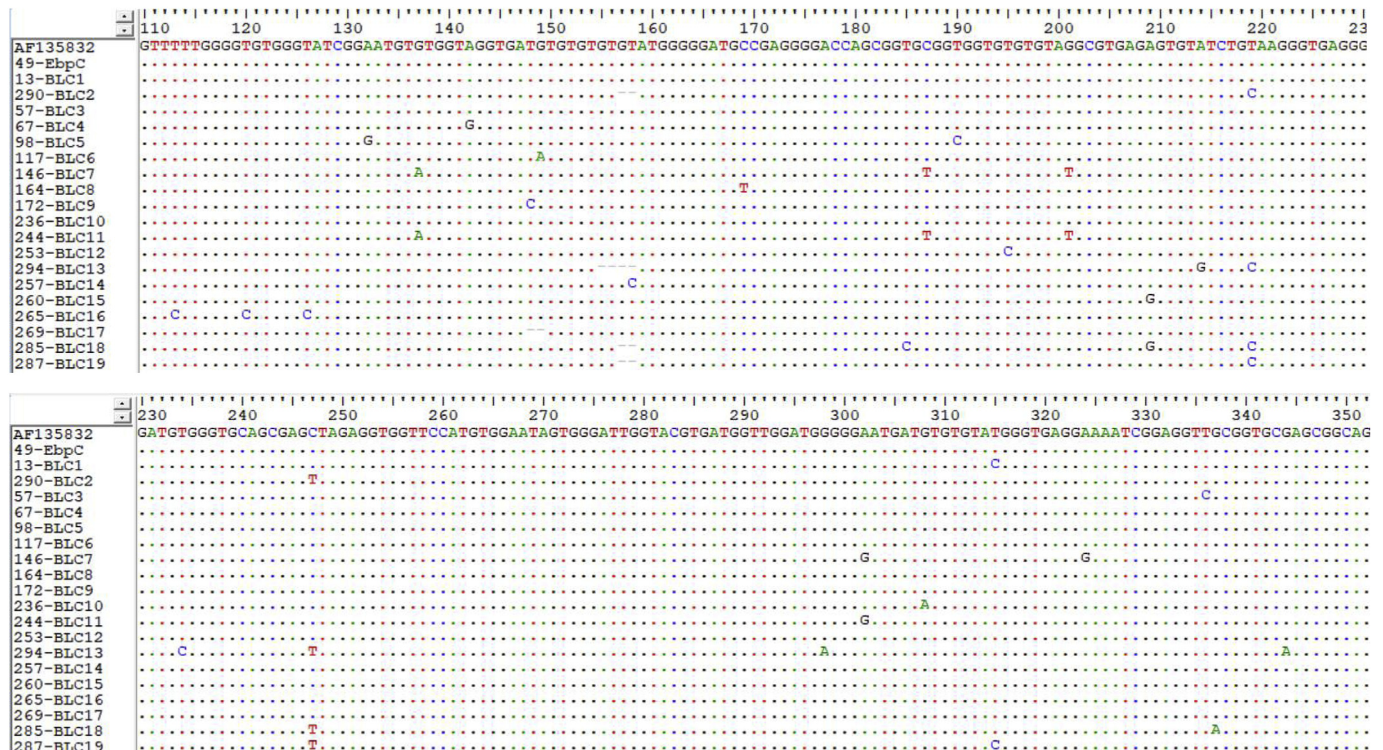


Fig. 1. Nucleotide sequence analysis of the newly identified *E. bieneusi* genotypes (BLC1 to BLC19) based on the ITS regions, as compared with the known genotype EbpC (AF135832) (synonym with genotype E).

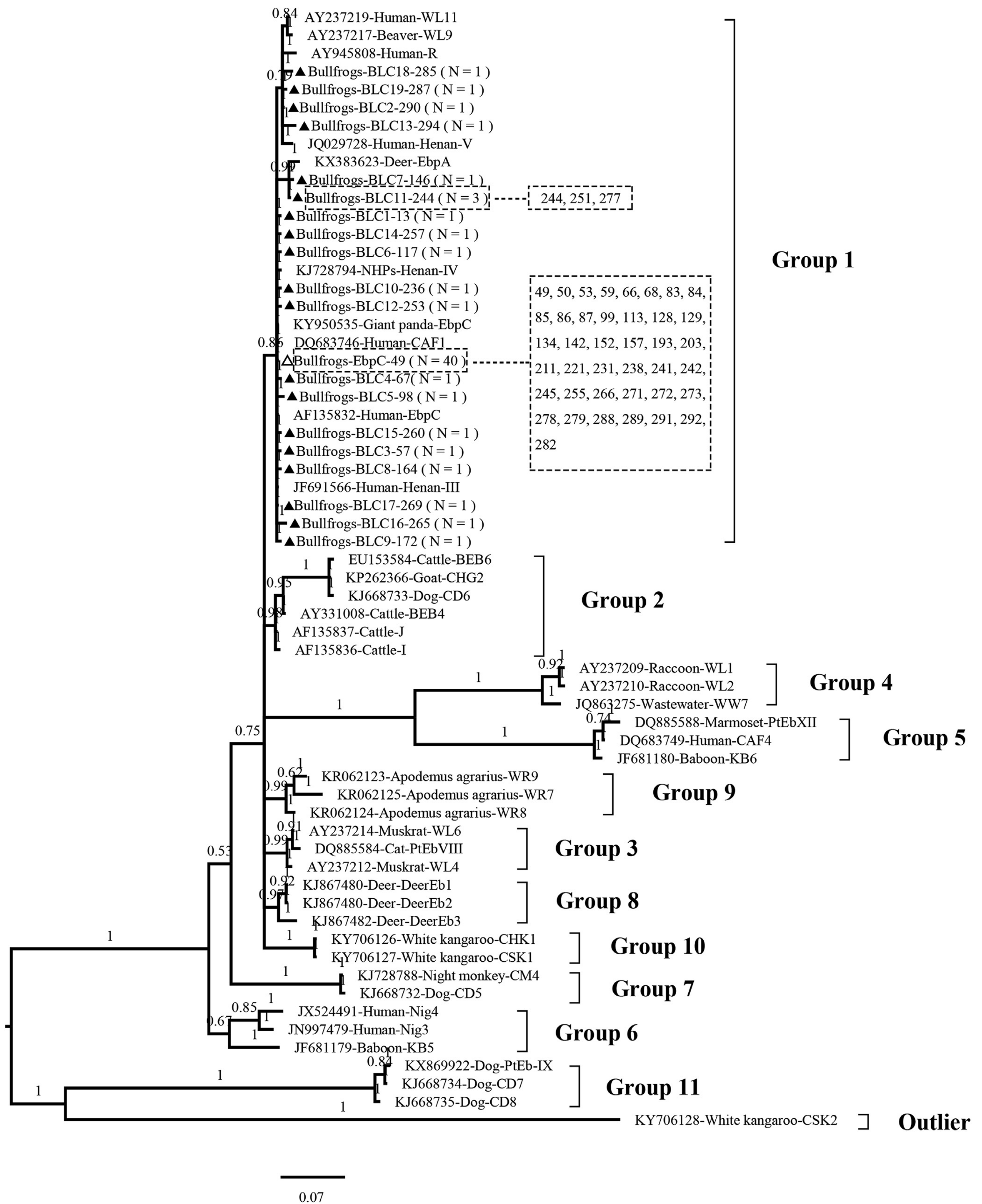


Fig. 2. Phylogenetic tree based on Bayesian inference (BI) analysis of *E. bieneusi* ITS sequences. Statistically significant posterior probabilities are indicated on the branches. Known and novel *E. bieneusi* ITS genotypes identified in the present study are indicated by hollow and filled triangles, respectively.

characteristics of this pathogen.

Declaration of competing interest

None.

Acknowledgments

This study was supported, in part, by the National Natural Science Foundation of China (31860699), the Program for Young and Middle-aged Leading Science, Technology, the Innovation of Xinjiang Production & Construction Group (2018CB034), and the National College Students Innovation and Entrepreneurship Training Program (201910757028). The funders played no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank Sandra Cheesman, PhD, from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

References

Chen, D., Wang, S.S., Zou, Y., Li, Z., Xie, S.C., Shi, L.Q., Zou, F.C., Zhu, X.Q., Yang, J.F., Zhao, G.H., 2018. Prevalence and multi-locus genotypes of *Enterocytozoon bieneusi* in black-boned sheep and goats in Yunnan Province, southwestern China. Infect. Genet. Evol. 65, 385–391.

da Cunha, M.J., Cury, M.C., Santín, M., 2016. Widespread presence of human-pathogenic *Enterocytozoon bieneusi* genotypes in chickens. Vet. Parasitol. 217, 108–112.

da Silva Fiuza, V.R., Lopes, C.W., de Oliveira, F.C., Fayer, R., Santin, M., 2016. New findings of *Enterocytozoon bieneusi* in beef and dairy cattle in Brazil. Vet. Parasitol. 216, 46–51.

Fiuza, V.R., Oliveira, F.C., Fayer, R., Santín, M., 2015. First report of *Enterocytozoon bieneusi* in pigs in Brazil. Parasitol. Int. 64, 18–23.

Gao, B., 2017. Current Status and Prospect of Bullfrog Industry in China. Jimei University.

Karim, M.R., Wang, R., Dong, H., Zhang, L., Li, J., Zhang, S., Rume, F.I., Qi, M., Jian, F., Sun, M., Yang, G., Zou, F., Ning, C., Xiao, L., 2014. Genetic polymorphism and zoonotic potential of *Enterocytozoon bieneusi* from non-human primates in China. Appl. Environ. Microbiol. 80, 1893–1898.

Li, D.F., Zhang, Y., Jiang, Y.X., Xing, J.M., Tao, D.Y., Zhao, A.Y., Cui, Z.H., Jing, B., Qi, M., Zhang, L.X., 2019c. Genotyping and zoonotic potential of *Enterocytozoon bieneusi* in pigs in Xinjiang, China. Front. Microbiol. 10, 2401.

Li, W., Feng, Y., Santin, M., 2019a. Host specificity of *Enterocytozoon bieneusi* and public health implications. Trends Parasitol. 35, 436–451.

Li, W., Feng, Y., Zhang, L., Xiao, L., 2019b. Potential impacts of host specificity on zoonotic or interspecies transmission of *Enterocytozoon bieneusi*. Infect. Genet. Evol. 75, 104033.

Li, W., Tao, W., Jiang, Y., Diao, R., Yang, J., Xiao, L., 2014. Genotypic distribution and phylogenetic characterization of *Enterocytozoon bieneusi* in diarrheic chickens and pigs in multiple cities, China: potential zoonotic transmission. PLoS One 9, e108279.

Liu, H., Jiang, Z., Yuan, Z., Yin, J., Wang, Z., Yu, B., Zhou, D., Shen, Y., Cao, J., 2017. Infection by and genotype characteristics of *Enterocytozoon bieneusi* in HIV/AIDS patients from Guangxi Zhuang autonomous region, China. BMC Infect. Dis. 17, 684.

Ma, J., Li, P., Zhao, X., Xu, H., Wu, W., Wang, Y., Guo, Y., Wang, L., Feng, Y., Xiao, L., 2015. Occurrence and molecular characterization of *Cryptosporidium* spp. and *Enterocytozoon bieneusi* in dairy cattle, beef cattle and water buffaloes in China. Vet. Parasitol. 207, 220–227.

Mirjalali, H., Mirhendi, H., Meamar, A.R., Mohebali, M., Askari, Z., Mirsamadi, E.S., Rezaeian, M., 2015. Genotyping and molecular analysis of *Enterocytozoon bieneusi* isolated from immunocompromised patients in Iran. Infect. Genet. Evol. 36, 244–249.

Mori, H., Mahittikorn, A., Watthanakulpanich, D., Komalamisra, C., Sukthana, Y., 2013. Zoonotic potential of *Enterocytozoon bieneusi* among children in rural communities in Thailand. Parasite 20, 14.

Santín, M., Fayer, R., 2011. Microsporidiosis: *Enterocytozoon bieneusi* in domesticated and wild animals. Res. Vet. Sci. 90, 363–371.

Shi, K., Li, M., Wang, X., Li, J., Karim, M.R., Wang, R., Zhang, L., Jian, F., Ning, C., 2016. Molecular survey of *Enterocytozoon bieneusi* in sheep and goats in China. Parasites Vectors 9, 23.

Thellier, M., Breton, J., 2008. *Enterocytozoon bieneusi* in human and animals, focus on laboratory identification and molecular epidemiology. Parasite 15, 349–358.

Udonsom, R., Prasertbun, R., Mahittikorn, A., Chiabchalard, R., Sutthikornchai, C., Palasuwan, A., Popruk, S., 2019. Identification of *Enterocytozoon bieneusi* in goats and cattle in Thailand. BMC Vet. Res. 15, 308.

Wang, S.S., Wang, R.J., Fan, X.C., Liu, T.L., Zhang, L.X., Zhao, G.H., 2018a. Prevalence and genotypes of *Enterocytozoon bieneusi* in China. Acta Trop. 183, 142–152.

Wang, S.S., Li, J.Q., Li, Y.H., Wang, X.W., Fan, X.C., Liu, X., Li, Z.J., Song, J.K., Zhang, L.X., Zhao, G.H., 2018b. Novel genotypes and multilocus genotypes of *Enterocytozoon bieneusi* in pigs in northwestern China: a public health concern. Infect. Genet. Evol. 63, 89–94.

Wang, X.T., Wang, R.J., Ren, G.J., Yu, Z.Q., Zhang, L.X., Zhang, S.Y., Lu, H., Peng, X.Q., Zhao, G.H., 2016. Multilocus genotyping of *Giardia duodenalis* and *Enterocytozoon bieneusi* in dairy and native beef (Qinchuan) calves in Shaanxi province, northwestern China. Parasitol. Res. 115, 1355–1361.

Zhao, W., Zhang, W., Yang, D., Zhang, L., Wang, R., Liu, A., 2015. Prevalence of *Enterocytozoon bieneusi* and genetic diversity of ITS genotypes in sheep and goats in China. Infect. Genet. Evol. 32, 265–270.

Zhou, H.H., Zheng, X.L., Ma, T.M., Qi, M., Cao, Z.X., Chao, Z., Wei, L.M., Liu, Q.W., Sun, R.P., Wang, F., Zhang, Y., Lu, G., Zhao, W., 2019. Genotype identification and phylogenetic analysis of *Enterocytozoon bieneusi* in farmed blackgoats (*Capra hircus*) from China's Hainan Province. Parasite 26, 62.

Zou, Y., Hou, J.L., Li, F.C., Zou, F.C., Lin, R.Q., Ma, J.G., Zhang, X.X., Zhu, X.Q., 2018. Prevalence and genotypes of *Enterocytozoon bieneusi* in pigs in southern China. Infect. Genet. Evol. 66, 52–56.