



Molecular Characterization of *Enterocytozoon bieneusi* in Domestic Rabbits (*Oryctolagus cuniculus*) in Northeastern China

Xiao-Xuan Zhang^{1,†}, Jing Jiang^{2,†}, Ya-Nan Cai¹, Chun-Feng Wang¹, Peng Xu³, Gui-Lian Yang^{1,*}, Quan Zhao^{1,*}

¹College of Animal Science and Technology, Jilin Provincial Engineering Research Center of Animal Probiotics, Jilin Agricultural University, Changchun, Jilin Province 130118, PR China; ²College of Animal Science and Technology, Changchun University of Science and Technology, Shuangyang, Jilin Province 130600, PR China; ³College of Animal Husbandry and Veterinary Medicine, Liaoning Medical University, Jinzhou, Liaoning Province 121001, PR China

Abstract: A study of 426 rabbits from 3 cities in Jilin province (Changchun City and Jilin City) and Liaoning province (Shenyang City) was conducted between May and June 2015. The overall prevalence of *E. bieneusi* in rabbits was 0.94% (4/426), with 0% (0/116), 1.72% (3/174), and 0.74% (1/136) in Jilin, Changchun, and Shenyang City, respectively. Only 3 farms (farm 1 and farm 3 in Changchun City, farm 8 in Shenyang City) were PCR-positive for *E. bieneusi*. Moreover, rabbits of more than 6 months (1.72%) had the highest *E. bieneusi* prevalence, followed by rabbits of 4-6 months (1.26%), 2-3 months (0.58%), and less than 1 month (0%). Analysis of ITS gene of *E. bieneusi* suggested that all 4 *E. bieneusi* isolates were genotype D, and were classified as group 1a. The present results first demonstrated the existence of zoonotic *E. bieneusi* in domestic rabbits in China. Effective control measures should be implemented to prevent *E. bieneusi* infection in domestic rabbits, other animals, and humans.

Key words: *Encephalitozoon bieneusi*, domestic rabbit, prevalence, internal transcribed spacer (ITS), China

China has a long history of breeding rabbits which have a close relationship with humans. With the improvement of living standards, rabbit meat became widely used for consumption. Thus, more people have pay attention to rabbit industry. Therefore, evaluating how pathogens influence animal health is essential to rabbit industry.

It is well known that the rabbit is a major host for many pathogens including microsporidia [1,2]. However, information regarding the prevalence and genotypes of *Encephalitozoon bieneusi* in China is scarce. The objectives of the present study were to estimate the prevalence of *E. bieneusi* in rabbits and to identify their genotypes in China. Moreover, the present study also aimed to provide the foundation data for further study of *E. bieneusi* genotypes distribution in China.

A total of 426 fecal samples were collected from Jilin (n=290) and Liaoning (n=136) province between May and June 2015.

At the time of sampling, all the rabbits were in good health. Fresh fecal sample was collected from each animal using sterile disposal PE gloves after defecation onto the ground. All samples were sent to the laboratory, and then genomic DNA was extracted using an EZNAR Stool DNA kit (OMEGA Biotec, Madison, Wisconsin, USA) according to the manufacturer instructions, immediately. All the extracted DNA samples were stored at -20°C until PCR analysis. Information concerning gender, geographic origin, and age were obtained by a questionnaire.

E. bieneusi genotypes were determined by nested PCR of the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene [3]. PCR reaction (25 µl) was composed of 200 µM each deoxy-ribonucleoside triphosphate (dNTP), 1 × Ex Taq buffer (Mg²⁺ free), 0.4 µM of each primer, 0.625 U of Ex Taq DNA polymerase (TAKARA, Kyoto, Japan), 2 mM MgCl₂, and 2 µl of DNA template. The cycling conditions were denatured at 95°C for 5 min, and then 35 cycles of 45 sec at 94°C, 45 sec at 55°C, and 1 min at 72°C, followed by 72°C for 10 min. Both positive and negative controls were included in each test. All the PCR products were electrophoresed on a 2% agarose gel, stained with GoldViewTM (Solarbio, Beijing, China), and then observed under UV light.

• Received 25 August 2015, revised 3 November 2015, accepted 4 November 2015.

* Corresponding author (yangguilian@jlau.edu.cn; zhaoquan0825@163.com)

† XXZ and JJ contributed equally to this work.

© 2016, Korean Society for Parasitology and Tropical Medicine

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Table 1. *Enterocytozoon bieneusi* genotypes identified in rabbits in different farms

Region	Farm ID	Age category (n)	No. positive/no. tested (%)	Genotype (n)
Changchun City	Farm 1	< 1 month (15), 2-3 months (21), 4-6 months (26)	2/62 (3.2)	D (n=2)
	Farm 2	>6 months (33)	0/33 (0)	-
	Farm 3	< 1 month (11), 2-3 months (16), 4-6 months (20)	1/47 (2.1)	D (n=1)
	Farm 4	4-6 months (32)	0/32 (0)	-
Jilin City	Farm 5	2-3 months (13), 4-6 months (50), >6 months (14)	0/77 (0)	-
	Farm 6	< 1 month (11), 2-3 months (28)	0/39 (0)	-
Shenyang City	Farm 7	2-3 months (21), 4-6 months (15)	0/36 (0)	-
	Farm 8	2-3 months (41), 4-6 months (16)	1/57 (1.8)	D (n=1)
	Farm 9	2-3 months (32), >6 months (11)	0/43 (0)	-
Total		< 1 month (37), 2-3 months (172), 4-6 months (159), >6 months (58)	3/426 (0.94)	D (n=4)

Table 2. *Enterocytozoon bieneusi* genotypes identified in rabbits in different age groups

Factor	Category	No. tested	No. positive	% (95% CI)	Genotype (n)
Age	< 1 month	37	0	0 (-)	-
	2-3 months	172	1	0.58 (0.00-1.72)	D (n=1)
	4-6 months	159	2	1.26 (0.00-2.99)	D (n=2)
	>6 months	58	1	1.72 (0.00-5.07)	D (n=1)

Positive secondary PCR products were sent to Genscript Company (Nanjing, China) for sequencing. The obtained sequences were aligned with reported sequences of *E. bieneusi* to determine the *E. bieneusi* genotypes using the BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). Mega 5.0 software was used to construct the phylogenetic trees using neighbor-joining (NJ) method (Kimura 2-parameter model, 1,000 replicates).

Differences in the prevalence of *E. bieneusi* in rabbits of different geographical locations, gender groups, and age groups were analyzed by the chi-square test using SAS version 9.1 (SAS Institute Inc., Cary, North Carolina, USA). If $P < 0.05$, the results were considered as statistically significant. Then, 95% confidence interval (CI) was provided in this study. Representative nucleotide sequences were deposited in GenBank with the accession no. KT325924.

A total of 4 (0.94%) out of 426 rabbit feces were *E. bieneusi*-positive, with 0% (0/116), 1.72% (3/174), and 0.74% (1/136) in Jilin, Changchun, and Shenyang City, respectively (Table 1). Only 3 farms (farm 1 and farm 3 in Changchun, farm 8 in Shenyang) were PCR-positive for *E. bieneusi*, with the highest infection rates in farm 1 (n=2, 3.2%), followed by farm 3 (n=1, 2.1%) and farm 8 (n=1, 1.8%) (Table 1). Moreover, rabbits of more than 6 months (1.72%, 95% CI 0.00-5.07) revealed the highest *E. bieneusi* prevalence, followed by rabbits of 4-6 months (1.26%, 95% CI 0.00-2.99), 2-3 months (0.58%, 95% CI 0.00-1.72), and less than 1 month (0%) (Table 2). Analysis of ITS gene of *E. bieneusi* revealed that all *E. bieneusi* isolates were geno-

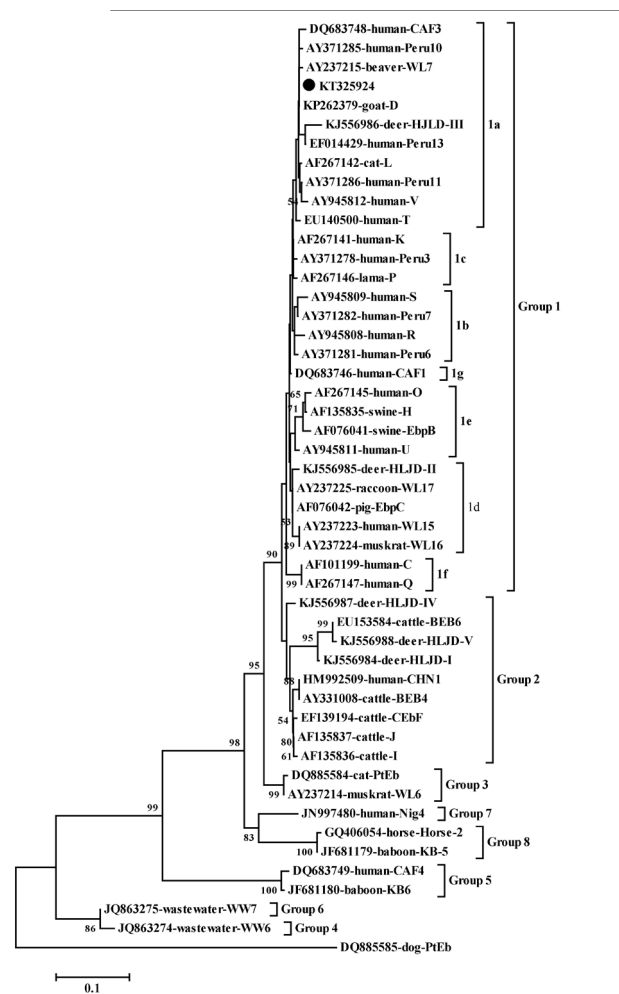


Fig. 1. Phylogenetic analysis of *Enterocytozoon bieneusi* using Neighbor-Joining (NJ) method based on sequences of the internal transcribed spacer (ITS) region. The numbers at nodes indicate bootstrap values (1,000 replicates, values below 50% are not shown). *E. bieneusi* isolate identified in the present study is indicated by solid circle.

type D (n=4), which belonged to group 1a (Fig. 1).

To date, information concerning distribution of genotypes

Table 3. *Enterocytozoon bieneusi* genotypes identified in different animals in northeastern China

Country	Hosts	No. tested	%	Microsporidia species	Reference
Heilongjiang and Jilin	Cattle	537	6.0	BEB4, BEB6, CM7, CS-4, EbpC, G, I, J, OEB1, and 11 new genotypes (NESH1 to NESH6 and NECA1 to NECA5)	[19]
	Sheep	498	13.9		
Heilongjiang	Dairy cattle	133	30.1	O, EbpA, I, J, D, BEB4, and three novel genotypes (CC-I to CC-III)	[14]
Heilongjiang	Sheep	138	22.5	Peru6, BeB6, D, EbpC, EbpA, COG-I	[13]
Heilongjiang	Goats	55	21.8	BeB6, Peru6, D, O, COS-I to COS-VII	
Heilongjiang	<i>Cervus nippon</i>	52	25.0	BeB6, HLJD-I-HLJD-V	
Heilongjiang	<i>Cervus elaphus</i>	5	20.0	HLJD-V	[4]
Jilin	<i>Cervus nippon</i>	34	44.1	BeB6, HLJD-V	
Jilin	Pigs	156	60.3	CHN7, CS-1, CS-4, CS-6, EbpA, EbpB, EbpC, EbpD, EBIT33, G, and Henan-I; one new: CS-9	[20]
Heilongjiang	Sheep	45	4.4	BEB6	
Heilongjiang	Pigs	95	89.5	H, HLJ-I, HLJ-II, HLJ-III, HLJ-IV, CS-1, O, LW1, EbpA, D	[15]
Heilongjiang	Cats	52	5.8	Human pathogenic genotypes D and IV	[12]
Heilongjiang	Dogs	267	6.7	D, EbpC, NED1, NED2, NED3, NED4, and PtEb IX	
Heilongjiang, Jilin, Inner Mongolia	Pigs	113	45.1	D, EbpA, EbpC, EbpD, H, Henan-I, Henan-III, Henan-IV, and O, and eight new genotypes (CS-1 to CS-8)	[21]
Jilin and Liaoning	Rabbits	426	0.94	D	This study

of *E. bieneusi* in China is scarce, especially in rabbits. In order to further improve the prevalence and distribution information, we conducted the present study. The overall prevalence of *E. bieneusi* infection in rabbits was 0.94%, which was considerably lower than that in other animals in northeastern China (Table 3). The lower infection rates may be related to different susceptibility of different animals, sample sizes and geo-ecological conditions, standardized management mode, and so on. Moreover, the prevalence of *E. bieneusi* in rabbits seemed to increase with the age in the present study, which agreed to previous studies; *E. bieneusi* was an opportunistic parasite and it may accumulate throughout the life time [4,5]. Moreover, probably because of similar climates in Changchun City, Jilin City, and Shenyang City, and the same management mode in these farms, the positive rates of *E. bieneusi* infection in rabbits among the 9 farms in northern China were not statistically different ($P > 0.05$).

In the present study, only genotype D ($n = 3$) was found in rabbits. This result was similar to a previous study conducted in Spain, in which also only genotype D was identified [6]. Genotype D not only has a wide geographical distribution but also can infect almost all host species, including humans, domestic animals, and wild animals [6]. In China, genotype D also has a broad host range. Besides some animals, e.g., golden takins (*Budorcas taxicolor bedfordi*) and panda in Shanxi [7,8], pet chinchillas in Anyang, Beijing, and Zhengzhou, China [9], nonhuman primates in Henan, Guangxi, Guangdong, Yunnan

[10], and Sichuan [11], dairy cattle, goats, sheep, pigs, cats, and dogs in northern China [12-15]. Genotype D infection was also reported in children in Shanghai [16] and HIV patients in Henan [17], which suggest that *E. bieneusi* genotype D could readily transmit between humans and animals. More importantly, genotype D was also present in wastewater which may lead to outbreaks of this disease [18]. Rabbits may play an important role and cannot be ignored during this pathogen infection process in China. Moreover, sequence analysis of the ITS rRNA gene showed that the obtained sequences were identical to the reference sequence from sheep and goat isolates (accession no. KP262379) in China, which revealed that the infectious source is more likely to be excreted from sheep and goats. Therefore, we should pay serious attention to nosocomial transmission among different animals.

In view of such situation, the following practice should be performed to prevent transmission of *E. bieneusi* among rabbits, other animals, and humans in China: first, different kinds of animals should be managed strictly separately; second, the prevalence of *E. bieneusi* should be examined periodically; third, feces should be cleaned timely; fourth, parasites should be expelled regularly; fifth, humans should have a habit of wash hands before meals and after contact with animals.

In conclusion, the present study first demonstrated existence (0.94%, 4/426) of *E. bieneusi* infection in rabbits in China, and the infection rate was increased with age. Moreover, only genotype D was identified in rabbits which should be consid-

ered as an important potential source for *E. bienersi* infection in China. Therefore, effective strategies should be taken to prevent and control of *E. bienersi* infection in rabbits, other animals, and humans in China.

ACKNOWLEDGMENTS

This work was supported by the National High-Tech R&D Program of China (863 Programs) (no. 2013AA102806, no. 2011AA10A215), National Natural Science Foundation of China (no. 31272552, no. 31272541), the Key Scientific and Technological Project of Jilin Province (no. 20140204068NY), Jilin Province Science and Technology Development Program of China (no. 20111816), and Jilin Province Quality and Safety of Agricultural Products Program by World Bank (no. 2011-Y07).

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

- Shin JC, Kim DG, Kim SH, Kim S, Song KH. Seroprevalence of *Encephalitozoon cuniculi* in pet rabbits in Korea. *Korean J Parasitol* 2014; 52: 321-323.
- Yang D, Ren Y, Fu Y, Xie Y, Nie H, Nong X, Gu X, Wang S, Peng X, Yang G. Genetic variation of *Taenia pisiformis* collected from Sichuan, China, based on the mitochondrial cytochrome B gene. *Korean J Parasitol* 2013; 51: 449-452.
- Hu Y, Feng Y, Huang C, Xiao L. Occurrence, source, and human infection potential of *Cryptosporidium* and *Enterocytozoon bienersi* in drinking source water in Shanghai, China, during a pig carcass disposal incident. *Environ Sci Technol* 2014; 48: 14219-14227.
- Zhao W, Zhang W, Wang R, Liu W, Liu A, Yang D, Yang F, Karim MR, Zhang L. *Enterocytozoon bienersi* in sika deer (*Cervus nippon*) and red deer (*Cervus elaphus*): deer specificity and zoonotic potential of ITS genotypes. *Parasitol Res* 2014; 113: 4243-4250.
- Thellier M, Breton J. *Enterocytozoon bienersi* in human and animals, focus on laboratory identification and molecular epidemiology. *Parasite* 2008; 15: 349-358.
- Galván-Díaz AL, Magnet A, Fenoy S, Henriques-Gil N, Haro M, Gordo FP, Miró G, del Águila C, Izquierdo F. Microsporidia detection and genotyping study of human pathogenic *E. bienersi* in animals from Spain. *PLoS One* 2014; 9: e92289.
- Tian GR, Zhao GH, Du SZ, Hu XF, Wang HB, Zhang LX, Yu SK. First report of *Enterocytozoon bienersi* from giant pandas (*Ailuropoda melanoleuca*) and red pandas (*Ailurus fulgens*) in China. *Infect Genet Evol* 2015; 34: 32-35.
- Zhao GH, Du SZ, Wang HB, Hu XF, Deng MJ, Yu SK, Zhang LX, Zhu XQ. First report of zoonotic *Cryptosporidium* spp., *Giardia intestinalis* and *Enterocytozoon bienersi* in golden takins (*Budorcas taxicolor bedfordi*). *Infect Genet Evol* 2015; 34: 394-401.
- Qi M, Luo N, Wang H, Yu F, Wang R, Huang J, Zhang L. Zoonotic *Cryptosporidium* spp. and *Enterocytozoon bienersi* in pet chinchillas (*Chinchilla lanigera*) in China. *Parasitol Int* 2015; 64: 339-341.
- Karim MR, Wang R, Dong H, Zhang L, Li J, Zhang S, Rume FI, Qi M, Jian F, Sun M, Yang G, Zou F, Ning C, Xiao L. Genetic polymorphism and zoonotic potential of *Enterocytozoon bienersi* from nonhuman primates in China. *Appl Environ Microbiol* 2014; 80: 1893-1898.
- Ye J, Xiao L, Li J, Huang W, Amer SE, Guo Y, Roellig D, Feng Y. Occurrence of human-pathogenic *Enterocytozoon bienersi*, *Giardia duodenalis* and *Cryptosporidium* genotypes in laboratory macaques in Guangxi, China. *Parasitol Int* 2014; 63: 132-137.
- Li W, Li Y, Song M, Lu Y, Yang J, Tao W, Jiang Y, Wan Q, Zhang S, Xiao L. Prevalence and genetic characteristics of *Cryptosporidium*, *Enterocytozoon bienersi* and *Giardia duodenalis* in cats and dogs in Heilongjiang province, China. *Vet Parasitol* 2015; 208: 125-134.
- Zhao W, Zhang W, Yang D, Zhang L, Wang R, Liu A. Prevalence of *Enterocytozoon bienersi* and genetic diversity of ITS genotypes in sheep and goats in China. *Infect Genet Evol* 2015; 32: 265-270.
- Zhao W, Zhang W, Yang F, Zhang L, Wang R, Cao J, Shen Y, Liu A. *Enterocytozoon bienersi* in Dairy Cattle in the Northeast of China: Genetic Diversity of ITS Gene and Evaluation of Zoonotic Transmission Potential. *J Eukaryot Microbiol* 2015; 62: 553-560.
- Zhao W, Zhang W, Yang F, Cao J, Liu H, Yang D, Shen Y, Liu A. High prevalence of *Enterocytozoon bienersi* in asymptomatic pigs and assessment of zoonotic risk at the genotype level. *Appl Environ Microbiol* 2014; 80: 3699-3707.
- Wang L, Xiao L, Duan L, Ye J, Guo Y, Guo M, Liu L, Feng Y. Concurrent infections of *Giardia duodenalis*, *Enterocytozoon bienersi*, and *Clostridium difficile* in children during a cryptosporidiosis outbreak in a pediatric hospital in China. *PLoS Negl Trop Dis* 2013; 7: e2437.
- Wang L, Zhang H, Zhao X, Zhang L, Zhang G, Guo M, Liu L, Feng Y, Xiao L. Zoonotic *Cryptosporidium* species and *Enterocytozoon bienersi* genotypes in HIV-positive patients on antiretroviral therapy. *J Clin Microbiol* 2013; 51: 557-563.
- Guo Y, Alderisio KA, Yang W, Cama V, Feng Y, Xiao L. Host specificity and source of *Enterocytozoon bienersi* genotypes in a drinking source watershed. *Appl Environ Microbiol* 2014; 80: 218-225.
- Jiang Y, Tao W, Wan Q, Li Q, Yang Y, Lin Y, Zhang S, Li W. Erratum for Jiang et al., Zoonotic and potentially host-adapted *Enterocytozoon bienersi* genotypes in sheep and cattle in northeast China and an increasing concern about the zoonotic importance of previously considered ruminant-adapted genotypes. *Appl Environ Microbiol* 2015; 81: 5278.
- Li W, Li Y, Li W, Yang J, Song M, Diao R, Jia H, Lu Y, Zheng J,

Zhang X, Xiao L. Genotypes of *Enterocytozoon bieneusi* in livestock in China: high prevalence and zoonotic potential. PLoS One 2014; 9: e97623.

21. Li W, Diao R, Yang J, Xiao L, Lu Y, Li Y, Song M. High diversity of human-pathogenic *Enterocytozoon bieneusi* genotypes in swine in northeast China. Parasitol Res 2014; 113: 1147-1153.

