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Research paper

Dominance of zoonotic genotype D of *Enterocytozoon bieneusi* in bamboo rats (*Rhizomys sinensis*)

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ABSTRACT

Enterocytozoon bieneusi is an emerging zoonotic intestinal pathogen that infects humans and various animal species. Here, we aimed to determine the infection rate and genetic characteristics of *E. bieneusi* from bamboo rats from different regions of China using nested polymerase chain reaction-based amplification of the internal transcribed spacer region of the rRNA gene. A total of 435 bamboo rats fecal samples were collected from individual tank from Guangdong, Hunan, Jiangxi, Chongqing, and Guangxi, southeastern China. *E. bieneusi* was detected on 22 tanks (5.1%, 22/435), with a higher infection rate being observed among samples from Guangdong Province (10.9%, 5/46) compared with those from Hunan (9.3%, 10/107), Jiangxi (6.7%, 6/90), Chongqing (2.0%, 1/50), and Guangxi (0%, 0/142) ($P < .01$). Six genotypes were identified, including four known genotypes (D, EbpA, J, and PigEBITS7) and two novel genotypes (named BR1 and BR2). Of these, zoonotic genotype D was the most prevalent in the present study ($n = 17$). Phylogenetic analysis revealed that genotypes D, EbpA, and PigEBITS7 were clustered into Group 1, while genotypes J, BR1, and BR2 were clustered into Group 2. To our knowledge, this is the first report of *E. bieneusi* in bamboo rats. The identification of zoonotic genotype D as the predominant genotype in bamboo rats suggests that these animals represent a potential zoonotic risk for the transfer of the pathogen in China.

1. Introduction

Enterocytozoon bieneusi is a zoonotic gastrointestinal pathogen with a broad host range including humans, livestock, companion animals, and wildlife (Santín and Fayer, 2011; Khanduja et al., 2017; Liu et al., 2017). Infection occurs via fecal-oral transmission following ingestion of infective spores, usually through water, food, or direct contact (Li et al., 2017). *E. bieneusi* has been listed as a candidate microbial contaminant of concern for waterborne transmission by the Environmental Protection Agency (Didier et al., 2009).

Molecular genotyping studies based on the rRNA gene internal transcribed spacer (ITS) region suggest that *E. bieneusi* comprises over 300 genotypes (Chen et al., 2018; Wang et al., 2018). These genotypes were clustered into at least 10 phylogenetic groups (Groups 1–9 and a so-called outlier) using strains isolated from wild dogs in Spain (Santín et al., 2018). Recently, more and more studies showed that it was difficult to assess the zoonotic potential through the division of groups. To date, over 70 zoonotic *E. bieneusi* genotypes have been identified in human samples, including genotypes D, EbpA, EbpC, Peru11, type IV in Group 1, and genotypes J, I, BEB4, and BEB6 in Group 2 (Zhang et al., 2011; Matos et al., 2012; Wang et al., 2013a, 2013b; Guo et al., 2014; Liu et al., 2014; Yang et al., 2014; Karim et al., 2015; Liu et al., 2017; Wang et al., 2017).

E. bieneusi has been reported in a wide range of hosts, including humans, non-human primates, domestic animals, wildlife, and wastewater, in China (Guo et al., 2014; Wang et al., 2018). Bamboo rat (*Rhizomys sinensis*), getting its name from eating bamboo, is widely distributed in southern Asia, such as southern China, India, Myanmar, and Thailand. The bamboo rat meat was recognised as valuable food in China because of its high protein content, low fat and cholesterol (Ma

Abbreviations: ITS, Internal transcribed spacer; SNPs, Single nucleotide polymorphisms

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Table 1
Distribution of *E. bieneusi* genotypes in bamboo rats from different farms in southeastern China.

Region	Farms	No. of tanks	No. of positive	% (95% CI)	Genotype (no.)
Hunan	Yongzhou 1	34	6	17.6 (4.1–31.1)	D (5), PigEBITS7 (1)
	Yongzhou 2	44	4	9.1 (0.2–17.9)	D (3), EbpA(1)
	Yueyang	29	0	0	–
	Subtotal	107	10	9.3 (3.7–15.0)	D (8), PigEBITS7 (1), EbpA(1)
Jiangxi	Ji'an	18	0	0	–
	Yugan	24	0	0	–
	Shinao	21	5	23.8 (3.9–43.7)	D (5)
	Pingxiang	27	1	3.7 (0.0–11.3)	J (1)
	Subtotal	90	6	6.7 (1.4–11.9)	D (5), J (1)
Guangdong	Luoding	24	1	4.2 (0.0–12.8)	BR1[a] (1)
	Huazhou	22	4	18.2 (0.7–35.7)	D (4)
	Subtotal	46	5	10.9 (1.5–20.2)	D (4), BR1[a] (1)
Guangxi	Hechi	34	0	0	–
	Fusui	60	0	0	–
	Chongzuo	48	0	0	–
	Subtotal	142	0	0	–
Chongqing	Subtotal	50	1	2.0 (0.0–6.0)	BR2[a] (1)
Total		435	22	5.1 (3.0–7.1)	D (17), J (1), BR1[a] (1), BR2[a] (1), EbpA (1), PigEBITS7 (1)

[a] Novel genotype of *E. bieneusi*.

et al., 2018). Information on the infection rate and genetic characteristics of *E. bieneusi* in bamboo rats remains unclear. Therefore, this study was conducted to determine the genotypes and assess the zoonotic potential of *E. bieneusi* from bamboo rats in China.

2. Materials and methods

2.1. Sample collection

In total, 435 fresh fecal samples were collected from seemingly healthy bamboo rats from 13 farms in 12 cities located in Hunan Province (three farms) (24°38′–30°08′ N, 108°47′–114°15′ E), Jiangxi Province (four farms) (24°29′–30°04′ N, 113°34′–118°28′ E), Guangdong Province (two farms) (3°28′–25°31′ N, 108°13′–119°59′ E), Guangxi Zhuang Autonomous Region (three farms) (22°13′–23°32′ N, 107°45′–108°51′ E), and Chongqing City (one farm) (28°10′–32°13′ N, 105°11′–110°11′ E), southeastern China. Each farm was sampled on one occasion between February 2017 and February 2018 (Table 1, Fig. 1). The rearing population of bamboo rats at each of the sampled farms ranged from 300 to 2200 individuals. At all of the farms, bamboo rats were housed in small concrete tanks, with 1–6 animals per tank. For sample collection, 5–10 g of fresh feces were immediately collected from the floors of the tanks using sterile gloves, with all deposits from each tank pooled as a single sample. Fecal samples were placed into a 15-mL centrifuge tube, to which 2.5% potassium dichromate was added. Information about the animals (location, age, sex and farm) was collected at the time of sampling. All fecal samples were immediately transported to the laboratory and stored at 4 °C until DNA extraction (within 1 week of collection).

2.2. DNA extraction

Prior to DNA extraction, each fecal sample (3–5 mL) was washed three times with distilled water by centrifugation at 3000 ×*g* for 10 min. Approximately 200 mg of cleaned fecal debris from each sample were then used for genomic DNA extraction using an E.Z.N.A. Stool DNA Kit (D4015–02, Omega Bio-tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions. DNA was eluted in 200 μL of Elution Buffer and stored at −20 °C until required for polymerase chain reaction (PCR)-based analyses.

2.3. PCR amplification

E. bieneusi was identified in the fecal samples by nested PCR amplification of an ~390 bp fragment of the ITS region of the rRNA gene using primers described previously (Buckholt et al., 2002). Each 25 μL PCR reaction mixture contained 0.3 μM of each primer, 12.5 μL of 2× EasyTaq PCR SuperMix (TransGene Biotech Co., Beijing, China), 1 μL of genomic DNA for the primary PCR or 1 μL of primary amplification product for the secondary PCR, and 10.9 μL of deionized water. Positive (dairy cattle-derived genotype I DNA) and negative controls were included in each assay.

2.4. Nucleotide sequencing and analysis

The ITS amplicon from positive samples identified in the secondary PCR assays was sent for bidirectional sequencing by GENEWIZ (Suzhou, China). Raw sequences were assembled and edited using Chromas Pro version 2.13 (Technelysium Pty., Ltd., Helensville, QLD, Australia). The resulting sequences were aligned against reference sequences downloaded from the National Center for Biotechnology Information GenBank database (https://www.ncbi.nlm.nih.gov/) to determine the genotypes of the *E. bieneusi* strains in each sample.

2.5. Phylogenetic analysis

Sequences of *E. bieneusi* genotypes ITS region obtained in the present study were aligned with previously reported reference sequences used by the Clustal X version 2.1 (http://clustal.org/). Bayesian inference (BI) and Monte Carlo Markov Chain (MCMC) methods was used to constructed phylogenetic trees in MrBayes v 3.2.6 (http://mrbayes.sourceforge.net/). The general time reversible model (GTR + G) was the best-fit nucleotide substitution model determined by ModelTest version 3.7 (http://www.molecularevolution.org/). The number of substitutions (Nst) was set at 6, with a proportion of invariable sites. Posterior probability values were estimated based on 1,000,000 generations with four simultaneous tree building chains, with trees being saved every 1000th generation. At the end of each run, the standard deviation of split frequencies was < 0.01, and the potential scale reduction factor approached one. A 50% majority rule consensus tree for each analysis was constructed based on the final 75% of trees generated by BI. Analyses were run three times to ensure convergence and insensitivity to priors. FigTree v 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize and edit the maximum clade credibility tree generated by these analyses.

Fig. 1. Specific locations where samples were collected in this study. ▲ Locations.

2.6. Statistical analysis

Chi-square tests carried out in SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) were used to compare the infection rate and distribution of predominant genotypes. All results were considered statistically significant at $p < .05$.

2.7. Nucleotide sequence accession numbers

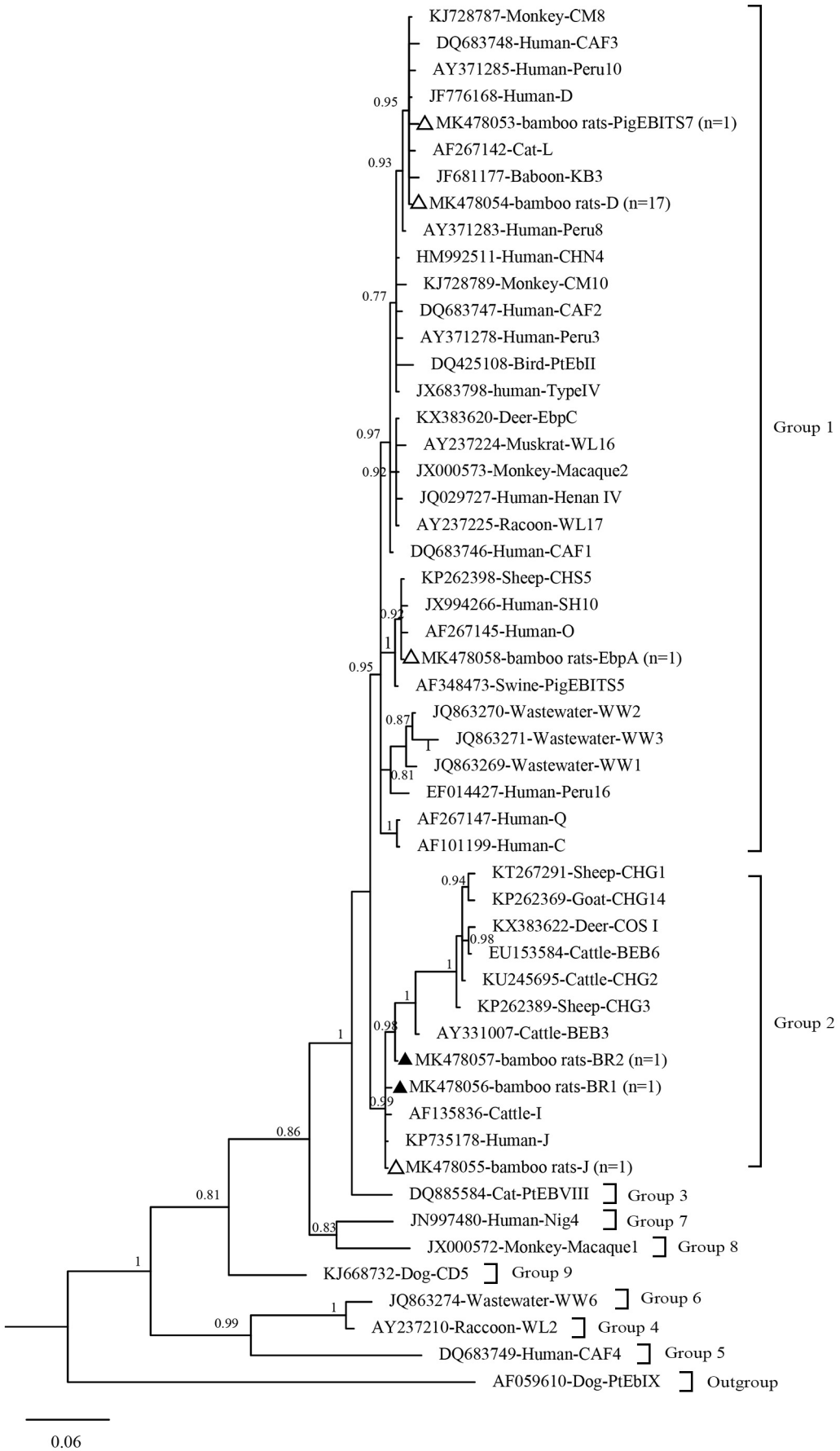
The nucleotide sequences of the ITS region from *E. bieneusi* obtained in the present study have been submitted to the GenBank database under accession numbers: MK478053 - MK478058.

3. Results and discussion

To our knowledge, this is the first report of the infection rate of *E. bieneusi* in bamboo rats. Of the 435 bamboo rats fecal samples collected from individual tank from 13 farms, 22 (5.1%) tanks from 7 (53.8%) farms were positive for *E. bieneusi*. The highest infection rate (10.9%, 5/46) was observed from Guangdong Province, followed by those from Hunan Province (9.35%, 10/107), Jiangxi Province (6.7%, 6/90) Chongqing City (2.0%, 1/50), and Guangxi Zhuang Autonomous Region (0%, 0/142), and the difference was significant ($\chi^2 = 16.357$, $df = 4$, $p < .01$). *E. bieneusi* infection is frequently reported in a wide range of hosts in China, including humans (0.2–22.5%), non-human primates (0–70.2%), cattle (1.3–37.6%), sheep and goats (4.4–69.3%), pigs (10.2–83.2%), horses (16.0–30.9%), donkeys (4.9–6.9%), rabbits (0.94–10.2%), dogs (6.0–15.5%), cats (5.6–11.5%), and wild animals (3.0–83.3%), as well as in water (31.5–100%) (Li et al., 2012; Wang et al., 2018). The infection rate (5.1%) in bamboo rats in the present study is within the range reported in previous studies.

A significant difference in infection rate was also detected among different farms ranging from 0 to 23.8% ($\chi^2 = 48.337$, $df = 12$, $p < .01$). The highest infection rate was recorded from farm Shinao, while *E. bieneusi* was not detected in any of the samples from farms in Guangxi Zhuang Autonomous Region (Table 1). The infection rate difference between farms may be due to different feeding model. Farms from Guangdong Province, Hunan Province and Jiangxi Province were free-ranging model with mainly fed with a complex food (e.g. bamboo branch, bamboo stem, bamboo root, maize stalk, sugarcane stalk, carrot) varying with the season. In contrast, farms from Guangxi Zhuang Autonomous Region and Chongqing City were intensive produced and mainly fed with concentrate (e.g. rice, corn) and bamboo branch. More studies should be undertaken to confirm whether the infection rate was related to season, location or management.

Six *E. bieneusi* genotypes, including four known genotypes (D, EbpA, J, and PigEBITS7) and two novel genotypes (designated BR1 and BR2), were identified among samples examined in this study (Table 1). Phylogenetic analysis revealed that the six *E. bieneusi* genotypes identified in the present study formed two genetic clusters, with genotypes D, EbpA, and PigEBITS7 were clustered into Group 1, while genotypes J, BR1, and BR2 were clustered into Group 2 (Fig. 2). Genotype D was the most prevalent genotype across the samples, accounting for 77.2% ($n = 17$) of all positive samples from four farms (Yongzhou, Yongzhou2, Shinao, and Huazhou). The identification of predominant zoonotic genotype D in the present study is in line with findings from studies conducted in non-human primates, foxes, dogs, raccoon dogs, and red-bellied tree squirrels in China (Table 2). More importantly, genotype D is reportedly responsible for most human infections. In a previous study conducted in human immunodeficiency virus (HIV)-infected adult patients in Thailand, 12 samples (36.4%) were *E. bieneusi* positive for genotype D (Leelayoova et al., 2006). A recent report from Guangxi Zhuang Autonomous Region, China, showed that genotype D was the most prevalent genotype (11 cases) among 33 HIV-positive



(caption on next page)

Fig. 2. Phylogenetic tree based on the Bayesian analysis of sequences. Statistically significant posterior probabilities are indicated at branches. Sample names include GenBank accession number followed by host and then genotype designation. The *E. bieneusi* genotypes PtEbIX (AF059610) from dogs were used as outgroup. Known and novel genotypes identified in this study are indicated by squares and triangles filled in black, respectively.

Table 2
Summary of data from studies identifying genotype D as the predominant *E. bieneusi* genotype in different hosts in China.

Hosts	No. of samples	No. of positive (%)	Genotype (no.)	Reference
Humans	285	33 (11.6)	D (11), type IV/K (7), PigEBITS7 (7), EbpC (4), GX25 (1), GX456 (1), GX458 (1)	Liu et al., 2017
NHPs	70	42 (60%)	D (33), J (5), CHG1 (1), CHG14 (1), CM19 (1), CM20 (1)	Yu et al., 2017
NHPs	75	28 (37.3%)	D (15), EbpC (4), O (3), CM12 (2), Type IV (1), BEB6 (1), CM13 (1), CM14 (1)	Zhang et al., 2016
Foxes	191	53 (27.7%)	D (44)	Yang et al., 2015
Blue foxes	110	18 (16.4%)	D (12), EbpC (5), CHN-F1 (1)	Zhao et al., 2015
Dogs	162	17 (10.5%)	D (14), CHN-DC1 (1), WildBoar3 (1)	Yang et al., 2015
Rabbit	426	4 (0.94%)	D (4)	Zhang et al., 2016
Cat	52	3 (5.8%)	D (2), IV (1)	Li et al., 2015
Cat	96	11 (11.5%)	D (3), BEB6 (2), I (1), PtEbIX (1), CC1(1), CC2 (1), CC3 (1), CC4 (1)	Karim et al., 2014
Donkey	48	3 (6.3%)	D (2), NCD-1 (1)	Yue et al., 2017
Squirrel	144	24 (16.7%)	D (18), EbpC (3), SC02 (1), 01CE (1), 02CE (1)	Deng et al., 2016
Goral	6	5 (83.3%)	D (5)	Du et al., 2016

patients infected with *E. bieneusi* (Liu et al., 2017). Additionally, genotype D was the dominant *E. bieneusi* genotype in wastewater from Shanghai, China (Huang et al., 2017). These results suggest that genotype D is an important zoonotic genotype.

Genotypes EbpA, J, PigEBITS7, BR1, and BR2 were only identified in one sample each. Two different genotypes were simultaneously identified in samples from two farms (Yongzhou and Yongzhou2), while the remaining positive samples only contained a single *E. bieneusi* genotype (Table 1). Three previously described *E. bieneusi* genotypes detected in the present study (EbpA, J, and PigEBITS7) also have zoonotic potential. Specifically, genotype EbpA was identified as the most dominant genotype in pigs in China (Zhao et al., 2014), while genotype EbpA *E. bieneusi* strains have also been reported in humans in the Czech Republic (Sak et al., 2011), China (Wang et al., 2013a), and Nigeria (Akinbo et al., 2012). Genotype PigEBITS7 *E. bieneusi* strains were originally identified in pigs in Switzerland (Breitenmoser et al., 1999), and later detected in HIV-positive patients in China and India and in immunocompromised patients in Thailand (Leelayoova et al., 2006; Li et al., 2013; Wang et al., 2013a, 2013b; Liu et al., 2017). Genotype J, previously regarded as ruminant-specific, was identified in three diarrhea samples from children in a study conducted in China (Zhang et al., 2011). These results suggest that bamboo rats may act as reservoirs of potentially zoonotic *E. bieneusi* strains in China.

Nucleotide sequence analysis revealed that novel genotypes BR1 (accession numbers: MK478056) and BR2 (accession numbers: MK478057) showed 99% sequence identity to genotype J, with only a single nucleotide difference at position 220 (G → A) and three nucleotide differences at positions 83 (A → G), 100 (A → G), and 321 (G → A) identified relative to genotype J (GenBank accession no. AF135837), respectively. The host range and potentially zoonotic of genotypes BR1 and BR2 need to be perfected by further epidemiological surveys.

In conclusion, to our knowledge, this is the first report of *E. bieneusi* in bamboo rats in China. Our study revealed the existence of zoonotic *E. bieneusi* genotypes in farmed bamboo rats, with zoonotic genotype D being identified as the predominant genotype among the samples. This suggests the potential for transmission of *E. bieneusi* from bamboo rats to humans, and provides further insight into the host range of *E. bieneusi*.

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