Parasitology Research (2019) 118:2729–2734
https://doi.org/10.1007/s00436-019-06398-4

PROTOZOOLOGY - SHORT COMMUNICATION

Occurrence of *Enterocytozoon bieneusi* in Chinese Tan sheep in the Ningxia Hui Autonomous Region, China

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Received: 25 January 2019 / Accepted: 11 July 2019 / Published online: 18 July 2019

Abstract

Enterocytozoon bieneusi is a zoonotic parasite which is considered to be an opportunistic pathogen of humans and animals. A number of studies have reported *E. bieneusi* infection in various animals. However, no information is available on the occurrence of *E. bieneusi* in Tan sheep, a unique indigenous sheep species in the Ningxia Hui Autonomous Region, China. The objectives of the present study were to examine the prevalence and identify the genotypes of *E. bieneusi* in Tan sheep in China. A total of 1014 fecal specimens of Tan sheep from six farms in the Ningxia Hui Autonomous Region were examined by nested PCR amplification of the internal transcribed spacer (ITS) of nuclear ribosomal DNA. The total prevalence of *E. bieneusi* was 12.2% (124/1014), ranging from 0.5 to 22.2% on six farms. Sequence analysis identified 10 genotypes of *E. bieneusi*, including three known genotypes, BEB6, COS-I, and CHG13, and seven novel genotypes designated as NX1 to NX7, which all belonged to group 2 by phylogenetic analysis. This is the first report describing the prevalence of *E. bieneusi* in Tan sheep, and the new genotypes identified in the current study expand the genotype distribution of *E. bieneusi*. These findings provide baseline data and have implications for the epidemiology and control of *E. bieneusi* infection in Tan sheep.

Keywords *Enterocytozoon bieneusi* · Tan sheep · Genotypes · Prevalence · Ningxia Hui Autonomous Region

Introduction

The microsporidia contain approximately 1300 formally described species in 160 genera (Keeling 2009), of which, 17 species have been documented to infect humans (Elizabeth and Louis 2011; Li et al. 2019). Microsporidiosis is mostly caused by *Enterocytozoon bieneusi* (Santín and Fayer 2011), which has emerged as an intestinal opportunistic pathogen of humans and a wide range of livestock and wild animals in China (Deng et al. 2018; Deng et al. 2016; Ye et al. 2015; Karim et al. 2014a; Karim et al. 2014b; Li et al. 2017; Liu et al. 2015; Yue et al. 2017; Zhang et al. 2018a; Zhao et al. 2016).

In addition, immunocompromised people, especially HIV-positive patients, have a higher risk of being infected by *E. bieneusi* than immunocompetent people (Elizabeth and Louis 2011; Tavalla et al. 2017). Chronic diarrhea is the most serious health problem caused by human-related microsporidia and is associated with weight loss, and *E. bieneusi* can be transmitted fecal-orally (Cama et al. 2007). *E. bieneusi* is also a waterborne pathogen, and spores can be detected in water (Izquierdo et al. 2011; Ma et al. 2016).

Section Editor: Yaoyu Feng

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Tan sheep, an indigenous sheep breed in the Ningxia Hui Autonomous Region, northern China, has been widely bred by the Ningxia people, especially Muslims. Tan sheep are famous for their meat and fur and are only raised in the Ningxia Hui Autonomous Region, which has a temperate continental semiarid climate, with an arid climate in the steppe and desert steppe areas. However, there are many managerial issues with respect to feeding Tan sheep, so they are likely to be affected by parasitosis, especially microsporidiosis. Ningxia Hui Autonomous Region is a place where water is limited. The poor husbandry management in Ningxia may lead to some diseases in Tan sheep. However, no data are available on the prevalence, genotype distribution, and epidemiology of *E. bieneusi* in Tan sheep in Ningxia. Therefore, the objectives of the present study were to investigate the prevalence of *E. bieneusi* in Tan sheep in Ningxia, northern China.

Materials and methods

Ethics statement

The present study was approved by the Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. All fecal samples were collected from the Chinese Tan sheep with the permission of farm owners or managers, and all procedures were performed strictly in accordance with the requirements of the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Fecal specimen collection

A total of 1014 fecal samples were collected from 6 farms in Ningxia (35° 14′ N–39° 23′ N, 104° 17′ E–107° 39′ E) in the northwest of China (Table 1). All the fecal specimens were collected from the rectum directly and then placed in a labeled sterile bag individually. Each animal's age, sex, and other useful information (such as drug usage) were recorded. All fecal specimens were sent to the laboratory and stored in 2.5% potassium dichromate at 4 °C.

DNA extraction

Genomic DNA was extracted by using an E.Z.N.A® Stool DNA kit (Omega Bio-tek Inc., Norcross, GA, USA) according to the manufacturer's instruction. Approximately 200 mg of each fecal specimen was washed at least three times with distilled water and collected by centrifugation at 13000×*g* for 5 min to remove the potassium dichromate; the remaining sediment was used for DNA extraction.

PCR amplification

A nested PCR was used to detect the prevalence and genotypes of *E. bieneusi* by amplifying the internal transcribed spacer (ITS) of nuclear ribosomal DNA. The cycling parameters and primers were used as previously described (Sulaiman et al. 2003). All the secondary PCR products were analyzed by 1.5% agarose gel electrophoresis, and ITS fragments of the expected size (~ 389 bp) were sent to Tsingke Biotechnology Company (Xi'an, China) for bidirectional sequencing.

Sequence analysis

All the sequences obtained in the present study were aligned with reference sequences downloaded from the GenBank database using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) and the Clustal X 1.83 software (http://www.clustal.org/) to determine *E. bieneusi* genotypes. If the sequence had 100% similarity with a previously described sequence, then, the sample was considered to be of a known genotype and thus named as firstly published. The sequences which had some base substitutions or mutations were considered to be novel genotypes. All of the genotypes were named based on the ITS gene region of *E. bieneusi* according to the established nomenclature system (Santín and Fayer 2009).

Phylogenetic analysis

The ITS sequences obtained in the present study and reference sequences available in GenBank were compared using Clustal X 1.83. A neighbor-joining tree was constructed using Mega 7 software based on the evolutionary distances calculated by a Kimura 2-parameter model. The reliability of these trees was assessed using bootstrap analysis with 1000 replicates.

Statistical analysis

The differences in *E. bieneusi* prevalence in different regions, ages, and sexes were examined using the chi-square (χ^2) test, which was implemented using SPSS Statistics 22.0 (IBM Corp., New York, NY, USA). Odds ratios (ORs) and their 95% confidence intervals (95% CIs) were estimated for the strength of the association between prevalence and the conditions tested, and the differences were considered significant when $P \leq 0.05$.

Results

Occurrence of *E. bieneusi* in Tan sheep

Of the 1014 fecal specimens collected from Tan sheep on six farms, 124 (12.2%) samples were positive for *E. bieneusi* by

Table 1 Prevalence and genotype distribution of *Enterocytozoon bieneusi* isolates from Tan sheep farms in different locations

Sampling site		Simple size	No. positive (%)	Genotype (*n*)
Ganyanchi, Haiyuan county	Farm 1	204	1 (0.5)	BEB6 (1)
Guanqiao, Haiyuan county	Farm 2	91	16 (17.6)	BEB6 (15), NX1 (1)
Xiamaguan, Tongxin county	Farm 3	119	1 (0.8)	BEB6 (1)
Yanchi county	Farm 4	83	7 (8.4)	BEB6 (6), NX2 (1)
	Farm 5	102	7 (6.9)	BEB6 (4), NX3 (1), NX4 (1), NX5 (1)
	Farm 6	415	92 (22.2)	BEB6 (84), COS-I (3), CHG13 (3), NX6 (1), NX7 (1)
Total		1014	124 (12.2)	BEB6 (111), COS-I (3), CHG13 (3), NX1 (1), NX2 (1), NX3 (1), NX4 (1), NX5 (1), NX6 (1), NX7 (1)

nested PCR-based sequencing of the ITS locus. The prevalence ranged from 0.5 to 22.2% among the six farms (Table 1). The prevalence on farm 6 (22.2%) was substantially higher than that on farm 1 (0.5%). Moreover, the prevalence of *E. bieneusi* in males (24.2%) was higher than that in females (8.5%), which was statistically significant ($\chi^2 = 41.7$, $P < 0.01$, Table 2).

The highest *E. bieneusi* prevalence of 26.5% (95% CI) was found in lambs (age ≤ 1 year), and the lowest prevalence was 2.0% (95% CI) in fatting sheep (1 ≤ year ≤ 4). The difference was statistically significant between these two group ($\chi^2 = 105.3$, $P < 0.01$).

ITS genotype distribution of *E. bieneusi* in Tan sheep

Ten *E. bieneusi* genotypes were identified among the 124 *E. bieneusi*–positive specimens from Tan sheep (Table 1), including 3 known genotypes (BEB6, COS-I, CHG13) and 7 novel genotypes (named NX1–NX7). Of these genotypes, BEB6 ($n = 111$, 89.5%) was the predominant genotype and was detected on all farms (Table 1), whereas COS-I ($n = 3$, 2.4%) and CHG13 ($n = 3$, 2.4%) were found only on farm 6. Genotype NX1 ($n = 1$, 0.8%) was found on farm 2, while NX2 ($n = 1$, 0.8%) was found on farm 4. Moreover, NX3 ($n = 1$, 0.8%), NX4 ($n = 1$, 0.8%), and NX5 ($n = 1$, 0.8%) were found on the same farm (farm 5), and the two remaining genotypes NX6 ($n = 1$, 0.8%) and NX7 ($n = 1$, 0.8%) were also found on the same farm (farm 6). All the genotypes belonged to zoonotic group 2 by phylogenetic analysis (Fig. 1).

Discussion

The present study is the first molecular investigation of *E. bieneusi* in Tan sheep, an indigenous sheep breed in Ningxia, China. As an important enteric pathogen, *E. bieneusi* has been reported in some ovine animals globally, and the prevalence of *E. bieneusi* in sheep ranges from 4.4 to 69.3% (Al-Herrawy and Gad 2016; Askari et al. 2015; Chen et al. 2018; Li et al. 2014; Lores et al. 2002; Shi et al. 2016; Stensvold et al. 2014; Wu et al. 2018; Yang et al. 2018; Ye et al. 2015; Zhao et al. 2015b). In this study, the prevalence of *E. bieneusi* in Tan sheep was 12.2%, similar to previous studies in sheep in Heilongjiang, China (13.9%) and in Spain (14.2%) (Jiang et al. 2015; Lores et al. 2002). The prevalence of *E. bieneusi* in Tan sheep was lower than that in sheep in Inner Mongolia (69.3%) and Sweden (68.0%) (Stensvold et al. 2014; Ye et al. 2015), but higher than that in sheep in Iran (10.6%) and Northeast China (4.4%) (Askari et al. 2015; Li et al. 2014). The differences in prevalence are likely the result of varying age groups, sample volumes, breeding management, and geographic or ecological elements. Furthermore, there are significant differences in *E. bieneusi* prevalence between lambs (age < 1 year) and fatting sheep (1 ≤ age ≤ 4 years) ($\chi^2 = 105.3$, $P < 0.01$) and between lambs and older sheep (age > 4 years) ($\chi^2 = 35.6$, $P < 0.01$) (Table 2). The prevalence of *E. bieneusi* in lambs (26.5%, 107/405) was similar to that found in a previous study (23.5%, 145/617) (Yang et al. 2018), higher than that reported in lambs in Heilongjiang (16.2%, 24/148) (Jiang et al. 2015), but lower than reported in lambs in Brazil (34.1%, 15/44) (Fiuza et al.

Table 2 Prevalence of *Enterocytozoon bieneusi* by age and sex in Tan sheep

Factor		No. examined	No. positive (%)	*P* value	OR (95% CI)
Sex	Female	774	66 (8.5)	0.001	0.3 (0.2-0.4)
	Male	240	58 (24.2)		Reference
Age	< 1 year	405	107 (26.5)		Reference
	1–4 years	438	9 (2.0)	0.001	17.1 (8.6–34.3)
	> 4 years	171	8 (4.7)	0.001	7.3 (3.5–15.4)
	Total	1014	124 (12.2)		

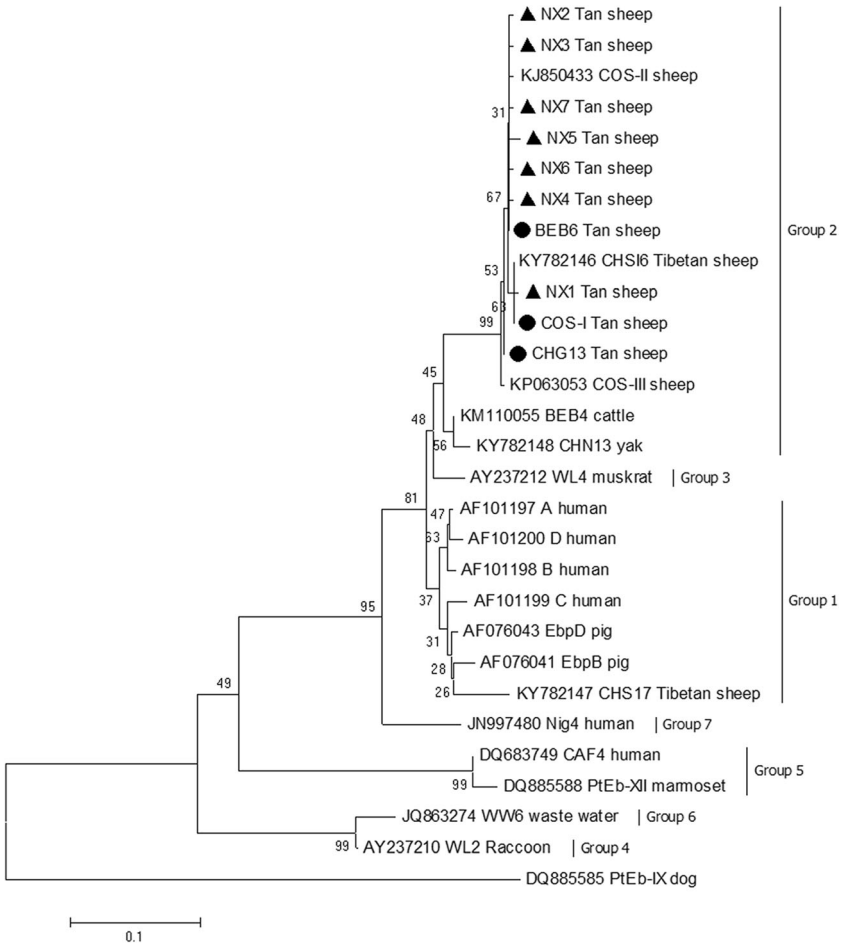

Fig. 1 Phylogenetic relationships of *E. bieneusi* genotypes identified in the present study and previously reported genotypes. Genotypes with black circles are known genotypes, and novel genotypes are marked with black triangles

2016), in Inner Mongolia (77.8%, 126/162) (Ye et al. 2015), and in Henan and Shaanxi (47.2%, 133/382) (Peng et al. 2016). These studies suggested that lambs are more easily infected with *E. bieneusi*, which is probably because the immune systems of lambs are weaker than those of mature sheep. Moreover, the *E. bieneusi* prevalence between female and male Tan sheep is significantly different ($\chi^2 = 41.7$, $P < 0.01$), which was not reported in several earlier reports (Chen et al. 2018; Fiuza et al. 2016; Yang et al. 2018).

Three known *E. bieneusi* genotypes (BEB6, CHG13, COS-I) and seven novel genotypes (NX1 to NX7) were identified in Tan sheep. The predominant genotype was BEB6 (89.5%, 111/124), which was first reported in cattle in the eastern USA (Fayer et al. 2007). It has also been reported in sheep in previous studies (Chen et al. 2018; Jiang et al. 2015; Li et al. 2014; Shi et al. 2016; Stensvold et al. 2014; Wu et al. 2018; Yang et al. 2018; Ye et al. 2015; Zhao et al. 2015b). In addition, the BEB6 genotype has also been reported in other animals, such as deer, alpaca, dairy cattle, duck, goose, NHPs (nonhuman primates), and golden takins (Huang et al. 2017; Karim et al. 2014a; Li et al. 2016a; Li et al. 2016b; Zhao et al. 2016; Zhao et al. 2015a; Zhong et al. 2017), and humans (reported as SH5) (Wang et al. 2013), which implies that Tan sheep could be a potential source of *E. bieneusi* infection for humans and other animals. Moreover, genotypes CHG13 and COS-I have been reported in goats in Henan (Shi et al. 2016) and Heilongjiang (Zhao et al. 2015b). In particular, COS-I has been reported in sheep in Henan, Shanghai, and Heilongjiang (Shi et al. 2016; Yang et al. 2018); in goats in Henan and Yunnan (Peng et al. 2016; Shi et al. 2016); and in Tibetan sheep in Qinghai (Zhang et al. 2018b), which indicated that these genotypes may be transmitted between different species.

Phylogenetic analysis showed that all the *E. bieneusi* genotypes found in this study were clustered in group 2 (Fig. 1), suggesting that Tan sheep may play a vital role in the transmission of distinct genotypes of *E. bieneusi* among sheep in Ningxia. Genotype BEB6 has also been detected in humans (Wang et al. 2013), which indicated that this genotype detected in the present study has zoonotic risk.

In conclusion, the present study revealed a 12.2% *E. bieneusi* prevalence with genetic diversity in Tan sheep. Age and sex are risk factors for *E. bieneusi* infections in Tan sheep. Ten genotypes (BEB6, CHG13, COS-I, NX1 to NX7) were detected through ITS sequencing. These findings have implications for the epidemiology and control of *E. bieneusi* infection in Tan sheep.

Funding information Project support was provided by the National Natural Science Foundation of China (Grant no. 31672549), the Open Fund of the Key Laboratory of Fujian Province Livestock Epidemic Prevention, Control and Biological Technology (2018KF02), the Agricultural Science and Technology Innovation Program (ASTIP) (Grant no. CAAS-ASTIP-2016-LVRI-03), and the Elite Program of the Chinese Academy of Agricultural Sciences.

Data availability The datasets supporting the conclusions in this article are included within the article. All the sequences of ITS genes obtained were deposited in GenBank with the following accession numbers: MK322754–MK322763.

Compliance with ethical standards

The present study was approved by the Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. All fecal samples were collected from the Chinese Tan sheep with the permission of farm owners or managers, and all procedures were performed strictly in accordance with the requirements of the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Conflict of interest The authors declare that they have no competing interests.

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