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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

First report on the molecular prevalence of *Enterocytozoon bieneusi* in horses in Turkey: genotype distributions and zoonotic potential

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Abstract

Horses might play an important role as reservoir hosts in the epidemiology of *Enterocytozoon bieneusi,* which is one of the most important zoonotic microsporidian pathogens, with a wide range of hosts. Nevertheless, limited information is available on the infection rates and genotypes of *E. bieneusi* in horses, and no data are available on the occurrence and molecular characteristics of *E. bieneusi* in horses in Turkey. We determined the prevalence of *E. bieneusi* among horses raised on farms from two provinces of Central Anatolia Region, by amplification of the partial small subunit ribosomal RNA gene using nested PCR. We identified the genotypes of *E. bieneusi* isolates by analyzing the ribosomal internal transcribed spacer (ITS) sequences. The overall prevalence of *E. bieneusi* was 18.7% (56/300), with no significant differences in infection rates among age groups or between genders of horses. Sequence analysis revealed eight genotypes: two known genotypes (ERUSS1, BEB6) and six novel genotypes (named ERUH2 to ERUH7). The genotype ERUSS1 was the most common and was found on all farms, age groups, and genders. Phylogenetic analysis clustered all the identified genotypes in ruminant-specific group 2. Our findings contribute to the molecular epidemiology of *E. bieneusi*.

Keywords *Enterocytozoon bieneusi* · Horse · Prevalence · Molecular characterization · Turkey

Introduction

Microsporidia, a diverse group of obligate unicellular eukaryotes closely related to fungi, cause gastroenteritis in invertebrates and vertebrate (Abe and Kimata 2010; Adl et al. 2019). The phylum Microsporidia is comprised of approximately 1500 species in 200 genera. At least 17 species in nine genera have been recognized as emerging human pathogens (Li et al. 2019; Zhao et al. 2015a). *Enterocytozoon bieneusi* is the most common species detected in healthy and immunocompromised individuals, with more than 90% of reported cases of human microsporidiosis (Matos et al. 2012). *Enterocytozoon bieneusi* has a wide range of hosts, and all animal phyla can be infected with this parasite (Santin and Fayer 2011). Therefore, many kinds of animals can act as potential reservoirs, contaminate the environment, and contribute to the continuous transmission of the disease (Galvan-Diaz et al. 2014).

Sequence analysis of the internal transcribed spacer (ITS) region of the rRNA gene is a standard tool for genotyping *E. bieneusi* and has been widely used for identification of genotypes from several animal and human hosts (Santin and Fayer 2009). More than 500 genotypes of *E. bieneusi* have been identified using ITS genotyping, and the majority have zoonotic potential, especially those in group 1 or group 2, highlighting the public health importance of cross-species transmission of *E. bieneusi* (Gong et al. 2019; Li et al. 2019; Liu et al. 2017; Prasertbun et al. 2019; Wang et al. 2018; Zhang et al. 2018b).

Horses are common hosts of *E. bieneusi* (Li et al. 2020; Zhao et al. 2019). Few genotyping studies have been conducted on *E. bieneusi* in equines, and the genotypes D, horse1, EbpA, and EbpC from zoonotic group 1 have been identified as the common genotypes (Deng et al. 2016; Li et al. 2020; Qi et al. 2016; Santin et al. 2010; Wagnerova et al. 2016). These

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findings draw attention to the importance of equines in zoonotic transmission of *E. bieneusi*. The genotypes of group 2, with a wide host range (e.g., J and BEB6), have also been reported from horses (Li et al. 2020; Qi et al. 2016).

Data on the distribution and genotypes of *E. bieneusi* in animals and humans in Turkey is limited. The zoonotic group 1 genotypes D, IV in cats (Pekmezci et al. 2019), and ERUNT1 in chickens (Ercan and Duzlu 2019) have been reported. The "ruminant-specific" group 2 genotypes ERUSS1-4, TREB1-6, BEB6, and N in ruminants (Bilgin et al. 2020; Yildirim et al. 2019) and chickens (ERUSS1) (Ercan and Duzlu 2019) have also been identified. Consequently, the genotypes of *E. bieneusi* in animal and human hosts in Turkey are not fully understood, and no data are available on the distribution and genotypes of *E. bieneusi* in horses. In Turkey, horses are used frequently for work and social activities. They commonly live in close association with humans, and possible shedding of *E. bieneusi* spores with their feces might be a potential risk for public health. We aimed to determine the prevalence and genotypes of *E. bieneusi* in horses on farms in Central Anatolia Region of Turkey, and reveal the potential risk of horses in the zoonotic transmission dynamics of *E. bieneusi*.

Materials and methods

Study area and specimens

From March to June 2018, 300 Arabian horses from five farms in Kayseri and Nevsehir provinces, Central Anatolia Region in Turkey (Fig. 1), were included in our survey. Sampling was

Fig. 1 Sampling locations for *Enterocytozoon bieneusi* in two provinces of Central Anatolia Region, Turkey. Image credit: Jimfbleak and TUBS, Wikimedia Commons

performed only after the consent of the owner, who was informed about the study and procedures in advance. Fecal samples from each horse were immediately collected as fresh droppings on the ground, using a sterile disposal latex glove, and placed in individual plastic containers. Age and gender of the horses were recorded. No clinical signs were obvious in the animals at the time of sampling. The specimens were transferred to the laboratory and stored at 4 °C before being used in DNA extraction.

Genomic DNA extraction and polymerase chain reactions

Genomic DNA (gDNA) was extracted from 200 mg of each fecal specimen, using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. The eluted nucleic acids were stored at − 20 °C until polymerase chain reaction (PCR) analyses. Concentrations of the gDNA isolates were measured by Qubit Fluorometric Quantitation (Thermo Fisher Scientific, USA) to optimize the amount used in the PCR mastermix.

The nested PCR protocol described by Buckholt et al. (2002), with the outer EBITS3/EBITS4 and inner EBITS1/EBITS2.4 primers, was used for amplifying a 390-bp fragment of the ITS rRNA gene of *E. bieneusi*. A gDNA of 10 to 30 ng for each specimen was used as a template in the commercial ready-to-use PCR Master Mix (Maxima Hot Start Green PCR Master Mix, Thermo Scientific, USA). The reactions were carried out in a C1000 Touch™ Thermal Cycler (Bio-Rad, USA), with previously described cycling parameters (Buckholt et al. 2002). The secondary PCR products were examined by 1.5% agarose gel electrophoresis and visualized using Fusion FX Gel Documentation System (Vilber Lourmat, France).

Genotyping of E. bieneusi

The ITS amplicons were purified from agarose gel, using a commercial kit (GeneJET Gel Extraction Kit, Thermo Scientific, USA). The amplified fragments were bi-directionally sequenced using inner PCR primers (Macrogen, Netherlands). Paired nucleotide sequences were assembled with Geneious Prime 2020.1.1 software (http://www.geneious.com). The finalized consensus sequences were aligned with reference ITS sequences downloaded from GenBank, using MUSCLE (Edgar 2004) implemented in Geneious prime.

The neighbor-joining method (NJ) implemented in Mega X (http://www.megasoftware.net/) was used to assess the phylogenetic relationships of the *E. bieneusi* genotypes, based on genetic distance by the Kimura-2-parameter model. A bootstrap analysis was done with 1000 replicates in NJ analyses. Representative ITS sequences characterized in the study were deposited in GenBank under accession numbers MT193676-MT193683.

Statistical analysis

Infection rates for *E. bieneusi* were compared among age groups and between genders, using the chi-square test and odd ratio (OR) calculation implemented in SPSS 20.0 (IBM Inc., Chicago, IL, USA). p values < 0.05 were considered significant.

Results

Prevalence of E. bieneusi

The DNA of *E. bieneusi* was identified in 56 out of 300 gDNA isolates of horse fecal specimens by ITS-nested PCR analysis, resulting in an overall prevalence of 18.7% (Table 1). The infection rates of *E. bieneusi* on the farms in Kayseri and Nevsehir provinces were 16.9% (31/183) and 21.4% (25/117), respectively (Table 1). The prevalence in horses 4–6 (20.0%) and 7–10 years of age (19.1%) was higher than that in horses < 1–3 years of age (16.3%) (Table 2). The OR

Table 1 Prevalence and distribution of *Enterocytozoon bieneusi* genotypes in horses in Central Anatolia Region of Turkey by location and farm

Location	Farm No.	Total no. of specimens	*Enterocytozoon bieneusi*	
			No. positive (%)	Genotypes (no. of specimens)
Kayseri	I	65	12 (18.5)	ERUSS1 (5), BEB6 (3), ERUH2 (2), ERUH3 (1), ERUH4 (1)
	II	62	10 (16.1)	ERUSS1 (6), BEB6 (3), ERUH2 (1)
	III	56	9 (16.1)	ERUSS1 (4), BEB6 (1), ERUH3 (3), ERUH4 (1)
Nevsehir	IV	60	13 (21.7)	ERUSS1 (5), ERUH2 (2), ERUH3 (1), ERUH4 (2), ERUH5 (1), ERUH6 (1), ERUH7 (1)
	V	57	12 (21.1)	ERUSS1 (4), BEB6 (1), ERUH2 (1), ERUH5 (3), ERUH6 (2), ERUH7 (1)
Total		300	56 (18.7)	ERUSS1 (24), BEB6 (8), ERUH2 (6), ERUH3 (5), ERUH4 (4), ERUH5 (4), ERUH6 (3), ERUH7 (2)

Table 2 Prevalence and distribution of *Enterocytozoon bieneusi* genotypes according to age and sex of horses

Factor		Total no. of specimens	*Enterocytozoon bieneusi*			
			No. positive (%)	χ^2	p	Genotypes (no. of specimens)
Age (years)	1–3	92	15 (16.3)	0.511	0.774	ERUSS1 (7), BEB6 (2), ERUH2 (1), ERUH3 (1), ERUH4 (1), ERUH5 (2), ERUH7 (1)
	4–6	140	28 (20.0)			ERUSS1 (12), BEB6 (5), ERUH2 (4), ERUH3 (3), ERUH4 (1), ERUH6 (2), ERUH7 (1)
	7–10	68	13 (19.1)			ERUSS1 (5), BEB6 (1), ERUH2 (1), ERUH3 (1), ERUH4 (2), ERUH5 (2), ERUH6 (1)
Gender	Female	185	36 (19.5)	0.655	0.761	ERUSS1 (14), BEB6 (6), ERUH2 (4), ERUH3 (3), ERUH4 (3), ERUH5 (2), ERUH6 (2), ERUH7 (2)
	Male	115	20 (17.4)			ERUSS1 (10), BEB6 (2), ERUH2 (2), ERUH3 (2), ERUH4 (1), ERUH5 (2), ERUH6 (1)

between 4–6 and < 1–3, 7–10, and < 1–3, and 4–6 and 7–10 years of age were 0.8 (95%CI = 0.4–1.6), 0.8 (95%CI = 0.4–1.9), and 1.1 (95%CI = 0.5–2.2), respectively. The differences among the age groups were not statistically significant ($p > 0.05$) (Table 2). Infection rates were similar in female and male horses, and the prevalence of *E. bieneusi* was not significantly associated with the gender of the horses (OR = 1.2; 95%CI = 0.6–2.1; $p > 0.05$) (Table 2).

Distribution of E. bieneusi genotypes

The secondary PCR products from all *E. bieneusi* positive specimens were sequenced successfully, and the reference genotyping ITS gene region (243 bp) was obtained. Through sequence analysis of 56 *E. bieneusi* isolates, eight ITS genotypes were identified, with seven polymorphic sites (Table 3) including two previously reported (ERUSS1 and BEB6) and six novel genotypes (named as ERUH2–ERUH7). The distributions of the detected genotypes across locations, ages, and genders are in Tables 1 and 2. Genotype ERUSS1 ($n = 24$) was the most common genotype in the horses, and was detected from all locations, age groups, and genders. In contrast, the novel genotype ERUH7 was the least frequent ($n = 2$), and was seen only in horses from farms of Nevsehir Province, age groups 1–3 and 4–6 years, and females.

Table 3 Variation in the ITS sequences of *Enterocytozoon bieneusi* from horses in Central Anatolia Region

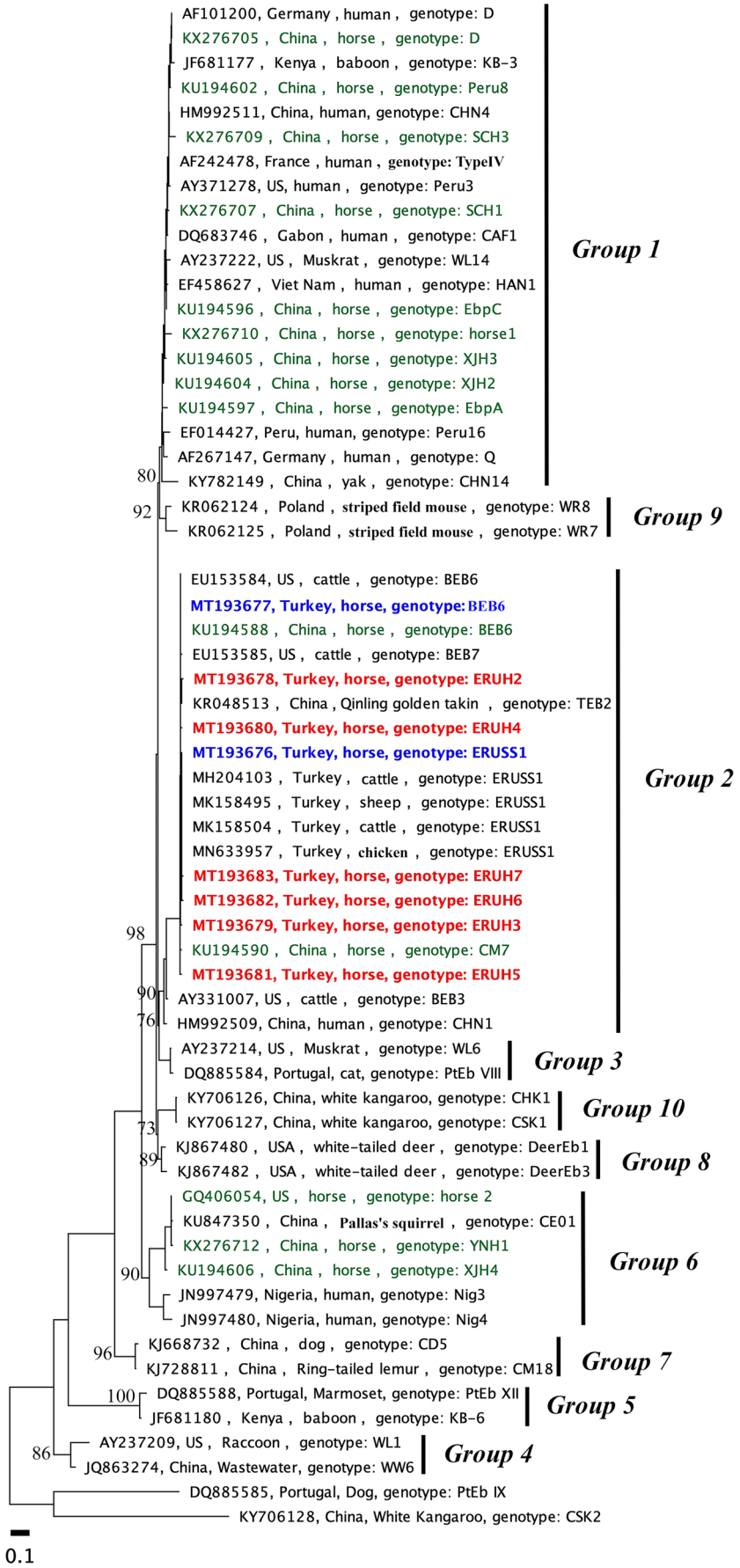
Genotypes	GenBank accession no.	Nucleotide at position						
		8	58	67	113	120	137	153
Known								
ERUSS1	MT193676	T	T	G	T	G	T	A
BEB6	MT193677	T	T	G	T	G	T	C
Novel								
ERUH2	MT193678	*C*	T	G	T	*A*	T	C
ERUH3	MT193679	T	T	*A*	T	G	T	C
ERUH4	MT193680	T	T	G	T	G	*C*	C
ERUH5	MT193681	T	*C*	G	T	G	T	C
ERUH6	MT193682	T	T	G	*C*	G	T	A
ERUH7	MT193683	*C*	T	G	T	G	T	A

Novel SNPs are shown with italic font

Phylogenetic analyses of E. bieneusi genotypes

The NJ tree of the detected haplotypes and the known genotypes from GenBank is presented in Fig. 2. The branches of the groups were supported by bootstrap values exceeding 70%. All genotypes identified in horses in the study were clustered in the so-called ruminant-specific group 2 (Fig. 2). The known genotypes BEB6 and ERUSS1 and all novel genotypes were genetically similar and clustered together in the NJ tree. The detected genotypes were also similar to genotypes BEB7, CM7, and TEB2 reported from cattle, horses, and Qinling golden takins,

Fig. 2 Phylogenetic relationships of identified *Enterocytozoon bieneusi* genotypes in horses (novel and known genotypes in red and blue, respectively) and other genotypes (genotypes from horses in green) deposited in GenBank, based on NJ analysis of ITS sequences. Numbers at the nodes represent the bootstrap values with 1000 replicates. The sequences are given as GenBank accession number, country, host, and genotype name. The *E. bieneusi* genotypes CSK2 from white kangaroo and PtEb IX from dog were used as outgroups


AF101200, Germany , human , genotype: D
KX276705 , China , horse , genotype: D
JF681177 , Kenya , baboon , genotype: KB-3
KU194602 , China , horse , genotype: Peru8
HM992511, China, human, genotype: CHN4
KX276709 , China , horse , genotype: SCH3
AF242478, France , human , genotype: TypeIV
AY371278, US, human , genotype: Peru3
KX276707 , China , horse , genotype: SCH1
DQ683746 , Gabon , human , genotype: CAF1
AY237222, US , Muskrat , genotype: WL14
EF458627 , Viet Nam , human , genotype: HAN1
KU194596 , China , horse , genotype: EbpC
KX276710 , China , horse , genotype: horse1
KU194605 , China , horse , genotype: XJH3
KU194604 , China , horse , genotype: XJH2
KU194597 , China , horse , genotype: EbpA
EF014427, Peru, human, genotype: Peru16
AF267147, Germany , human , genotype: Q
KY782149 , China , yak , genotype: CHN14
Group 1
80
KR062124 , Poland , striped field mouse , genotype: WR8
KR062125 , Poland , striped field mouse , genotype: WR7
92
Group 9
EU153584, US , cattle , genotype: BEB6
MT193677, Turkey, horse, genotype: BEB6
KU194588 , China , horse , genotype: BEB6
EU153585, US , cattle , genotype: BEB7
MT193678, Turkey, horse, genotype: ERUH2
KR048513 , China , Qinling golden takin , genotype: TEB2
MT193680, Turkey, horse, genotype: ERUH4
MT193676, Turkey, horse, genotype: ERUSS1
MH204103 , Turkey , cattle , genotype: ERUSS1
MK158495 , Turkey , sheep , genotype: ERUSS1
MK158504 , Turkey , cattle , genotype: ERUSS1
MN633957 , Turkey , chicken , genotype: ERUSS1
MT193683, Turkey, horse, genotype: ERUH7
MT193682, Turkey, horse, genotype: ERUH6
MT193679, Turkey, horse, genotype: ERUH3
KU194590 , China , horse , genotype: CM7
MT193681, Turkey, horse, genotype: ERUH5
Group 2
98
90
AY331007, US , cattle , genotype: BEB3
76
HM992509, China, human , genotype: CHN1
AY237214, US , Muskrat , genotype: WL6
DQ885584, Portugal, cat, genotype: PtEb VIII
Group 3
KY706126, China, white kangaroo, genotype: CHK1
KY706127, China, white kangaroo, genotype: CSK1
Group 10
73
KJ867480 , USA , white-tailed deer , genotype: DeerEb1
89
KJ867482 , USA , white-tailed deer , genotype: DeerEb3
Group 8
GQ406054, US , horse , genotype: horse 2
KU847350 , China , Pallas's squirrel , genotype: CE01
KX276712 , China , horse , genotype: YNH1
KU194606 , China , horse , genotype: XJH4
90
JN997479, Nigeria, human, genotype: Nig3
JN997480, Nigeria, human, genotype: Nig4
Group 6
96
KJ668732 , China , dog , genotype: CD5
KJ728811 , China , Ring-tailed lemur , genotype: CM18
Group 7
100
DQ885588, Portugal, Marmoset, genotype: PtEb XII
JF681180 , Kenya , baboon , genotype: KB-6
Group 5
86
AY237209, US , Raccoon , genotype: WL1
JQ863274, China, Wastewater, genotype: WW6
Group 4
DQ885585, Portugal, Dog, genotype: PtEb IX
KY706128, China, White Kangaroo, genotype: CSK2
0.1

respectively, with identities of 98.8 to 99.6% (Fayer et al. 2007; Zhao et al. 2015; Qi et al. 2016).

Discussion

Our study revealed a high prevalence of *E. bieneusi* in horses in Central Anatolia Region of Turkey. The infection rates on the farms of two neighboring provinces were similar. The overall prevalence (18.7%) of *E. bieneusi* in horses in our study is similar to the 17.3% infection rate in the Czech Republic (Wagnerova et al. 2012) and 22.5% in southwestern China (Deng et al. 2016). The mean prevalence was higher than in northern China (7.4%) (Li et al. 2020), Colombia (10.8%) (Santin et al. 2010), and Algeria (6.8%) (Laatamna et al. 2015), and lower than in Xinjiang, China (30.9%) (Qi et al. 2016). The differences among infection rates of *E. bieneusi* in horses in our study and the rates in other countries were statistically significant. Variations, such as in the study design, sample sizes, horse ages, detection methods, and animal management practices, might affect the infection rates of *E. bieneusi* in different geographical areas (Li et al. 2020).

Although horses > 4 years of age had higher *E. bieneusi* infection rates than did younger animals (1–3 years of age), this difference was not statistically significant. Thus, we found no age-dependent distribution of *E. bieneusi* in our study. This finding is consistent with previous reports for horses (Deng et al. 2016; Laatamna et al. 2015; Qi et al. 2016; Wagnerova et al. 2012). On the other hand, Li et al. (2020) reported significantly higher infection rates in horses > 1 year of age than in those < 1 year of age. We also found no significant difference in infection rates according to gender of the horses, which is consistent with several studies (Deng et al. 2016; Laatamna et al. 2015; Qi et al. 2016).

All the genotypes identified in our study are in group 2 of *E. bieneusi,* referred to as "ruminant-specific" group (Fayer et al. 2003; Li et al. 2016; Santin et al. 2012; Wang et al. 2019; Wang et al. 2016; Zhang et al. 2018a; Zhao et al. 2015b), suggesting the possibility of zoonotic transmission. The most common genotype, ERUSS1, in horses in our study might be an example of host specificity reduction. This genotype was previously found to be the common genotype in cattle (2.5–16.0%), sheep (9.5%), and chickens (0.3%) from locations of Central Anatolia Region (Bilgin et al. 2020; Ercan and Duzlu 2019; Yildirim et al. 2019). Further studies with human sampling are required for exploring the zoonotic potential of this genotype in the Central Anatolia Region. The BEB6 genotype, previously considered to be adapted to ruminants, was also the second most prevalent genotype in horses in our study. This genotype was previously identified in cattle and sheep milk in the same region in Turkey, with 1.5% and 5.5% prevalence, respectively (Yildirim et al. 2019). BEB6 was also reported in horses in China (Qi et al. 2016). Several studies indicated the presence and high prevalence of BEB6 in non-ruminant hosts, such as cats, chinchillas, rhesus macaques, and humans (Jiang et al. 2015; Karim et al. 2015; Karim et al. 2014; Qi et al. 2015; Wang et al. 2013). We identified six novel genotypes in group 2, referred to as ERUH2 to ERUH7, which are closely related to each other, and also to ERUSS1 and BEB6 genotypes. These novel genotypes have one to two nucleotide substitutions in their ITS sequences compared with each other and to ERUSS1 and BEB6 genotypes. The host specificity of these new genotypes needs further investigation by large-scale sampling from several hosts, along with horses, in the region. Several studies indicated the dominance of horse1 genotype of zoonotic group 1 in horses in China, USA, Czech Republic, Colombia, and Algeria (Laatamna et al. 2015; Qi et al. 2016; Santin et al. 2010; Wagnerova et al. 2012; Wagnerova et al. 2016). However, this genotype was not found in our study.

Conclusions

Our study provides the first data on the prevalence of *E. bieneusi* infections in horses raised in Turkey, and presents the phylogenetic relationships of the responsible genotypes. The findings indicate high prevalence of two known (ERUSS1 and BEB6) genotypes and the presence of six novel (ERUH2-ERUH7) genotypes in group 2 of *E. bieneusi* in horses. As host range expansion in some group 2 genotypes is well known, the zoonotic potential of the detected genotypes should not be ignored. Further studies with increased sample sizes from a wider variety of animal species, as well as from humans, are needed to better understand the molecular epidemiology and zoonotic potential of *E. bieneusi* in Turkey.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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