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Molecular detection and genotyping of microsporidia species in chickens in Turkey

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ABSTRACT

Microsporidia are obligate intracellular pathogens that infect various hosts including invertebrates and vertebrates. Despite the importance, knowledge on the prevalence and molecular characteristics of microsporidia in chickens is limited, and no data are available for Turkey. A total of 300 fecal samples from chickens in the Central Anatolia Region of Turkey were analyzed by using a nested polymerase chain reaction assay targeting the rRNA internal transcribed spacer (ITS) region for the common microsporidia species. Corresponding PCR amplicons from the positive samples were sequenced for genotyping. *Enterocytozoon bieneusi* was identified in 22 (7.3 %) samples, whereas *Encephalitozoon* spp. was not detected. The prevalence of *E. bieneusi* was 63.6 % in Kayseri and 36.4 % in Nevsehir provinces, and 8.8 % in soft fecal samples and 9.7 % in diarrhoeic samples. No infections were found in Kirsehir Province. Significant differences were found for the distribution of *E. bieneusi* among provinces and fecal conditions. Infections were found only in free-range chickens. As a result of ITS region sequencing, two genotypes were characterized. The novel genotype ERUNT1 (n = 21), belonging to zoonotic group 1, was the most common genotype throughout the study area. The other known genotype, ERUSS1 (n = 1), had a restricted distribution and was previously detected in cattle and sheep in the same region. Our study provides the first data on microsporidia species from chickens in Turkey. None of these genotypes have been reported in humans; thus, the risk potential for public health is limited but needs further investigation.

1. Introduction

Microsporidia are spore-forming, intracellular, obligate fungi infecting humans and animals [1–3]. They represent approximately 200 genera and more than 1300 species [4]. At least 14 species are pathogenic for humans, including the most common species *Enterocytozoon bieneusi, Encephalitozoon cuniculi, En. intestinalis*, and *En. hellem*, which all have zoonotic potential [5–7]. *Enterocytozoon bieneusi* is the most common species among the four zoonotic microsporidia and is widely detected in HIV-positive or immunosuppressed individuals and in asymptomatic immunocompetent individuals such as the elderly, children, contact lens wearers, and travelers [8].

To date, more than 500 genotypes, including 142 from humans and 49 from both humans and animals have been identified by internal transcribed spacer (ITS) region genotyping [4,9–14], and these genotypes have been clustered in 11 phylogenetic groups (groups 1–11) [11]. Among the recognized genotypes to date, 132 of the 142 human pathogenic and 47 of the 49 zoonotic genotypes belong to group 1 or group 2, emphasizing the public health importance and the nature of cross-species transmission [4,9–14].

Although *E. bieneusi* might cause clinical manifestation characterized by chronic to acute diarrhea and malabsorption or respiratory tract inflammation [15,16], little is known about the pathogenesis and clinical manifestation of this microsporidium on avian hosts. In addition, limited surveys of birds have mainly focused on genotype characterization and distribution and possible risks for zoonotic transmission to humans [17–20]. The first case of *E. bieneusi* in birds (also first in a non-mammalian host), was diagnosed in two chickens in Germany [21]. Since then, limited surveys have been conducted in chickens, and the occurrence, prevalence, and genotypes of *E. bieneusi* have been reported in countries such as Peru [22], China [23], and Brazil [24]. Although a limited number of genotypes have been identified, the recognized zoonotic genotypes in group 1 of *E. bieneusi* indicate that birds could be a source for human infections [19].

To date, little is known about microsporidian infections in humans and animals in Turkey. In the limited surveys of animal hosts, zoonotic group 1 of *E. bieneusi*, including genotypes D and IV in the domestic cat, has been reported [7]. The *E. bieneusi* group 2, the so called “ruminant-

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specific" group genotypes ERUSS1-4, TREB1-6, BEB6 and N, have been identified in cattle and sheep [25,26]. Genotype III of *En. cuniculi* and *En. intestinalis* have been reported from dogs in Turkey [27]. A few serological and molecular surveys without genotype characterization indicate the prevalence of *En. intestinalis* is in the range of 8.5%–26.0% in humans [28,29].

There have been limited reports on the microsporidia infections in poultry and their genetic characterization. The distribution and genotypes of *E. bieneusi* in both animal and human hosts in Turkey are not fully understood. To fill this knowledge gap, we investigated the presence of microsporidia in chickens for the first time in Turkey, using molecular tools. Sequence analysis of ITS was used to reveal the genotypes of *E. bieneusi.* We also assessed the potential role of chickens in the transmission dynamics of microsporidia.

2. Materials and methods

2.1. Sample collection

A total of 300 fecal samples from chickens, including 244 free-range, 45 layer, and 11 broiler chickens, were randomly collected in Kayseri, Kirsehir, and Nevsehir provinces in the Central Anatolia Region between May 2018 and August 2019 (Fig. 1). Due to mixed breeding, the age and breed distribution of the animals could not be determined. Fecal samples were collected using sterile latex gloves, placed in sterile individual containers, and labeled. All samples were stored at -20 ˚C until DNA extraction.

2.2. DNA extraction and PCR amplification

Genomic DNA (gDNA) was isolated from fecal samples, using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The final nucleic acids were precipitated in 50 µL elution buffer and stored at -20 ˚C. To determine the gDNA quality and concentrations of the isolates, randomly selected isolates were assessed by Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Waltham, USA). Nested PCR analysis were performed using EBITS (first-round: EBITS3, 5′-GGTCATAGGGATGAAGAG-3′ and EBITS4, 5′-TTCGAGTTCTTTCGCGCTC-3′; second-round: EBITS1, 5′-GCTCTGAATATCTATGGCT-3′ and EBITS2.4, 5′ATCGCCGACGGATC CAAGTG-3′) and MSP primers (first-round: MSP-1, 5′-TGAATGKGTC-CCTGT-3′ and MSP-2A, 5′-TCACTCGCCGCTACT-3′; second-round: MSP-3, 5′-GGAATTCACACCGCCCGTCRYTAT-3′ and MSP-4A, 5′−CCAAGCTTATGCTTAAGTYMA ARGGGT-3′), targeting the internal transcribed spacer (ITS) of *E. bieneusi* and *Encephalitozoon* spp., respectively [30,31]. The reaction mixes contained 12.5 µL of ready to use 2x Maxima Hot Start Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 1.25 µL from each primer (10 µM), 8 µL of ddH2O, and 1 µL of template DNA in a final reaction volume of 25 µL. In the second round PCRs, 1 µL of first PCR products was used as template DNA. The PCR amplifications were performed in a C1000 Touch Thermal Cycler (BioRad, CA, USA). Cycling conditions for EBITS primers were as follows: 95 ˚C for 4 min, followed by 35 cycles, each consisting of 95 ˚C for 30 s, 47 ˚C for 30 s, 72 ˚C for 1 min, and a final extension at 72 ˚C for 10 min. Nested amplification cycling conditions were the same as for the primary amplification, except the annealing step was at 46 ˚C for 30 s and 30 cycles of amplification. For MSP primers, the same conditions were used except the annealing step was at 40 ˚C, and the nested cycling annealing step was at 57 ˚C and 34 cycles of amplification. The gDNAs of *Encephalitozoon* species (*En. intestinalis* and *En. cuniculi* from dogs and *En. hellem* from budgerigars (1A genotype)) and *E. bieneusi* (Cattle, ERUSS1 genotype) that were previously identified from several animal hosts and stored in our laboratory were used as positive controls, and nuclease-free water was used as negative control in each analysis. To avoid potential contamination, DNA extractions and all PCR setups were carried out in separate rooms and laminar flow cabinets. The PCR products were separated by 1.5 % agarose gel electrophoresis and visualized using Fusion FX Gel Documentation System (Vilber Lourmat, France).

2.3. DNA sequencing and phylogenetic analysis

All secondary PCR products were purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced in both directions with the inner primers (Macrogen, Amsterdam, The Netherlands). The forward and reverse sequences were paired, assembled, and then aligned with reference sequences downloaded from GenBank, using MUSCLE plugin available with Geneious 11.1.5 software [32]. The final consensus sequences were aligned and compared, and sequence similarity was determined. The best DNA model was determined according to the Akaike Information Criterion (AIC) algorithm by using jModeltest v.0.1.1 [33]. The phylogenetic tree was built using Maximum Likelihood (ML) inference based on the GTR + G+I model. Branch support was assessed with 1000 bootstrap replicates. Representative ITS sequences were deposited in GenBank under accession numbers MN633956 and MN633957.

2.4. Statistical analysis

Pearson's chi-square test implemented in SPSS 20.0 (IBM Inc., Chicago, IL, USA) was performed to compare prevalence of infection among fecal conditions (formed, soft, and diarrhea) and sampling provinces. Differences were considered significant when $P < 0.05$.

3. Results

A total of 22 (7.3 %) fecal samples were positive for *E. bieneusi,* and no infection with *Encephalitozoon* spp. was detected. The prevalence of *E. bieneusi* infections in chickens in Kayseri and Nevsehir provinces, respectively, was 63.6 % and 36.4 %. No infections were found in Kirsehir province. Prevalence differed significantly with regard to sampling location ($P < 0.05$) (Table 1). The prevalence of *E. bieneusi* was 8.8 % in soft fecal samples and 9.7 % in diarrhoeic samples. No positivity was found in formed fecal samples. These differences were significant ($P < 0.05$) (Table 1). Infections were found only in free-range chickens (Table 1).

Sequence analysis of the complete ITS region (243 bp) of 22 *E. bieneusi* isolates indicated that 21 were wholly identical to each other, representing the presence of a novel genotype, here named ERUNT1. The one other isolate was identified as the known genotype ERUSS1.

The ML tree, including the detected genotypes and the known genotypes from GenBank, is presented in Fig. 2. The branches of the groups were supported by bootstrap values exceeding 64.0 %. Phylogenetic analysis clustered the genotypes ERUNT1 and ERUSS1 into group 1 and group 2 of *E. bieneusi*, respectively (Fig. 2). The novel genotype ERUNT1 exhibited the highest identity (99.2 %) to genotypes identified from USA (BEB5, AY331009), Peru (Type: IV, KC860884), China (CHN4, HM992511), and Nigeria (Type: IV, JX683799) in GenBank.

4. Discussion

Little has been known of the presence of zoonotic microsporidia in chickens. Thus, our study contributes to the knowledge of microsporidia in chickens. The overall prevalence of *E. bieneusi* in chickens in our study is 7.3 %. Previous studies in Germany, Peru, and China examined low numbers of animals and found *E. bieneusi* infections in 2/6, 1/26, and 3/14 chickens, respectively [21–23]. In studies with larger samples, prevalence of 15.9 % and 1.94 % of *E. bieneusi* was determined in 151 and 206 chickens from Brazil [24] and China [34], respectively. A few experimental studies demonstrate the host competence of chickens for *En. hellem* and development of an immune response

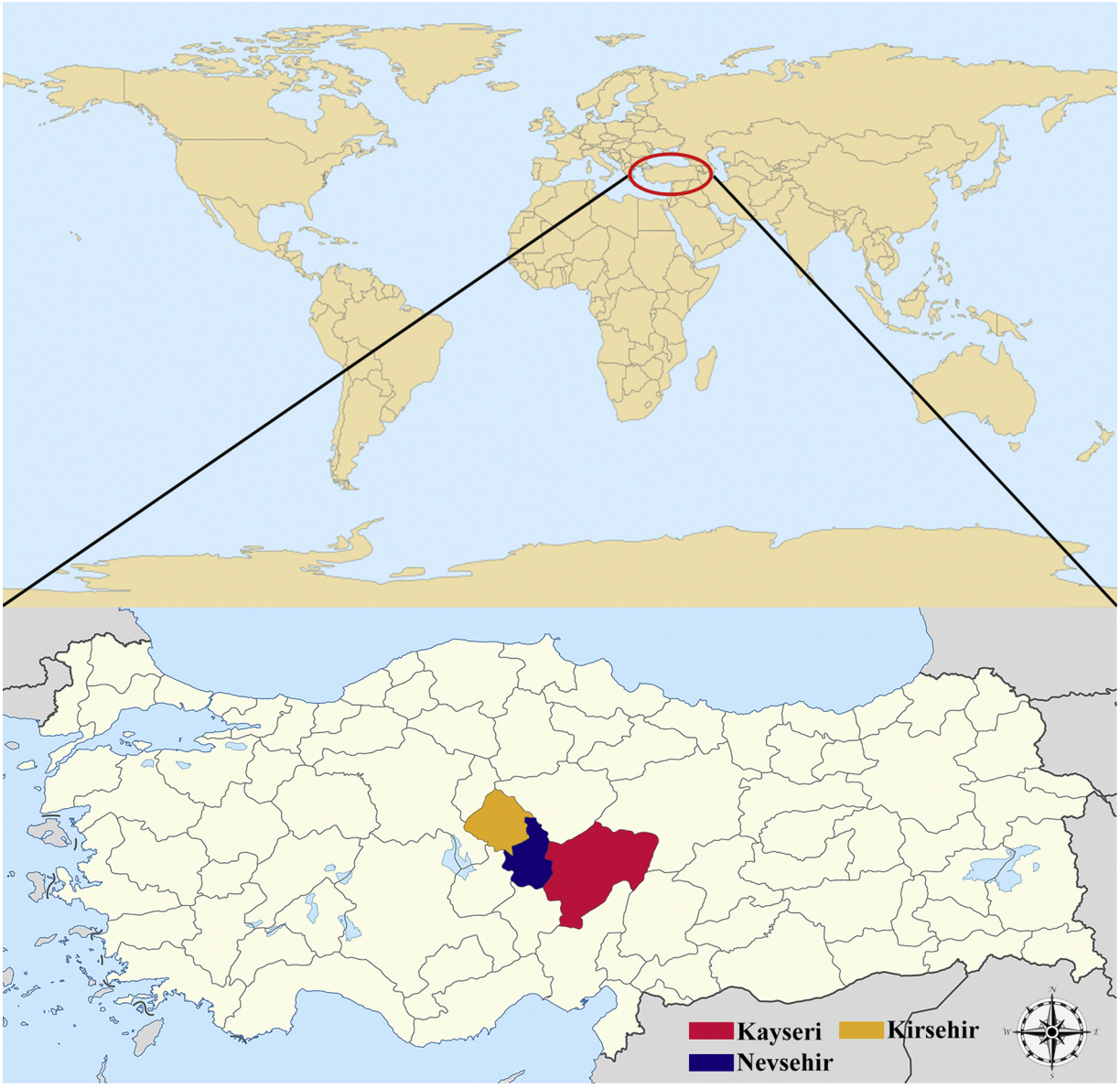

Fig. 1. Sampling locations for microsporidia in chickens in the three provinces of Central Anatolia Region, Turkey.

Table 1
Prevalence and distribution of microsporidia species and genotypes of *E. bieneusi* in chickens by province, living conditions, and fecal condition in Turkey.

		Enterocytozoon bieneusi			*Encephalitozoon* spp.	
Factor	Number examined	No. positive	Prevalence (%)	Genotypes (no. of isolates)	No. positive	*P* value
Province						< 0.05
Kayseri	105	14	13.3	ERUNT1 (13), ERUSS 1 (1)	–	
Kirsehir	92	–	0.0	–	–	
Nevsehir	103	8	7.8	ERUNT1 (8)	–	
Living condition						NS
Free-range	244	22	7.3	ERUNT1 (21), ERUSS 1 (1)	–	
Layer	45	–	–	–	–	
Broiler	11	–	–	–	–	
Fecal condition						< 0.05
Formed	61	–	–	–	–	
Soft	147	13	8.8	ERUNT1 (12), ERUSS 1 (1)	–	
Diarrhea	92	9	9.7	ERUNT1 (9)	–	
Total	300	22	7.3	ERUNT1 (21), ERUSS 1 (1)		

NS: Not significant.

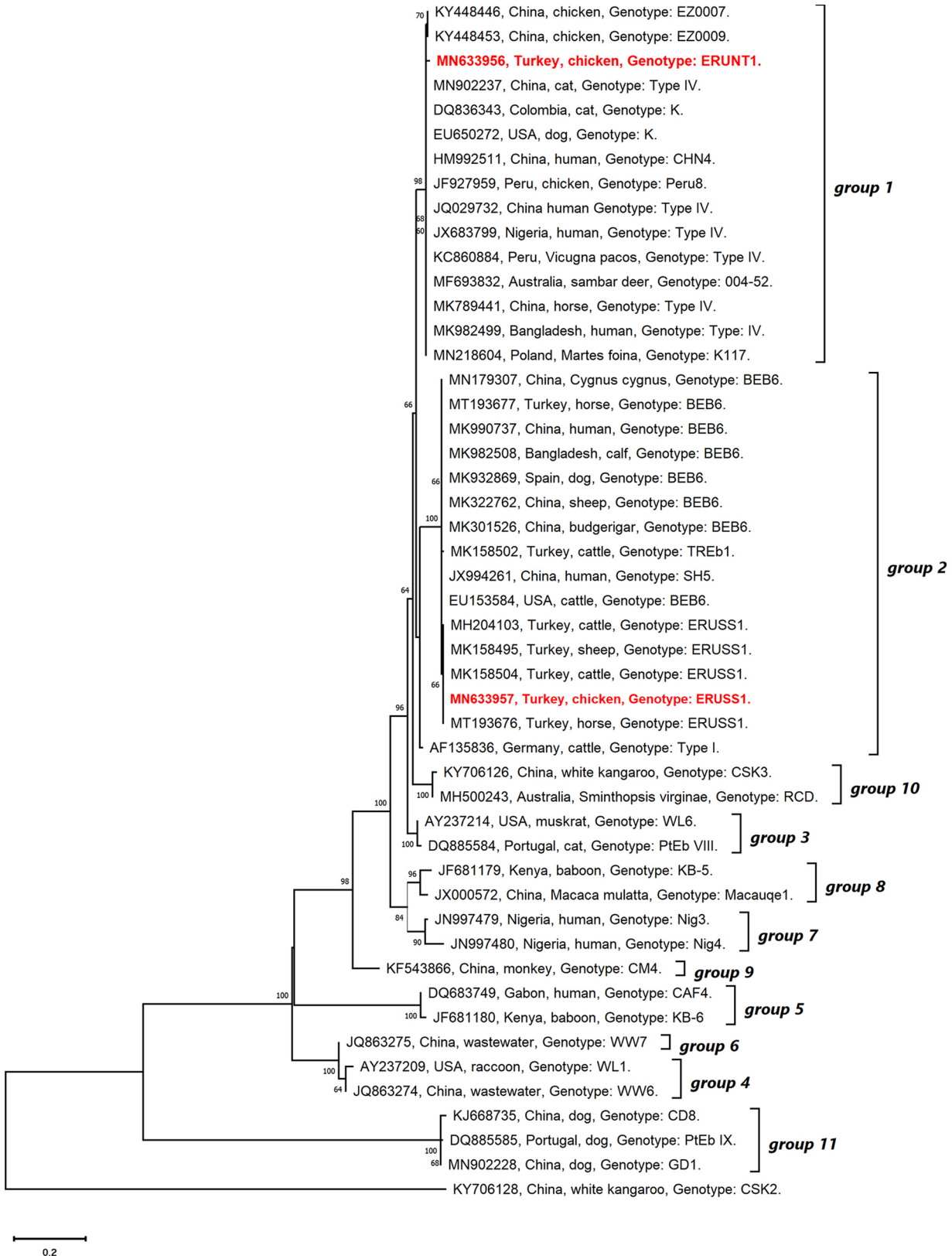

Fig. 2. Phylogenetic relationships of *E. bieneusi* isolates from chickens in this study (in red) and known genotypes previously reported from different countries and hosts. The tree was constructed using Maximum Likelihood analyses of internal transcribed spacer (ITS) sequences. The isolates are displayed with GenBank accession numbers, country, and host. Numbers at the branches indicate bootstrap values (1000 replicates). The *E. bieneusi* genotype CSK2 from the white kangaroo is used as an outgroup.

[35,36]. Reetz et al. (1993) [37] reported naturally occurring infections with *En. cuniculi* in two chicken embryos. However, no infection with *Encephalitozoon* species was detected in the chickens in our study. The prevalence and distribution of microsporidia in many kinds of livestock could be affected by various factors, such as differences in animal management systems, sample sizes, climatic and environmental conditions, potential pollution of feed and water, and health and immune status of the animals [38,39].

We found that all the infections were in free-ranging chickens, with none in layer chickens or broilers. Similarly, da Cunha et al. [24] reported the prevalence of *E. bieneusi* infections in chickens with free-ranging characteristics before they were brought to markets. Low infection rates in layer chickens and broilers kept in cages were also reported from China [23,34]. The symptoms of diarrhea were diagnosed in chickens infected with *E. bieneusi* in Germany and China [21,23]. Similarly, we detected significantly higher infection rates in chickens with diarrhoeic or soft feces. On the other hand, da Cunha et al. [24] reported *E. bieneusi* infections in apparently healthy chickens and highlighted their role as asymptomatic carriers.

Genotypes of *E. bieneusi* include a novel genotype ERUNT1 in zoonotic group 1 and a known genotype ERUSS1 in group 2, referred to as "ruminant-specific" group [38,40,41]. The most common genotype ERUNT1 has high identity with some genotypes found in chickens from Peru (Peru8, JF927959) and China (CHN4, HM992511) as well as genotypes identified in humans in China (CHN4, HM992511) and Nigeria (Type: IV, JX683799), indicating possible public health significance. The known genotype ERUSS1 was identified in a fecal sample of only one chicken. ERUSS1 has been previously identified as the common genotype in cattle [25,26], horses (unpublished data), and sheep [25] from several locations in the Central Anatolia Region. Reduced host specificity and host switching of ruminant-specific genotypes have been reported by previous researchers [19,21,42–45], and identifying the genotype ERUSS1 in several host species supports this observation. Sporadic human infections with group 2 of *E. bieneusi* genotypes have also been reported from several countries [44,46–48]. Therefore, further studies with human samples are required for exploring the possible zoonotic potential of ERUSS1. Several other zoonotic genotypes of *E. bieneusi* such as J in Germany, Peru8 in Brazil, Henan-IV and CC-1 in China, and Peru6, Peru11, Type IV, and D in Brazil [21–24] have been identified in both humans and chickens. Nevertheless, none of these genotypes were found in chickens in our study.

In conclusion, we provide unique data on the occurrence and genotypes of *E. bieneusi* in chickens raised in Turkey. Our findings indicate high prevalence of a novel genotype, ERUNT1, in zoonotic group 1 and the presence of the known genotype ERUSS1 in group 2 of *E. bieneusi*. Further studies on a wider variety of animal species, as well as humans, are needed to better understand the molecular epidemiology and zoonotic potential of microsporidian species in Turkey.

Ethical statement

Ethical approval was not required for performing animal experiments, according to the Erciyes University Animal Experiments Committee.

Declaration of Competing Interests

The authors declare that they have no competing interests.

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