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Molecular prevalence and genetic characterization of *Enterocytozoon bieneusi* in cattle in a dairy farm in Türkiye

Tülay Öncü Öner[1], Hüseyin Can[2,3,4*], Aysu Değirmenci Döşkaya[3,4,5], Mervenur Güvendi[2], Sedef Erkunt Alak[3], Ecem Sürgeç[6], Cemal Ün[2,3,4], Mert Döşkaya[3,4,5], Adnan Yüksel Gürüz[3,4,5] and Muhammet Karakavuk[3,4,7]

Abstract

Background Microsporidia, which are unicellular and obligate intracellular eukaryotic pathogens, can infect many animal species and humans. Cattle are known to be an important source for the spread of *Enterocytozoon bieneusi* (*E. bieneusi*) to humans and animals. Therefore, this study aimed to investigate *E. bieneusi* in fecal samples collected from cattle and environmental samples within a dairy farm and to genotype *E. bieneusi*-positive samples. For this purpose, a Nested PCR targeting a region of the *E. bieneusi* ribosomal DNA internal transcribed spacer (ITS) was applied to the DNA samples extracted from fecal ($n=47$) or environmental samples ($n=41$). Later, sequencing data belonging to *E. bieneusi*-positive samples were analyzed by BLAST and phylogenetic analysis for genotyping.

Results Nested PCR screening detected 15 *E. bieneusi*-positive samples and among them six (12.8%; 6/47) were detected in fecal samples while nine (21.9%; 9/41) were detected in environmental samples (including drinking water containers, mangers, feeding bottle, milk heating tank and towel). When cattle were categorized by their age, the molecular prevalence of *E. bieneusi* for cattle < 2 months old was detected as 19.2% (5/26). However, in cattle group > 2 months old, the molecular prevalence of *E. bieneusi* was 10% (1/10). Surprisingly, remaining cattle > 2 years old ($n=11$) were found to be *E. bieneusi*-negative. BLAST and phylogenetic analyses revealed type IV as well as 11 new genotypes designated as NG1 to NG11.

Conclusion These findings point out that cattle and farms are important sources for the transmission of *E. bieneusi* spores to humans or animals.

Keywords *Enterocytozoon bieneusi*, Genotype, Type IV, New genotypes

*Correspondence:
Hüseyin Can
huseyin.can@ege.edu.tr
[1]Faculty of Engineering, Department of Bioengineering, Manisa Celal Bayar University, Manisa, Türkiye
[2]Faculty of Science, Department of Biology, Molecular Biology Section, Ege University, İzmir, Türkiye
[3]Vaccine Development Application and Research Center, Ege University, İzmir, Türkiye
[4]Department of Vaccine Studies, Ege University Institute of Health Sciences, İzmir, Türkiye
[5]Faculty of Medicine, Department of Parasitology, Ege University, İzmir, Türkiye
[6]Faculty of Science, Department of Biology Zoology Section, Ege University, İzmir, Türkiye
[7]Ödemis Vocational School, Ege University, İzmir, Türkiye

Background

Microsporidia, which are unicellular and obligate intracellular eukaryotic pathogens related to fungi, can infect many animal species [1]. They mainly cause diarrhea but may also lead to pulmonary and systemic disseminated microsporidiosis in immunocompromised patients [2–5]. Microsporidia comprise more than 200 genera and 1500 species, in which *Enterocytozoon bieneusi* (*E. bieneusi*) is the most frequently diagnosed type in humans [6]. *E. bieneusi*, which is a common etiological cause of diarrhea, can also lead to high mortality and morbidity in immunocompromised individuals [6].

Detection of *E. bieneusi* in wild and domestic animals, including livestock, and in water sources has been associated with higher risk of waterborne, foodborne, and zoonotic transmission [4]. Zoonotic transmission, which is the main source of infection, can occur through direct contact with infected humans or animals in farms with inadequate sanitation, or indirectly through consumption of *E. bieneusi* contaminated food or water [6].

Nucleic acid-based diagnostics are superior to traditional methods in terms of specificity, sensitivity, reproducibility, and speed up the detection of pathogens [7]. Molecular analyses of *E. bieneusi*'s 243-bp ITS (Internal Transcribed Spacer) of the rRNA gene have showed remarkable genetic variation within *E. bieneusi* strains isolated from animals and humans, supporting the possibility of zoonotic transmission [8]. According to the phylogenetic analysis, more than 500 *E. bieneusi* ITS genotypes have been classified into at least 11 major groups [9–11]. Among these genotypes, genotype D, EbpC and type IV within group 1 are the most frequently detected genotypes, which have been detected not only in humans but also in various animal species worldwide [9]. A recent study also proposed Group 12, Group 13, Group 14 and Group 15 [11].

E. bieneusi in cattle was first reported in 8 calves in Germany [12], and has been described as a common pathogen in dairy and beef cattle. Later, *E. bieneusi* has been identified in cattle from various countries. According to the results of these studies, more than 40 *E. bieneusi* genotypes have been identified in cattle, in which the majority belongs to group 2 genotypes [13].

To date, a limited number of studies have been conducted in Türkiye which have reported the presence of *E. bieneusi* and its genotype profile in cattle. Accordingly, in raw milk samples collected from cattle in Türkiye, ERUSS1, BEB6 and a new genotype which were classified within group 2 have been detected [14]. In another study, ERUSS1 to ERUSS4 and N genotypes were detected in cattle [15]. However, the available data on *E. bieneusi* in animals, including cattle, in Turkey are not sufficient to know the zoonotic importance of *E. bieneusi* and its genotype profile in animals and therefore further studies are needed. Based on this, in this study *E. bieneusi* were investigated in fecal samples of cattle ($n = 47$) in a dairy farm in Türkiye by Nested PCR and *E. bieneusi*-positive samples were genotyped by sequencing the ITS region as previously described [16]. In addition, drinking water and environmental samples ($n = 41$) that could be contaminated with environmentally resistant spores of *E. bieneusi* were collected from the dairy farm and analyzed for the presence of *E. bieneusi*.

Materials and methods

Samples

The fecal, water, and environmental samples analyzed in this study were collected in our previous study [17] and DNA samples were stored under appropriate conditions. DNA was extracted from these samples by different protocols according to the sample type [17]. During the collection of samples, the permission was obtained from the general manager of the dairy farm.

Briefly, a total of 47 DNA samples extracted from fecal samples were analyzed in this study. As we categorize these samples by age of the cattle, 26 of them were collected from cattle < 2 months old while the 10 samples were collected from cattle > 2 months old. The remaining 11 samples were collected from cattle > 2 years old. In addition to fecal DNA samples, a total of 41 DNA samples were extracted from different sources. To elaborate, 31 of them were collected by a sterile cotton swab from environmental surfaces in the dairy farm. Among the remaining 10 DNA samples, two of them were extracted from water samples which belonged to two different artesian well used as main drinking water source while the remaining eight were extracted from water samples in drinking water containers found in paddocks.

Nested PCR

A nested PCR approach was used to amplify a region of the *E. bieneusi* ITS gene as described by Buckholt et al. (2002) [16]. For the first amplification reaction the outer primers, EBITS3 (5'-GGTCATAGGGATGAAGAG-3') and EBITS4 (5'-TTCGAGTTCTTTCGCGCTC-3'), were used to amplify a 435-bp region. PCR was carried out in a 25 µL reaction mixture including 5 µL 5× PCR master mix (GeneMark, Taichung, Taiwan), 2 µL template DNA, 1 µL primers (10 µM each) and 16 µL distilled water. The PCR cycle consisted of 35 cycles of 94 °C for denaturation (30 s), 57 °C for annealing (30 s) and 72 °C for elongation (40 s), with an initial 94 °C denaturation (5 min) and a 72 °C extension (10 min). For the second round of amplifications, the reaction mixture was prepared as described above, except that the inner primers, EBITS1 (5'-GCTCT GAATATCTATGGCT-3') and EBITS2.4 (5'-ATCGCCG ACGGATCCAAGTG-3') which amplified 390-bp region, were used and 2 µl of the amplified product from the

first PCR was used as the DNA template. The following PCR cycle was used: 5 min initial denaturation at 94 °C, followed by 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C, 40 s extension at 72 °C with a 10 min final extension at 72 °C. All secondary PCR products were separated by electrophoresis on 1% agarose gel containing SafeView Classic (Abm, Canada) in 1×TAE buffer, and then visualized using a UV light source. Positive and negative controls were used in each experiment.

Genotyping and phylogenetic analysis

The PCR products were purified using Qiaquick PCR Purification Kit (Qiagen, USA) and then sequenced by ABI3730XL for genotyping. To sequence the purified products, the inner forward primer (EBITS1) was used. MEGA X software was used to align and compare the nucleotide sequences with each other [18] and with the referenced *E. bieneusi* sequences obtained from GenBank database (http://www.ncbi.nlm.nih.gov). The phylogenetic tree belonging to *E. bieneusi* isolates was constructed via MrBayes v.3.2.3 using Monte Carlo Markov Chain (MCMC) and Bayesian methods [19–20]. Also, the constructed phylogenetic tree was visualized by FigTree v.1.4.4 [21].

Results

In this study, a total of 88 DNA samples extracted from different sources in a dairy farm were examined for the presence of *E. bieneusi* by Nested PCR and 15 of them were *E. bieneusi*-positive. Among the 47 fecal DNA samples, 6 of them were *E. bieneusi*-positive. Accordingly, the molecular prevalence of *E. bieneusi* was 12.8% (6/47) within the dairy farm. When cattle were categorized by their age, *E. bieneusi* was found in five cattle < 2 months old and the molecular prevalence of *E. bieneusi* for this cattle group increased to 19.2% (5/26). In cattle group > 2 months old, only one sample was found to be *E. bieneusi*-positive and the molecular prevalence of *E. bieneusi* for this cattle group was 10% (1/10). On the other hand, all cattle > 2 years old was *E. bieneusi*-negative (Table 1).

Table 1 The prevalence values detected in different age groups and genotype profiles detected in fecal samples

Cattle age profile	Prevalence (%)	Genotypes
All cattle ($n=47$)	12.8	Type IV, NG1, NG2, NG5, NG6, NG7
Cattle < 2 months old ($n=26$)	19.2	Type IV, **NG1**, **NG2**, NG5, NG7
Cattle > 2 months old ($n=10$)	4.76	NG6
Cattle > 2 years old ($n=11$)	-	-

NG indicates the new genotypes

Bold indicated type IV related genotypes

Among 41 environmental samples, 9 of them were *E. bieneusi*-positive. Interestingly, drinking water containers, mangers, feeding bottle, milk heating tank and towels which are used within dairy farm were found to be contaminated with *E. bieneusi* spores.

According to the phylogenetic analysis, type IV as well as new genotypes of *E. bieneusi* were identified (Fig. 1). Among these genotypes of *E. bieneusi*, 7 of them were classified within Group 1 while 8 of them were classified within Group 2 (Fig. 1). In group 1 isolates, only three samples, genotyped as type IV, showed 100% similarity with type IV genotype with GenBank accession number of AF242478.1. The remaining 4 showed an approximately 99% similarity with type IV genotype with GenBank accession number of AF242478.1 and genotyped as new genotype 1 to 4 (NG1 to NG4) as they did not show 100% similarity with any other known genotypes and each other. In group 2 isolates, none of them had 100% similarity with *E. bieneusi* isolates deposited in NCBI or with each other except two *E. bieneusi* isolates (Enviromental_sample_1 and 2). However, seven of them were closely grouped with *E. bieneusi* isolate with GenBank accession number of MK559495.1 while one was grouped with *E. bieneusi* isolate with GenBank accession number of OM101104.1 (Fig. 1). Depending on these data, these new *E. bieneusi* isolates were genotyped as genotype 5 to 11 (NG5 to NG11). Remarkably, within all detected genotypes, the prevalence value of type IV which is zoonotic genotype detected frequently in humans was found to be 20% (3/15).

Discussion

The results of this study point to three noteworthy findings. The prevalence differences detected among cattle categorized by age was one of them. Accordingly, a prevalence value of 19.2% was detected in cattle < 2 months old whereas a prevalence value of 4.76% was detected in cattle > 2 months old. Surprisingly *E. bieneusi* was not found in cattle > 2 years old. Moreover, the prevalence value (19.2%) detected in cattle < 2 months old was higher than the prevalence value (12.8%) detected in all cattle analyzed (Table 1). In line with this result, Juránková et al. (2013) also reported that the prevalence value of *E. bieneusi* was higher in calves up to three months (26.66%) compared to the prevalence value detected in calves 6–8 months old (18.33%), heifers of 14–16 months (6.6%) and cows 28–30 months (10%) [22]. Similar findings showing higher prevalence value in pre-weaned calves < 3 months old (10%) compared to the post-weaned calves 3–12 months old (7.7%), juveniles 13–24 months old (4.5%) and adults > 24 months old (3.9%) were also reported [23]. In another study, the prevalence of *E. bieneusi* was found to be higher in pre-weaned calves < 2 months old (17.7%) than the prevalence value detected in post-weaned calves

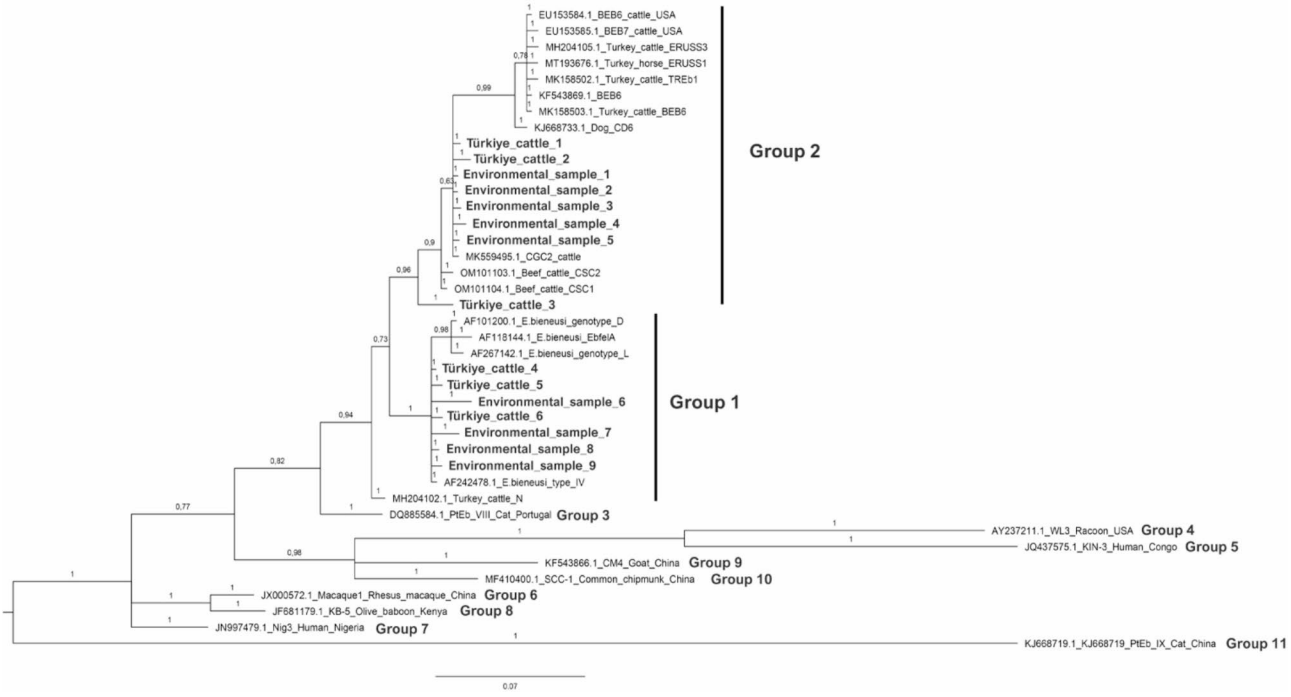

Fig. 1 The phylogenetic tree shows the genotype profile of *E. bieneusi*-positive samples. Accordingly, Türkiye_cattle_4, Enviromental_sample_8 and 9 were genotyped as type IV. Türkiye_cattle_5 and 6 as well as Enviromental_sample_6 and 7 were designed as NG1, NG2, NG3, and NG4, respectively. Türkiye_cattle_1, 2 and 3 as well as Enviromental_sample_1–2,3,4, and 5 were designed as NG5, NG6, NG7, NG8, NG9, NG10, and NG11, respectively

2–5 months old (15.5%) [24]. The relatively high prevalence values detected in previous studies as well as our study indicate that cattle < 2 months old are more prone to opportunistic infections like *E. bieneusi* and are important reservoir for transmission of zoonotic genotypes of *E. bieneusi* such as type IV, D, BEB4, and BEB6 to humans and animals. In addition, the susceptibility of young cattle to opportunistic infections can be explained by their less developed immune system [22].

In addition to this finding which is important for both veterinary and public health, total prevalence value (12.8%) detected in this study was comparable with previous studies conducted in Türkiye and other countries although relatively limited samples were analyzed in this study (Table 1). Accordingly, the reported prevalence values including our study's prevalence value changed from 0.59 to 34.80% (Table 2). It was thought that the prevalence values can change depending on number of samples analyzed, geographic location as well as probable hygiene precautions applied in farm.

The second remarkable finding of this study was the presence of type IV genotype which is a zoonotic genotype detected frequently in humans. The prevalence value of type IV within all detected genotypes was 20% (3/15) (Türkiye_cattle_4; Enviromental_sample_8 and 9). Importantly, this zoonotic genotype was not only found in fecal samples but also found in mangers found in two different paddock. Since the mangers are places where workers are constant contact with feces of infected cattle, these contaminated places can become additional source for the transmission of *E. bieneusi* to workers. Depending on this finding, it was thought that treating infected cattle and improving hygiene conditions may be useful for clearance of *E. bieneusi* in the dairy farm analyzed in this study. In other words, it is crucial to use the one health approach to tackle zoonotic pathogens including *E. bieneusi* infections.

The third important finding of this study was the presence of many new genotypes of *E. bieneusi* in addition the prevalent zoonotic genotypes of *E. bieneusi.* Some of them (Türkiye_cattle_5 and 6; Enviromental_sample_6 and 7) were associated with type IV which is known as zoonotic genotype. Among new genotypes classified in group 2, some of them (Türkiye_cattle_1 and 2; Enviromental_sample_1,2,3,4 and 5) were associated with MK559495.1 isolate which is a known genotype called CGC2 [59] while one (Türkiye_cattle_3) was associated with OM101104.1 isolate detected in cattle in Shanxi Province, North China [57]. The OM101104.1 isolate was reported to be close with MN728943 genotyped as BEB6. The presence of these new genotypes associated with different genotypes demonstrates that cattle harbor a high genetic diversity for *E. bieneusi* and that more cattle should be examined for *E. bieneusi* in order to reveal more accurate genotype profile of *E. bieneusi* in our study region.

Table 2 The prevalence of *E. bieneusi* and genotypes identified in fecal samples collected from cattle in different countries

Country	No. positive/ No. of sample tested	Prevalence	Genotypes (no.)	References
America	13/413	3.15%	Genbank accession no AY257180 (13)	[25]
America	59/452	13.05%	Genbank accession no AY257180 (59)	[26]
America	32/338	9.47%	BEB1 (9), BEB4 (8), BEB2 (7), BEB5 (6), BEB3 (2)	[27]
America	131/571	22.94%	BEB2 (66), BEB1 (27), BEB4 (27), BEB3/BEB4 (4), BEB1/BEB2 (2), BEB1/BEB4 (1), BEB2/BEB4 (2), Peru 6 (1), Peru 9 or D (1)	[28]
America	24/541	4.44%	BEB1 (8), BEB2 (8), BEB4 (5), BEB6 (2), BEB7 (1)	[29]
Korea	80/538	14.87%	CEbE (3), CEbD (2), CEbB (2), CEbA (1), CEbF (1), CEbC (1)	[30]
South America	9/121	7.44%	BEB3-like (4), BEB4 (3), I (1), D (1)	[31]
America	285/819	34.80%	J (108), BEB4 (65), I (59), BEB8 (41), BEB9 (6), mixed I/BEB4 (3), J/BEB4 (2), Type IV (1)	[32]
Czech Republic	37/240	15.42%	I (6)	[22]
Argentina	10/70	14.29%	J (4), I (2), BEB4 (1), EbpC (1), BEB10 (1), D (1)	[33]
China	32/537	5.96%	J (7), CS-4 (3), I/Ja (3), BEB4 (1), EbpC (1), G (1), BEB4/Ja (1), CS-4/EbpCa (1), CS-4/NECA1a (1), CS-4/NECA3a (1), EbpC/NECA2a (1), NECA4/NECA5a (1)	[34]
China	40/133	30.08%	O (26), EbpA (2), I (2), J (2), CC-I (2), CC-II (2), CC-III (2), D (1), BEB4 (1)	[35]
Egypt	13/214	6.07%	-	[36]
Brazil	79/452	17.48%	I (33), BEB8 (19), BEB4 (5), BEB12 (5), D (4), BEB11 (3), BEB13 (2), EbpA (1), BEB8/BEB13 (1), BEB4/BEB16 (1), I/BEB13 (1), BEB8/BEB13/BEB15 (1), BEB4/I (1), BEB8/BEB13/BEB17 (1), BEB8/BEB13/BEB14 (1)	[37]
China	214/879	24.34%	J (77), I (61), CM8 (18), BEB6 (17), BEB4 (15), EbpC (6), COS-1 (5), EbpA (2), D (2), BEB8 (1), CD6 (1), CHC1 (1), CHC2 (1), CHC3 (1), CHC4 (1), CHC5 (1), CHG2 (1), CHG3 (1), H (1), O (1)	[38]
China	73/371	19.68%	I (40), J (30), CHN1 (1), CSX1 (1), CSX2 (1)	[39]
Algeria	11/108	10.8%	BEB4 (4), BEB6 (2), BEB3 (1), I (1), J(1), PtEb XI (1), mixed (1)	[40]
China	202/1040	19.42%	I (87), J (83), BEB4 (18), CHC8 (7), BEB6 (3), N (1), Ebpc (1), CHC6 (1), CHC7 (1),	[41]
China	85/514	16.54%	J (57), I (19), BEB4 (4), EbpC (2), D (2), CC4 (1)	[24]
Iran	36/256	14.06%	D (22), J (9), M (5)	[42]
China	214/809	26.45%	J (145), BEB4 (59), CHN4 (4), Type IV and BEB4 (4), CHN15 (1), mixed (1)	[43]
China	61/388	15.72%	J (57), D (3), J/D (1)	[9]
China	118/1440	8.19%	-	[44]
Thailand	3/60	5.00%	D (3)	[45]
China	177/1366	12.96%	J (138), I (21), BEB4 (10), Type IV (1), CHC17 (1)	[13]
China	33/277	11.91%	BEB6(10), COS-1(6), I(6), CHG2(1), CHG3(1), J(1), CHC9(1), CHC10(1), CHC11(1), CHC12(1), CHC13(1), CHC14(1), CHC15(1), CHC16(1)	[46]
Australia	49/471	10.40%	I (18), J (14), BEB4 (6), TAR_fc2 (6), TAR_fc1 (1), TAR_fc3 (1), BEB8/TAR_fc5 (1), BEB8/TAR_fc5/TAR_fc6/TAR_fc7 (1), I/TAR_fc4 (1)	[47]
Türkiye	29/150	19.33%	ERUSS1 (24), N (2), ERUSS2 (1), ERUSS3 (1), ERUSS4 (1)	[15]
Korean	53/314	16.88%	BEB8 (21), J (16), BEB4 (12), BEB8-like (2), KCALF1 (1), KCALF2 (1)	[48]
China	93/321	28.97%	J (40), I (31), BEB4 (22)	[49]
China	11/513	2.14%	I (7), CHC8 (2), EbpC (2)	[50]
China	30/423	7.09%	J (23), I (5), BEB4 (2)	[51]
China	31/314	9.87%	EbpC (14), BEB4 (12), J (2), I (1), CHG5 (1), HNC-I (1)	[10]
Northern Spain	2/168	1.2%	BEB4 (2)	[52]
China	5/841	0.59%	I (1), J (1), BEB4 (1), YNDCEB-90 (1), YNDCEB-174 (1)	[53]
Iran	10/32	31.25%	I (7), BEB6 (1), D (2)	[54]
Bangladesh	55/699	7.9%	PigEBITS7 (4), BEB4 (9), BEB6 (5), D (6), I (18), J (13)	[55]
China	28/108	25.9%	J (14), I (9), BEB4 (3), COS-I (2)	[56]
North China	90/401	22.44%	I (50), BEB4 (20), J (10), BEB6 (3), BEB8 (4), PigSpEb2 (1), CSC1 (1), CSC2 (1)	[57]

Table 2 (continued)

Country	No. positive/ No. of sample tested	Prevalence	Genotypes (no.)	References
Southeastern China	30/556	5.4%	D (10), I (5), J (4), IV (4), N (1), BEB4 (1), JX-I (1), JX-II (1), JX-III (1), JX-IV (1), JX-V (1)	[58]
China	40/955	4.2%	J (26), AHDC1 (3), AHYC1 (1), AHYC2 (2), AHYC3 (2), AHYC4 (1), AHYC5 (1), AHYC6 (2), AHYC7 (1), CHN11 (1)	[59]
Austria	16/351	4.6%	J (2), I (12), BEB4 (3), BEB8 (1)	[60]
Türkiye	6/47	12.8%	Type IV, NG1, NG2, NG5, NG7 NG6	This study

Conclusion

In this study, a higher prevalence rate of 19.2% among cattle < 2 months old were detected. Also, within the dairy farm, some places where workers are constant contact with each other were found to be contaminated with *E. bieneusi* spores. Moreover, type IV as well as new genotypes were detected. These findings show the importance of cattle and farms as a source for spreading *E. bieneusi* spores to humans or animals.

Author contributions

Conceptualization: H.C., M.D., M.K. Methodology: H.C., M.D., M.K. Formal analysis and investigation: T.Ö.Ö., M.G., S.E.A., E.S., A.D.D. Writing-original draft preparation: H.C., T.Ö.Ö., Writing- review and editing: H.C., C.Ü., M.D., A.D.D., A.Y.G., M.K. Resources: H.C., C.Ü., A.Y.G., M.D., Supervision: H.C., M.D., A.D.D., M.K., A.Y.G.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethical approval

All experiments were performed under the instructions and approval of the Institutional Animal Care and Use Committee (IACUC) of Ege University for animal ethical norms (Permit number: 2019–047). All methods were carried out in accordance with relevant guidelines and regulations.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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