Genetic diversity within dominant Enterocytozoon bieneusi genotypes in pre-weaned calves

Chuanxiang Tang 1,2, Min Cai 3, Lin Wang 1, Yaqiong Guo 2, Na Li 2, Yaoyu Feng 1,2*, and Lihua Xiao 4

Abstract

Background: Cattle are commonly infected with the microsporidian parasite Enterocytozoon bieneusi. Sequence characterization of E. bieneusi in these animals at the ribosomal internal transcribed spacer (ITS) locus had identified I, J and BEB4 as the dominant genotypes. However, current studies on E. bieneusi in dairy cattle are mostly on infection rates and genotype distribution. This study aims to examine the intragenotypic diversity within dominant E. bieneusi genotypes in pre-weaned dairy calves in Shanghai, China.

Methods: Enterocytozoon bieneusi genotypes and subtypes were identified by PCR sequence analysis of ITS and multilocus sequence typing (MLST), based on material from farms. Chi-square test was used to examine differences in E. bieneusi infection rates between farms or age groups.

Results: The overall infection rate of E. bieneusi was 26.5% (214/809), ranging from 12.6% (Farm 5) to 38.5% (Farm 4). Infection rates increased with age during early life, with the peak infection rate (43.0%; 43/100) occurring at six weeks. Four genotypes were present, including J (n = 145, 67.8%), BEB4 (n = 59, 27.6%), CHN4 (n = 4, 1.9%) and CHN15 (n = 1, 0.5%), with the former two belonging to Group 2 and the latter two belonging to Group 1. Differences were detected in the distribution of the dominant genotypes J and BEB4 among five study farms. Altogether, 10 multilocus genotypes (MLGs) were identified in the two dominant ITS genotypes, including MLG-J1 to MLG-J8 of genotype J and MLG-B1 to MLG-B2 of genotype BEB4. MLG-B1 and MLG-B2 were recovered in Farms 1, 2 and 5, whereas MLG-J1 to MLG-J5 and MLG-J6 to MLG-J8 were found in Farms 3 and 4, respectively.

Conclusions: There is extensive genetic heterogeneity within the dominant E. bieneusi genotypes J and BEB4 in dairy calves in Shanghai, China, and MLST should be used in molecular epidemiological studies of E. bieneusi in cattle.

Keywords: Enterocytozoon bieneusi, Transmission, Dairy calves, Genetic diversity, Multilocus sequence typing

Background

Microsporidia are obligate intracellular parasites with a wide range of vertebrate and invertebrate hosts such as humans, farm and companion animals, and wildlife [1, 2]. Of approximately 17 human-pathogenic microsporidian species, Enterocytozoon bieneusi is the most frequently detected [2, 3]. In immunocompromised patients (HIV-positive patients or organ transplant recipients), E. bieneusi usually causes chronic diarrhea and wasting syndrome [3–5], but in immunocompetent humans and animals, E. bieneusi infection can be asymptomatic [6, 7].

Based on sequence analysis of the internal transcribed spacer (ITS) of the rRNA gene (~243 bp), more than 200 E. bieneusi genotypes have been identified [1, 8]. Phylogenetic analyses revealed that they belong to nine groups [9, 10]. Group 1, which contains most genotypes found in humans, is considered a zoonotic group, with the remaining groups being largely host-specific. To date, over 40 E. bieneusi genotypes have been detected in cattle, most of which belong to Group 2 [11–13]. Among them, at least 15 genotypes, including eight genotypes in Group 1 and seven genotypes in Group 2,
have been reported in humans [11, 12, 14], suggesting that cattle may be potential reservoirs for human infections.

Genotypes I, J and BEB4 are common E. bieneusi genotypes found in pre-weaned dairy calves worldwide [8, 11, 12, 15–21] and have been further detected in at least 13 human cases [6, 22]. However, current studies on E. bieneusi in dairy calves are mostly on infection rates and genotype distribution. Little is known about the age distribution of E. bieneusi infection in pre-weaned dairy calves. In addition, genetic diversity within the dominant E. bieneusi genotypes has not been examined thoroughly using advanced molecular diagnostic tools such as multilocus sequence typing (MLST).

MLST has been used in investigations of E. bieneusi transmission in humans [23, 24], non-human primates [25–27], giant pandas [26, 28, 29], red pandas [26, 28], bears [26], lions [26], golden cats [26], deer [26], alpacas [26], blackbucks [26], raccoons [26], golden takins [30], horses [31], raccoon dogs [32], foxes [32, 33] and red-bellied tree squirrels [34]. Thus far, there has been only one study on multilocus characterization of E. bieneusi in cattle in Shaanxi, China, and the data were not analyzed for intra-genotypic variations and transmission among farms [17]. In this study, MLST was used to assess genetic heterogeneity within dominant E. bieneusi genotypes of Group 2 in pre-weaned calves, and the age pattern of E. bieneusi infection during early life of cattle was examined.

Methods

Specimen collection

From April 2015 to March 2016, 809 specimens, each of approximately 25 g fresh fecal material, were collected from pre-weaned Holstein calves in five farms in Shanghai, China. These farms are located in Fengxian (Farms 1, 2, 3 and 4) and Jinshan (Farm 5), two neighboring districts in suburban Shanghai. They were ranked A to E by combined farm quality score based on hygiene status, animal density, and facility condition, with A representing “excellent” and E representing “poor” [35]. Each farm was visited 2–5 times at 2–3 months intervals, for a total of five times for Farm 3, four times for Farm 1, and twice for Farms 2, 4 and 5. These fecal specimens were collected directly from the rectum by using disposable gloves into 50 ml centrifuge tubes, transported to the laboratory in coolers with ice packs, and stored in 2.5% potassium dichromate at 4 °C before DNA extraction.

DNA extraction

Genomic DNA was extracted by using the Fast DNA SPIN Kit for soil (MP Biomedical, Santa Ana, CA, USA) from approximately 200 mg of each fecal specimen, which was washed three times with distilled water by centrifugation at 2000×g for 10 min. The obtained DNA was stored at -20 °C until being used in PCR analysis.

PCR analysis

The occurrence and genotype distribution of E. bieneusi were determined by PCR and sequence analyses of the ITS as previously described [36]. For subtyping the dominant E. bieneusi ITS genotypes J and BEB4, the MLST technique targeting microsatellite loci MS1, MS3 and MS7 and minisatellite locus MS4 was used [24]. In a pre-study analysis, 90 of 98 E. bieneusi-positive specimens yielded the expected PCR products at the MS3 locus. Therefore, PCR analysis of the MS3 locus was used for screening of the 204 specimens positive for ITS genotypes J and BEB4. Among them, 84 MS3-positive specimens of five different MS3 subtypes were further analyzed at the MS1, MS7 and MS4 loci. Duplicate nested PCR was used in the analysis of the specimens at each genetic locus. The secondary PCR products obtained were identified by agarose gel electrophoresis.

Sequence analysis

All secondary PCR products of the expected size were bi-directionally sequenced using the secondary PCR primers on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were assembled using ChromasPro 2.1.5.0 (http://technelysium.com.au/ChromasPro.html), edited manually for sequence miscalls using BioEdit 7.1.3.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and aligned with reference sequences from the GenBank database using ClustalX 2.0.11 (http://clustal.org). Only sequence data from specimens that were successfully subtyped at all four MLST loci were used in the determination of multilocus genotypes (MLGs). MLGs were named according to the ITS genotypes: MLG-J1 to MLG-J8 for ITS genotype J and MLG-B1 to MLG-B2 for ITS genotype BEB4.

Statistical analysis

Differences in E. bieneusi infection rates between farms or age groups were examined by using the Chi-square test implemented in SPSS Statistics v.20.0 for Windows (IBM Corp., New York, NY, USA). Differences were considered significant at P ≤ 0.05.

Results

Occurrence of E. bieneusi in pre-weaned dairy calves

Among the 809 specimens collected from pre-weaned calves in five farms, 214 (26.5%) were positive for E. bieneusi in PCR analysis of the ITS locus. All five farms had E. bieneusi, with infection rates ranging between 12.6–38.5%. The highest infection rate (38.5%; 15/39) was in Farm 4, while the lowest (12.6%; 26/206) was in Farm 5 (Table 1). Farms with good management (such as Farms 2 and 5)
had lower E. bieneusi infection rates than farms with relatively poor management (such as Farm 4). In the former, the infection rates were 16.1% (9/56) on Farm 2 and 12.6% (26/206) on Farm 5, whereas in the latter the infection rate was 38.5% (15/39) on Farm 4 (χ² = 6.104, df = 1, P = 0.013 between Farms 2 and 4; and χ² = 15.714, df = 1, P < 0.0001 between Farms 5 and 4).

**Age distribution of E. bieneusi infection**

By age in weeks, E. bieneusi infection rates increased gradually during the first four weeks of life, with the highest infection rate (43.0%; 43/100) reached at six weeks (Fig. 1). The overall infection rate at 4–7 weeks of age was 35.2% (143/406), which was significantly higher than the infection rate of 11.7% (31/266) at 1–3 weeks of age (χ² = 46.519, df = 1, P < 0.0001).

**Enterocytozoon bieneusi infection and occurrence of diarrhea**

The specimens in this study were from three groups of animals: calves with watery diarrhea (G1, n = 85), moderate diarrhea (G2, n = 346), or no diarrhea (G3, n = 378). G1 specimens had a slightly higher E. bieneusi infection rate (30.6% or 26/85) than G2 (25.1% or 87/346) and G3 (26.7% or 101/378) specimens. The differences among the three groups were not significant (χ² = 0.522, df = 1, P = 0.470 between G1 and G3; and χ² = 0.233, df = 1, P = 0.629 between G2 and G3).

**ITS genotypes of E. bieneusi by farm**

DNA sequencing of ITS PCR products was successful for 209 of 214 PCR-positive specimens. The remaining five specimens generated ITS sequences with underlying signals because of the presence of mixed E. bieneusi genotypes. Four E. bieneusi genotypes were identified among the 209 successfully genotyped specimens, including J, BEB4, CHN4 and CHN15, with the latter being identical to an unnamed genotype (JF909995) in wastewater from Tunisia [37]. The dominant genotypes in calves were J (n = 145, 67.8%) and BEB4 (n = 59, 27.6%), which both belong to Group 2. The remaining two genotypes,

| Farm | Farm rank* | Sampling point | Sample size | No. positive for E. bieneusi (%) | ITS genotype (No.) | MLG (No.) |
|------|------------|----------------|-------------|---------------------------------|---------------------|-----------|
| 1    | D          | 1              | 36          | 12 (33.3)                       | BEB4 (11), Mixed infection (1) | MLG-B1 (2) |
|      |            | 2              | 12          | 3 (25.0)                        | BEB4 (3)            | –         |
|      |            | 3              | 46          | 13 (28.3)                       | BEB4 (13)           | MLG-B1 (2), MLG-B2 (1) |
|      |            | 4              | 25          | 5 (20.0)                        | BEB4 (5)            | MLG-B2 (1) |
| Subtotal |      | 119         | 33 (27.7)  | BEB4 (32), Mixed infection (1)  | MLG-B1 (4), MLG-B2 (2) |
| 2    | A          | 1              | 47          | 6 (12.8)                        | CHN4 (4), Type IV and BEB4 (2) | –         |
|      |            | 2              | 9           | 3 (33.3)                        | BEB4 (1), Type IV and BEB4 (2) | MLG-B1 (1) |
| Subtotal |      | 56           | 9 (16.1)   | CHN4 (4), BEB4 (1), Type IV and BEB4 (4) | MLG-B1 (1) |
| 3    | B          | 1              | 112         | 44 (39.3)                       | J (42), BEB4 (1), CHN15 (1) | MLG-J1 (1), MLG-J2 (1), MLG-J4 (1) |
|      |            | 2              | 43          | 17 (39.5)                       | J (17)              | MLG-J3 (1), MLG-J5 (1) |
|      |            | 3              | 81          | 24 (29.6)                       | J (24)              | MLG-J2 (4) |
|      |            | 4              | 84          | 19 (22.6)                       | J (19)              | MLG-J2 (1) |
|      |            | 5              | 69          | 27 (39.1)                       | J (27)              | MLG-J2 (4) |
| Subtotal |      | 389          | 131 (33.7) | J (129), BEB4 (1), CHN15 (1)   | MLG-J2 (10), MLG-J1 (1), MLG-J3 (1), MLG-J4 (1), MLG-J5 (1) |
| 4    | E          | 1              | 29          | 12 (41.4)                       | J (12)              | MLG-J6 (3), MLG-J7 (1), MLG-J8 (1) |
|      |            | 2              | 10          | 3 (30.0)                        | J (3)               | MLG-J6 (1) |
| Subtotal |      | 39           | 15 (38.5)  | J (15)                          | MLG-J6 (4), MLG-J7 (1), MLG-J8 (1) |
| 5    | C          | 1              | 109         | 21 (19.3)                       | BEB4 (20), J (1)    | MLG-B1 (7), MLG-B2 (1) |
|      |            | 2              | 97          | 5 (5.2)                         | BEB4 (5)            | –         |
| Subtotal |      | 206          | 26 (12.6)  | BEB4 (25), J (1)                | MLG-B1 (7), MLG-B2 (1) |
| Total |          | 809          | 214 (26.5) | J (145), BEB4 (59), CHN4 (4), Type IV and BEB4 (4), CHN15 (1), Mixed infection (1) | MLG-B1 (12), MLG-J2 (10), MLG-J6 (4), MLG-B2 (3), MLG-J1 (1), MLG-J3 (1), MLG-J4 (1), MLG-J5 (1), MLG-J7 (1), MLG-J8 (1) |

*Farm ranks A-E were ranking scores used to evaluate the hygiene status, animal density and facility condition, with A representing “excellent” and E representing “very poor” (see [35] for details)

**Table 1** Occurrence and distribution of Enterocytozoon bieneusi ITS genotypes and multilocus genotypes (MLGs) in pre-weaned dairy calves on five farms in Shanghai, China

---

**Tang et al. Parasites & Vectors (2018) 11:170**
CHN4 and CHN15, were seen in only four (1.9%) and one (0.5%) *E. bieneusi*-positive calves, respectively (Table 1). Among the five farms, Farms 1 and 4 each had only one genotype, whereas Farms 2, 3, and 5 each had two or three genotypes. The dominant genotype in farms with higher infection rates (Farms 3 and 4) was genotype J, compared with genotype BEB4 in farms with lower infection rates (Farms 1 and 5). Among the five specimens with mixed *E. bieneusi* genotypes, four from Farm 2 had concurrence of genotypes Type IV and BEB4.

**Distribution of MLST subtypes**

MLST analysis was conducted on 84 specimens of the two dominant ITS genotypes J (Farms 3 and 4) and BEB4 (Farms 1, 2 and 5) to assess intra-genotypic variations in pre-weaned dairy calves, after screening all 204 specimens that were positive for the two ITS genotypes by using MS3 PCR. The overall amplification efficiency at the MS1, MS3, MS4 and MS7 loci was 92.9% (78/84), 69.6% (142/204), 94.0% (79/84) and 41.7% (35/84), respectively (Table 2). The amplification efficiency of ITS genotypes J and BEB4 was similar at the MS1 (92.5 and 93.5%, respectively), MS4 (92.5 and 96.8%, respectively) and MS7 (37.7 and 48.4%, respectively) loci. However, at the MS3 locus, there was an obvious difference (58.6 vs 96.6%) in amplification efficiency between these two dominant genotypes. In addition, all 15 genotype J-positive specimens from Farm 4 were negative in MS3 PCR, while those from Farm 3 produced the expected MS3 PCR products.

Altogether, there were eight, five, two and four subtypes at the MS1, MS3, MS4 and MS7 loci, respectively. The diversity of subtype was different between genotypes J and BEB4, with seven, five, two, and three subtypes being detected in genotype J, compared to one, one, one, and two subtypes in BEB4, respectively.

As expected, the dominant subtype at each locus was different between genotypes J and BEB4 (Table 3). At the MS1 locus, the dominant subtype was MS1-3 in genotype J (28/53), while it was MS1-1 in BEB4 (29/31). At the MS3 locus, the dominant subtype was MS3-1 in genotype J (29/53), while it was MS3-2 in BEB4 (31/31). At the MS4 locus, MS4-1 (24/53) and MS4-2 (24/53) were the dominant subtypes in genotype J, while MS4-2 was the only subtype in BEB4 (30/31). At the MS7 locus, the dominant subtype was MS7-1 both in genotypes J (18/53) and BEB4 (12/31).

**Table 2** PCR amplification efficiency of DNA from *Enterocytozoon bieneusi* ITS genotypes J and BEB4 at the MLST loci

| ITS genotype | Farm ID | No. of specimens | No. of specimens amplified/No. of specimens analyzed |
|--------------|---------|------------------|-----------------------------------------------------|
|              |         | MS1          | MS3          | MS4          | MS7          |
| J            | 1       | 0             | –           | –           | –            |
|              | 2       | 0             | –           | –           | –            |
|              | 3       | 129           | 35/38       | 85/129      | 35/38        | 14/38        |
|              | 4       | 15            | 14/15       | 0/15        | 14/15        | 6/15         |
|              | 5       | 1             | –           | 0/1         | –            | –            |
| BEB4         | 1       | 32            | 13/15       | 31/32       | 14/15        | 6/15         |
|              | 2       | 1             | 1/1         | 1/1         | 1/1          | 1/1          |
|              | 3       | 1             | –           | 0/1         | –            | –            |
|              | 4       | 0             | –           | –           | –            | –            |
|              | 5       | 25            | 15/15       | 25/25       | 15/15        | 8/15         |
| Total        |         | 204           | 78/84       | 142/204     | 79/84        | 35/84        |
The distribution of subtypes in genotype J differed between Farms 3 and 4. The main subtypes on Farm 3 were MS1-3, MS3-1, MS4-2 and MS7-1 at the four loci, while the main subtypes on Farm 4 were MS1-8, no MS3 amplification, MS4-1 and MS7-1. In contrast, the main subtypes in genotype BEB4 at the four loci were the same (MS1-1, MS3-2, MS4-2 and MS7-1) on all BEB4-positive farms (Farms 1, 2 and 5).

Of the 84 specimens analyzed by MLST, only 29 were positive at all four genetic loci, with seven MLGs obtained, including five genotype J MLGs (MLG-J1 to MLG-J5) and two genotype BEB4 MLGs (MLG-B1 to MLG-B2). To compare subtype diversity of genotype J among farms, six additional specimens from Farm 4, which were successfully subtyped at MS1, MS4 and MS7 loci but were PCR-negative at the MS3 locus, were

Table 3 Occurrence and distribution of subtypes from *Enterocytozoon bieneusi* ITS genotypes J and BEB4 at four MLST loci

| ITS genotype | Farm ID | Subtype | MS1   | MS3   | MS4   | MS7   | No. of positive specimens |
|--------------|---------|---------|-------|-------|-------|-------|---------------------------|
| J            | 3       | MS1-3<sup>a</sup> | MS3-1 | MS4-2 | –     | 12    |
|              |         | MS1-3<sup>a</sup> | MS3-1 | MS4-2 | MS7-1 | 10    |
|              |         | MS1-3<sup>a</sup> | MS3-1 | MS4-1 | –     | 1     |
|              |         | MS1-3<sup>a</sup> | MS3-1 | –     | –     | 1     |
|              |         | MS1-3<sup>a</sup> | MS3-1 | MS4-1 | MS7-1 | 1     |
|              |         | MS1-3<sup>a</sup> | MS3-1 | MS4-2 and MS4-3 | – | 1     |
|              |         | MS1-3<sup>a</sup> | MS3-2 | MS4-1 | –     | 1     |
|              |         | MS1-2     | MS3-2 | MS4-1 | –     | 1     |
|              |         | MS1-4<sup>a</sup> | MS3-3<sup>a</sup> | MS4-1 | – | 1     |
|              |         | MS1-5<sup>a</sup> | MS3-2 | MS4-1 | MS7-1 | 1     |
|              |         | MS1-5<sup>a</sup> | MS3-5<sup>a</sup> | MS4-1 | MS7-1 | 1     |
|              |         | MS1-6<sup>a</sup> | MS3-5<sup>a</sup> | MS4-1 | – | 1     |
|              |         | MS1-7<sup>a</sup> | MS3-1 | MS4-1 | – | 1     |
|              |         | –          | MS3-1 | MS4-2 | – | 1     |
|              |         | –          | MS3-1 | – | – | 1     |
|              |         | –          | MS3-2 | MS4-1 | – | 1     |
|              | 4       | MS1-8<sup>a</sup> | – | MS4-1 | – | 6     |
|              |         | MS1-8<sup>a</sup> | – | MS4-1 | MS7-1 | 4     |
|              |         | MS1-8<sup>a</sup> | – | MS4-1 | MS7-2<sup>a</sup> | 1     |
|              |         | MS1-8<sup>a</sup> | – | MS4-1 | MS7-4<sup>a</sup> | 1     |
|              |         | MS1-8<sup>a</sup> | – | – | – | 1     |
|              |         | –          | MS3-2 | MS4-1 | – | 1     |
|              |         | –          | MS3-2 | MS4-1 | – | 1     |
|              |         | –          | – | MS4-1 | – | 1     |
|              |         | Noisy     | – | MS4-1 | – | 1     |
| BEB4         | 1       | MS1-1     | MS3-2 | MS4-2 | – | 7     |
|              |         | MS1-1     | MS3-2 | MS4-2 | MS7-1 | 4     |
|              |         | MS1-1     | MS3-2 | MS4-2 | MS7-3<sup>a</sup> | 2     |
|              |         | –          | MS3-2 | MS4-2 | – | 1     |
|              |         | –          | MS3-2 | – | – | 1     |
|              | 2       | MS1-1     | MS3-2 | MS4-2 | MS7-1 | 1     |
|              |         | MS1-1     | MS3-2 | MS4-2 | MS7-1 | 7     |
|              |         | MS1-1     | MS3-2 | MS4-2 | – | 7     |
|              |         | MS1-1     | MS3-2 | MS4-2 | MS7-3<sup>a</sup> | 1     |
| Total        |         |           |       |       |       | 84    |

Abbreviation: <sup>a</sup> novel subtype


included in the MLGs analysis. They were assigned the MLG-J6 to MLG-J8 because of the likely presence of a unique MS3 sequence (Table 4). The dominant MLGs were MLG-B1 \( (n = 12) \) and MLG-J2 \( (n = 10) \). Between them, MLG-B1 was the dominant MLG in three farms (Farms 1, 2 and 5), while MLG-J2 was the predominant MLG in only one farm (Farm 3). In addition, the distribution of genotype J MLGs was different among Farms 3 (MLG-J1 to MLG-J5) and 4 (MLG-J6 to MLG-J8), whereas the distribution of genotype BEB4 MLGs was similar among Farms 1 (MLG-B1 and MLG-B2), 2 (MLG-B1) and 5 (MLG-B1 and MLG-B2) (Table 1).

**Discussion**

In the present study, *E. bieneusi* was found in 26.5% (214/809) of pre-weaned dairy calves in Shanghai. This is similar to the infection rate of 29.3% (127/434) reported in one study in Henan and Ningxia, but higher than rates reported in other studies in Henan and Shandong (10.0% or 1/10), Heilongjiang (7.7% or 20/259), Shaanxi (19.5% or 39/200) and Xinjiang (17.7% or 42/237) in China [12, 15–21]. Similar differences (3.1–35.4%) in infection rates in pre-weaned calves have been reported in studies in the USA, Brazil, Argentina and the Czech Republic [8, 11, 18–20, 38]. Variations in *E. bieneusi* infection rates in pre-weaned dairy calves among studies could be due to differences in detection methods, age and management of animals, and climate.

Pre-weaned dairy calves appear to have peak *E. bieneusi* infection around 4–7 weeks of age. In this study, although calves were infected by *E. bieneusi* from one to nine weeks of age, *E. bieneusi* occurrence in newborn animals increased gradually with age, with the peak infection rate (43.0%) being reached at six weeks of age. Thus, the mean infection rate at 4–7 weeks of age was significantly higher than at 1–3 weeks. This agrees with the result of the only other study of the age pattern of *E. bieneusi* infection in pre-weaned dairy calves in the USA [8]. Previously in China, only slightly higher *E. bieneusi* infection rates were reported in pre-weaned dairy calves than in post-weaned dairy calves: 17.7% and 15.5% in Xinjiang [16], 29.3 and 23.9% in Henan and Ningxia [15], 10.0 and 7.3% in Henan and Shandong [21], and 7.4 and 4.3% in Northeast China [12], respectively. Lumping all pre-weaned calves into one group could be responsible for the small differences in *E. bieneusi* infection rates between pre-weaned and post-weaned calves.

Four genotypes (J, BEB4, CHN4 and CHN15) were identified among 214 *E. bieneusi*-positive specimens at the ITS locus. Genotype J was the dominant one among the four genotypes and the main genotype reported in pre-weaned dairy calves worldwide [11, 12, 15–17]. Another common genotype in the study (27.6% or 59/214), BEB4, was a genotype with lower prevalence in Xinjiang (9.5% or 4/42), Heilongjiang (5.0% or 1/20), Shaanxi (2.6% or 1/39), Henan and Ningxia (2.4% or 3/127) within China. This was also the case in the USA (10.0% or 1/10), Argentina (10.0% or 1/10) and Brazil (5.3% or 1/19) [8, 11, 12, 15–17, 20]. Between the remaining two *E. bieneusi* genotypes found in the study, CHN4 was reported in cattle in Jilin, China [22], while CHN15 was reported in wastewater in Tunisia [37]. In contrast, Genotype I, a common *E. bieneusi* genotype in pre-weaned dairy calves worldwide [8, 11, 15–21], was not detected in the present study. In the present study, the distribution of the two dominant *E. bieneusi* genotypes, J and BEB4, is different among five study farms; genotype BEB4 occurred on farms with lower infection rates of *E. bieneusi* (Farms 1, 2 and 5), whereas genotype J occurred on farms with higher infection rates (Farms 3 and 4).

Results of the MLST analysis support the existence of differences in the transmission of the two dominant *E.

---

**Table 4** Multilocus sequence types of *Enterocytozoon bieneusi* in pre-weaned dairy calves by ITS genotype in Shanghai, China

| MLG | ITS Genotype | Multilocus type | No. of MLGs | Farm (no. of specimens) |
|-----|--------------|-----------------|-------------|------------------------|
|     |              | MS1 MS3 MS4 MS7 |             |                        |
| J1  | J            | MS1-3n MS3-4n   | MS4-2 MS7-1 | 1                      |
| J2  | J            | MS1-3n MS3-1    | MS4-2 MS7-1 | 10                     |
| J3  | J            | MS1-3n MS3-1    | MS4-1 MS7-1 | 1                      |
| J4  | J            | MS1-5n MS3-1    | MS4-1 MS7-1 | 1                      |
| J5  | J            | MS1-5n MS3-2    | MS4-1 MS7-1 | 1                      |
| J6  | J            | MS1-8n –        | MS4-1 MS7-1 | 4                      |
| J7  | J            | MS1-8n –        | MS4-1 MS7-2 | 1                      |
| J8  | J            | MS1-8n –        | MS4-1 MS7-4 | 1                      |
| B1  | BEB4         | MS1-1 MS3-2     | MS4-2 MS7-1 | 12                     |
| B2  | BEB4         | MS1-1 MS3-2     | MS4-2 MS7-3 | 3                      |

**Abbreviation:** n novel subtype
bienuei ITS genotypes. All dominant subtypes in genotype BEB4 at the four individual loci, such as MS1-1, MS3-2, MS4-2 and MS7-1, and the most common MLG (MLG-B1) in genotype BEB4 were present on all farms with ITS genotype BEB4 (Farms 1, 2 and 5). In contrast, the dominant subtypes of ITS genotype J at each locus were different between Farms 3 and 4. In fact, genotype J on Farm 4 was so divergent from the one on Farm 3 at the MS3 locus that none of the 15 genotype J-positive specimens from Farm 4 generated the expected MS3 PCR product whereas 85 of the 129 genotype J-positive specimens from Farm 3 generated it. The most common genotype J MLG (MLG-J2) was only seen on Farm 3, and all other genotype J MLGs identified in this study, were exclusively present on Farm 3 or 4. Therefore, although all five farms are owned by the same dairy enterprise and located in two neighboring districts of suburban Shanghai, there is extensive genetic heterogeneity within the dominant E. bienuei genotypes, especially ITS genotype J.

Conclusions

Results of this study indicate that E. bienuei infection is common in pre-weaned dairy calves in suburban Shanghai, China, with animals of 4–7 weeks of age having the highest occurrence of the pathogen. Data of MLGs among farms suggest that there are apparent differences in the distribution of dominant E. bienuei genotypes among farms and extensive genetic heterogeneity within ITS genotypes. Molecular epidemiologic studies involving advanced pathogen characterization should be conducted to improve understanding of the population genetics of E. bienuei in cattle and relationship among infection rates, age-associated infection patterns, genotype distribution, farm management, and transmission of the pathogen.

Abbreviations

ITS: Internal transcribed spacer; MLGs: Multilocus genotypes; MLST: Multilocus sequence typing; PCR: Polymerase chain reaction

Acknowledgements

We thank the farm owners and staff for their cooperation in sample collection during this study.

Funding

This work was supported by the National Natural Science Foundation of China (grants 31630078, 31425025 and 31502055). The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the US. Centers for Disease Control and Prevention. We thank the farm owners and staff for their cooperation in sample collection during this study.

Availability of data and materials

The data supporting the conclusions of this article are included within the article. Representative nucleotide sequences generated in this study were submitted to the GenBank database under accession numbers MF592787-MF592790 (ITS locus), MF592777-MF592784 (MS1 locus), MF592772-MF592776 (MS3 locus), MF592785-MF592786 (MS4 locus), and MF592768 to MF592771 for the MS7 locus.

Authors’ contributions

YF and LX conceived and designed the experiments. CT, MC and LW performed the experiments. CT, YF, QQ, NL and LX analyzed the data. CT, YF and LX wrote the paper. All authors read and approved the final manuscript.

Ethics approval

This study was approved by the Ethics Committee of the East China University of Science and Technology. The dairy calves were handled in compliance with the Animal Ethics Procedures and Guidelines of the People’s Republic of China. Permissions were obtained from the owners or managers of dairy farms before specimen collections.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher’s Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. Santín M, Fayer R. Microsporidiosis: Enterocytozoon bienuei in domesticated and wild animals. Res Vet Sci. 2011;90(3):363–71.
2. Didier ES, Weiss LM. Microsporidiosis: current status. Curr Opin Infect Dis. 2006;19(5):485–92.
3. Didier ES, Weiss LM. Microsporidiosis: not just in AIDS patients. Curr Opin Infect Dis. 2011;24(5):490–5.
4. Lobo ML, Xiao LH, Antunes F, Matos O. Microsporidia as emerging pathogens and the implication for public health: a 10-year study on HIV-positive and -negative patients. Int J Parasitol. 2012;42(2):197–205.
5. Galvan AL, Sanchez AM, Valentin MAP, Henriques-Gil N, Izquierdo F, Fenoy S, et al. First cases of microsporidiosis in transplant recipients in Spain and review of the literature. J Clin Microbiol. 2011;49(4):1301–6.
6. Sak B, Brady D, Pelikanova M, Kvetonova D, Rost M, Kostka M, et al. Unapparent microsporidial infection among immunocompetent humans in the Czech Republic. J Clin Microbiol. 2011;49(3):1064–70.
7. Nkinin SW, Asonganyi T, Didier ES, Kaneshio ES. Microsporidial infection is prevalent in healthy people in Cameroon. J Clin Microbiol. 2007;45(9):2841–6.
8. Santín M, Fayer R. A longitudinal study of Enterocytozoon bienuei in dairy cattle. Parasitol Res. 2009;105(1):141–4.
9. Karim MR, Dong H, Li T, Yu F, Li D, Zhang L, et al. Predomination and new genotypes of Enterocytozoon bienuei in captive non-human primates in 2015 in China: high genetic diversity and zoonotic significance. PLoS One. 2015;10(2):e0117991.
10. Guo YQ, Alderisio KA, Yang WL, Cama V, Feng YQ, Xiao LH. Host specificity and source of Enterocytozoon bienuei genotypes in a drinking source watershed. Appl Environ Microbiol. 2014;80(1):218–25.
11. Del Coco VF, Cordoba MA, Bilbao G, Castro PD, Basualdo JA, Santín M. First report of Enterocytozoon bienuei from dairy cattle in Argentina. Vet Parasitol. 2014;199(1–2):112–5.
12. Jiang YX, Tao W, Wan Q, Li Q, Yang YQ, Lin YC, et al. Zoonotic and potentially host-adapted Enterocytozoon bienuei genotypes in sheep and cattle in northeast China and an increasing concern about the zoonotic importance of previously considered ruminant-adapted genotypes. Appl Environ Microbiol. 2015;81(10):3326–35.
13. Zhao W, Zhang W, Yang F, Zhang L, Wang R, Cao J, et al. *Enterocytozoon bieneusi* in dairy cattle in the Northeast of China: genetic diversity of ITS gene and evaluation of zoonotic transmission potential. J Eukaryot Microbiol. 2015;62(4):533–60.

14. Matos O, Lobo ML, Xiao L. Epidemiology of *Enterocytozoon bieneusi* infection in humans. J Parasitol Res. 2012;2012:681424.

15. Li JQ, Luo NN, Wang CR, Qi M, Cao JK, Cui ZH, et al. Occurrence, molecular characterization and predominant genotypes of *Enterocytozoon bieneusi* in dairy cattle in Henan and Ningxia, China. Parasit Vectors. 2016;9:142.

16. Qi M, Jing B, Jian FC, Wang RJ, Zhang SM, Wang HY, et al. Dominance of *Enterocytozoon bieneusi* genotype I in dairy calves in Xinjiang, Northwest China. Parasitol Int. 2017;66(1):960–3.

17. Wang YT, Wang RJ, Ren GJ, Yu QZ, Zhang LX, Zhang SY, et al. Mult locus genotyping of *Giardia duodenalis* and *Enterocytozoon bieneusi* in dairy and native beef (Qinchuan) calves in Shaanxi province, northwestern China. Parasitol Res. 2016;115(3):1355–61.

18. Fayer R, Santín M, Macarisin D. Detection of concurrent infection of dairy cattle with * Blastocystis, Cryptosporidium, Giardia*, and *Enterocytozoon* by molecular and microscopic methods. Parasitol Res. 2012;111(3):1349–55.

19. Juránková J, Kamler M, Koudela B. *Enterocytozoon bieneusi* in bovine viral diarrhea virus (BVDV) infected and noninfected cattle herds. Res Vet Sci. 2013;94(1):100–4.

20. Fiuza VRD, Lopes CWG, de Oliveira FCR, Fayer R, Santín M. New findings of *Enterocytozoon bieneusi* in beef and dairy cattle in Brazil. Vet Parasitol. 2016;216:46–51.

21. Ma JB, Li P, Zhao XP, Yu HL, Wu WX, Wang YF, et al. Occurrence and molecular characterization of *Cryptosporidium* spp. and *Enterocytozoon bieneusi* in dairy cattle, beef cattle and water buffaloes in China. Vet Parasitol. 2015;207(3–4):220–7.

22. Zhang X, Wang Z, Su Y, Liang X, Sun X, Peng S, et al. Identification and genotyping of *Enterocytozoon bieneusi* in China. J Clin Microbiol. 2011;49(5):2066–8.

23. Li W, Cama V, Akinbo FO, Ganguly S, Kiulia NM, Zhang XC, et al. Multilocus sequence typing of *Enterocytozoon bieneusi*: lack of geographic segregation and existence of genetically isolated sub-populations. Infect Genet Evol. 2013;14:111–9.

24. Feng YY, Li N, DREAMS, Lobo ML, Matos O, Cama V, et al. Development of a multilocus sequence typing tool for high-resolution genotyping of *Enterocytozoon bieneusi*. Appl Environ Microbiol. 2011;77(14):4822–8.

25. Zhong ZJ, Li W, Deng L, Song Y, Wu KJ, Tian YN, et al. Multilocus genotyping of *Enterocytozoon bieneusi* derived from nonhuman primates in southwest China. PLoS One. 2017;12(5):e0176926.

26. Li W, Deng L, Yu XM, Zhong ZJ, Wang Q, Liu XH, et al. Multilocus genotypes and broad host-range of *Enterocytozoon bieneusi* in captive wildlife at zoological gardens in China. Parasitol Vectors. 2016;9:395.

27. Kerem MR, Wang RJ, He XY, Zhang LX, Li J, Rume FL, et al. Multilocus sequence typing of *Enterocytozoon bieneusi* in nonhuman primates in China. Vet Parasitol. 2014;200(1–2):13–23.

28. Tian GR, Zhao GH, Du SZ, Hu XF, Wang HB, Zhang LX, et al. First report of *Enterocytozoon bieneusi* from giant pandas (*Ailuropoda melanoleuca*) and red pandas (*Alulas fulgens*) in China. Infect Genet Evol. 2015;34:32–5.

29. Li W, Song Y, Zhong ZJ, Huang XM, Wang CD, Li CW, et al. Population genetics of *Enterocytozoon bieneusi* in captive giant pandas of China. Parasitol Vectors. 2017;10:499.

30. Zhao GH, Du SZ, Wang HB, Hu XF, Deng MJ, Yu SK, et al. First report of zoonotic *Cryptosporidium* spp., *Giardia intestinalis* and *Enterocytozoon bieneusi* in golden takins (*Budorcas taxicolor bedfordi*). Infect Genet Evol. 2015;34:394–401.

31. Deng L, Li W, Zhong ZJ, Gong C, Liu XH, Huang XM, et al. Molecular characterization and multilocus genotypes of *Enterocytozoon bieneusi* among horses in southwestern China. Parasitol Vectors. 2016;9:561.

32. Li W, Wan Q, Yu QL, Yang YQ, Tao W, Jiang YX, et al. Genetic variation of mini- and microsatellites and a clonal structure in *Enterocytozoon bieneusi* population in foxes and raccoon dogs and population differentiation of the parasite between fur animals and humans. Parasitol Res. 2016;115(7):2899–904.

33. Zhang XX, Cong WC, Lou ZL, Ma JG, Zheng WB, Yao QX, et al. Prevalence, risk factors and multilocus genotyping of *Enterocytozoon bieneusi* in farmed foxes (*Vulpes lagopus*), northern China. Parasitol Vectors. 2016;9:72.

34. Deng L, Li W, Yu XM, Gong C, Liu XH, Zhong ZJ, et al. First report of the human-pathogenic *Enterocytozoon bieneusi* from red-bellied tree squirrels (*Callosciurus erythraeus*) in Sichuan, China. PLoS One. 2016;11(9):e0163605.

35. Cai M, Guo Y, Pan B, Li N, Wang X, Tang C, et al. Longitudinal monitoring of *Cryptosporidium* species in pre-weaned dairy calves on five farms in Shanghai, China. Vet Parasitol. 2017;241:14–9.

36. Sulaiman IM, Fayer R, Lal AA, Trout JM, Schaefer FW, Xiao LH. Molecular characterization of microsporidia indicates that wild mammals harbor host-adapted *Enterocytozoon* spp. as well as human-pathogenic *Enterocytozoon bieneusi*. Appl Environ Microbiol. 2003;69(8):4495–501.

37. Ben Ayed L, Yang WL, Widmer G, Cama V, Ortega Y, Xiao LH. Survey and genetic characterization of wastewater in Tunisia for *Cryptosporidium* spp., *Giardia duodenalis*, *Enterocytozoon bieneusi*, *Cyclospora cayetanensis* and *Enterobius* spp. J Water Health. 2012;10(3):431–44.

38. Fayer R, Santín M, Trout JM. First detection of microsporidia in dairy calves in North America. Parasitol Res. 2003;90(5):383–4.