Genotyping and zoonotic potential of *Enterocytozoon bieneusi* in cattle farmed in Hainan Province, the southernmost region of China

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Received 7 October 2020, Accepted 9 November 2020, Published online 24 November 2020

Abstract – *Enterocytozoon bieneusi* is an intestinal pathogen that infects a wide range of species, including humans. Cattle constitute an important host for *E. bieneusi*; however, there is a scarcity of information on the prevalence and genotyping of *E. bieneusi* in cattle in the Hainan Province of China. In this study, PCR analysis of 314 fecal samples from cattle in six cities of Hainan was performed for genotype identification. The average prevalence of *E. bieneusi* in these animals was 9.9% (31/314), and ranged from 0.0% (0/12) to 20.5% (8/39). Five known genotypes – EbpC (*n* = 14), BEB4 (*n* = 12), J (*n* = 2), I (*n* = 1), and CHG5 (*n* = 1) – and a novel genotype: HNC-I (*n* = 1) – were identified. Genotypes EbpC and HNC-I were placed in zoonotic Group 1, and the remaining four genotypes (BEB4, J, I, and CHG5) were placed in Group 2. Since 93.5% of the genotypes found in the cattle (29/31) (EbpC, BEB4, J, and I) have previously been found in humans, these genotypes are probably involved in the transmission of microsporidiosis to humans.

Key words: *Enterocytozoon bieneusi*, Cattle, Genotyping, Hainan (China).

Résumé – Génotypage et potentiel zoonotique d’*Enterocytozoon bieneusi* chez les bovins élevés dans la province de Hainan, la région la plus au sud de la Chine. *Enterocytozoon bieneusi* est un pathogène intestinal qui infecte un large éventail d’espèces, y compris les humains. Le bétail constitue un hôte important pour *E. bieneusi*, mais les informations sur la prévalence et le génotypage d’*E. bieneusi* chez les bovins de la province de Hainan en Chine sont rares. Dans cette étude, une analyse PCR de 314 échantillons fécaux provenant de bovins dans six villes de Hainan a été réalisée pour l’identification du génotype. La prévalence moyenne d’*E. bieneusi* chez ces animaux était de 9,9 % (31/314), et variait de 0,0 % (0/12) à 20,5 % (8/39). Cinq génotypes connus, EbpC (*n* = 14), BEB4 (*n* = 12), J (*n* = 2), I (*n* = 1) et CHG5 (*n* = 1), et un nouveau génotype, HNC-I (*n* = 1) ont été identifiés. Les génotypes EbpC et HNC-I ont été placés dans le groupe zoonotique 1, et les quatre génotypes restants (BEB4, J, I et CHG5) ont été placés dans le groupe 2. Puisque 93,5 % (29/31) (EbpC, BEB4, J et I) des génotypes trouvés chez les bovins ont déjà été trouvés chez l’homme, ces génotypes sont probablement impliqués dans la transmission de la microporidiose à l’homme.

Introduction

*Enterocytozoon bieneusi*, a zoonotic intestinal pathogen, infects a wide range of species worldwide [20, 24]. Microsporidiosis occurs through the ingestion of infectious spores of *E. bieneusi* through contaminated soil, feces, surfaces, water, as well as by improper farming practices, such as using untreated animal manure as fertilizer directly on open crops or tillage land [20]. *Enterocytozoon bieneusi* has received considerable attention due to its known propensity to cause both water- and food-borne outbreaks of illness [44]. Sequence analysis of the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene has revealed more than 500 genotypes (142 in humans, of which 49 were also
identified in animals) [11, 20, 54]. Phylogenetic comparative analyses clustered all genotypes into eleven major genetic groups. Human cases have been reported to show infection with E. bieneusi in cattle from six cities of Hainan Province, the southernmost region of China, where, local yellow cattle breeding is very popular. Here, we evaluated the prevalence, genetic characteristics, and zoonotic potential of E. bieneusi in cattle from six cities of Hainan Province.

**Materials and methods**

**Ethics statement**

The study was initiated after obtaining written informed consent for animal use by farm owners. All animal experiments were reviewed and approved by the Ethics Committee of Hainan Medical University.

**Fecal specimen collection**

In all, 314 fecal samples were gathered from 10 cattle farms in six cities of Hainan Province between March and December 2019 (Fig. 1 and Table 2). The cattle farms were selected based only on the owners’ willingness to participate and the accessibility of animals for sampling. Samples were obtained from 30 months (n = 296). Cattle were divided into two groups: young aged ≤ 12 months (n = 18) and adults aged > 12 months (n = 296). Cattle were in good health at the time of sampling. Within 24 h of sampling, the labeled fecal bags were transported and stored in the laboratory at 4 °C and were processed within 48 h.

| Country            | Positive/examined (%) | Genotypesa (n)                          | Ref.       |
|--------------------|-----------------------|----------------------------------------|------------|
| Algeria            | 11/102 (10.8)         | BEB4 (4), BEB6 (2), BEB3 (1), I (1), J (1), PtEb XI (1), mixed (1) | [2]        |
| Argentina          | 10/70 (14.3)          | BEB4 (1); I (2), J (4); EbpC (1); BEB10 (1); D (1) | [5]        |
| Australia          | 49/471 (10.4)         | I (18), J (14), BEB4 (6), TAR_fc2 (6), TAR_fcI (1), TAR_fc3 (1) | [48]       |
| Brazil             | 79/452 (17.5)         | I (35), BEB8 (23), BEB4 (7), BEB13 (7), BEB12 (5), D (4), BEB11 (3), EbpA (1), BEB14–BEB17 (1 each) | [10, 12, 13, 19, 23, 26, 37, 38, 41–43, 46, 47, 49, 50] |
| China              | 1817/10504 (17.3)     | J (904); I (519); BEB4 (151), BEB6 (31), O (27), CM8 (18), COS-I (14), EbpC (14), CHN3 (14), D (13), CHN1 (11), CGC3 (11), CGC2 (8), CHC8 (7), CGC1 (6), CHN4 (6), CS-4 (6), Type IV (5), CM19 (5), BEB10 (3), CHG2 (2), CHG3 (2), CHN–DC1 (2), CHN–DC2 (2), CHN–DC3 (2), G (2), NECA1–NECA5, CHC1–CHC7, CHC9–CHC17, N, BEB8, CD6, H, CC4, CSX1–CSX2, CHN15, CM21, PN, and mixed (1 each) | [10, 12, 13, 19, 23, 26, 37, 38, 41–43, 46, 47, 49, 50] |
| Czech Republic     | 3797/240 (15.4)       | I (6) | [14]        |
| Germany            | 10/88 (11.4)          | I (2); J (4); F (1); M (1); N (1), I/J (1) | [6, 27]    |
| Iran               | 48/256 (18.8)         | D (22), J (9), M (5) | [16]        |
| Korea              | 805/538 (14.9)        | CebE (3), CebD (2), CebB (2), CebA (1), CebF (1), CebC (1) | [17]        |
| Portugal           | 2/2* (100.0)          | PtEbX (1), PtEbhXI (1) | [22]        |
| South Africa       | 9/50 (18.0)           | BEB4 (3); I (1); BEB3-like (4); D (1) | [1]        |
| United States      | 706/3306 (21.4)       | J (110); BEB4 (120); BEB2 (85); I (79), BEB1 (47); BEB8 (41); BEB5 (8); BEB9 (6); BEB3 (6); Peru 6 (1); D (1), BEB7 (1), Type IV (1) | [8, 9, 31–33, 36] |
| Slovakia           | 2/100 (2.0)           | I (2) | [40]        |
| Thailand           | 3/60 (5.0)            | D (3) | [39]        |

a The names of genotypes are from publications.

b The number of genotypes is not consistent with the number of positives because only some E. bieneusi isolates were genotyped in the Czech Republic and Korea.

c Only two isolates positive for E. bieneusi by microscopy after staining were genotyped in Portugal.

The genotypes previously found in humans are shown in bold.

**Table 1. ITS genotypes of Enterocytozoon bieneusi of natural infection identified in cattle worldwide.**
DNA extraction

All fecal specimens were filtered through sieve in distilled water, followed by centrifugation at 1500 × g for 10 min. A QIAamp DNA stool mini kit (QIAGen, Germany) was used to isolate the genomic DNA of each processed specimen (approximately 200 mg), following the manufacturer’s instructions. A total of 200 mL AE elution buffer was used to elute the DNA, followed by storage at −20 °C before PCR analysis.

Polymerase chain reaction (PCR) amplification

Enterocytozoon bieneusi-specific nested primers and cycle parameters designed by Hamed Mirjalal were used to amplify...
a 410 bp sequence in the ITS region of the rRNA gene using TaKaRa Taq DNA Polymerase [25]. The PCR products were analyzed using 1.5% agarose gel electrophoresis, followed by GelRed (Biotium Inc., USA) staining.

**Nucleotide sequencing and analysis**

The sequence accuracy of all *E. bieneusi*-positive PCR products (sequenced by Sangon Biotech Co., Ltd., China) was confirmed through bidirectional sequencing and the sequencing of additional PCR products. The Basic Local Alignment Search Tool (BLAST) and ClustalX 1.83 were used to compare the published GenBank sequences with the ones identified in this study to identify the genotypes of *E. bieneusi*. Genotypes that were identical to the genotypes deposited in the GenBank database were given the nomenclature system [30].

**Phylogenetic analysis**

A neighbor-joining phylogenetic tree was built using Mega X software, and the Kimura-2-parameter model with 1000 replications to evaluate the relationship between the novel ITS genotype and the known genotypes, and to confirm the gene group designation.

**Statistical analysis**

Fisher’s exact test and a Chi-square test were used to evaluate the difference in infection rates among different locations and ages, respectively, using SPSS v22.0 (IBM Corp., USA). A *p*-value < 0.05 was regarded as statistically significant.

**Nucleotide sequence accession numbers**

The GenBank database accession number of the identified nucleotide sequence was MT193626.

**Results and discussion**

Of the 314 fecal samples, 31 (9.9%) were *E. bieneusi*-positive, based on sequence analysis of the ITS region of the

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**Table 3.** ITS genotypes of natural *Enterocytozoon bieneusi* infections identified in cattle in China.

| Regions              | Positive/examined (%) | Genotypes (n)          | Ref. |
|----------------------|-----------------------|------------------------|------|
| Gansu                | 320/1414 (22.6)       | J (155), I (126), CGC3 (11), CGC2 (8), CGC1 (6), BEB4 (5), CM19 (5), BEB10 (3), CM21 (1) | [43] |
| Guangdong            | 160/1440 (11.1)       | I (91), J (60), D (4), BEB4 (3), EbpC (2) | [43] |
| Hebei and Tianjin    | 202/1040 (19.4)       | I (87), J (83), BEB4 (18), CHS8 (7), BEB6 (3), N (1), EbpC (1), CHC6 (1), CHC7 (1) | [12] |
| Henan                | 28/44 (6.0)           | I (16), J (7), BEB4 (5) | [23] |
| Hebei and Ningxia    | 214/879 (24.3)        | J (77), I (61), CM8 (18), BEB6 (17), BEB4 (15), EbpC (6), COS-1 (5), EbpA (2), D (2), BEB8, CD6, CHC1-CHC5, CHG2, CHG3, H, and O (1 each) | [19] |
| Henan and Ningxia    | 31/526 (5.9)          | J (10), CS-4 (7), I (3), BEB4 (2), EbpC (3), G (1), NECA1 - NECA5 (1 each) | [13] |
| Heilongjiang         | 40/133 (30.1)         | O (26), EbpA (2), J (2), I (2), CHN-DC1-CHN-DC3 (2 each), BEB4 (1), D (1) | [50] |
| Jiangsu              | 93/321 (29.0)         | BEB4 (22), J (40), I (31) | [38] |
| Jilin                | 177/1366 (13.0)       | J (144), I (26), BEB4 (11), Type IV (1), CHC17 (1) | [42] |
| Liaoning             | 35/93 (37.6)          | CHN3 (14); CHN1 (10); J (9); I (8); CHN4 (2) | [47] |
| Qingha and Yunnan    | 1/11 (9.1)            | J (1) | [13] |
| Shandong             | 50/105 (17.5)         | J (5), COS I (3), PN (1), BEB6 (1) | [49] |
| Shaanxi              | 39/198 (19.7)         | I (21), J (16), CHN1 (1), CSX1 (1) | [41] |
| Shanghai             | 21/653 (3.12)         | J (18), BEB4 (2), I (1) | [41] |
| Xinjiang             | 3/148 (2.0)           | I (1), J (2) | [23] |

The genotypes previously found in humans are shown in bold.

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A significant difference in the rate of occurrence of *E. bieneusi* was observed in cattle from the six cities (p < 0.05), with 20.5% (8/39) in Danzhou, 19.4% (6/31) in Wanning, 12.5% (12/96) in Lingshui, 4.9% (4/82) in Chengmai, 1.9% (1/54) in Haikou, and an absence of this parasite (0/12) in Ledong (Table 2).

Since the first report of *E. bieneusi* in calves in Germany, there have been 38 published epidemiological reports on *E. bieneusi* conducted in 14 countries, and the average infection rates in these countries range from 2.0% to 21.4% (Table 1). The infection rate of *E. bieneusi* based on cattle from 16 provinces of China, falls in the range of 2.0–37.6% (Table 3). This study reports the occurrence of *E. bieneusi* in cattle from Hainan Province. The differences in prevalence might be related to the sensitivity and specificity of detection methods, the health status of hosts, the experimental design, the overall sample size, animal practices, and so on. Like in other animals and humans, age appears to be a significant factor affecting the occurrence of *E. bieneusi* in cattle [51]. In the present study, the prevalence of *E. bieneusi* was 22.2% (4/18) in young animals /C20 12 months and 9.1% (27/296) in adult animals > 12 months. Although the infection rates in calves were higher than those in adults, the differences were not significant ($\chi^2 = 1.966, p > 0.05$) (Table 2). A study by Ma et al. revealed

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**Figure 2.** Phylogenetic tree based on neighbor-joining (N-J) analysis of ITS sequences. Phylogenetic relationships between the *E. bieneusi* genotypes identified in cattle here and other known genotypes deposited in GenBank were inferred by an N-J analysis of ITS sequences based on genetic distance by the Kimura two-parameter model. The numbers on the branches are percent bootstrapping values from 1000 replicates. Each sequence is identified by its accession number, host origin, and genotype designation. *Enterocytozoon bieneusi* genotype CSK2 (KY706128) was used as the outgroup. The squares and triangles filled in black indicate novel and known genotypes identified in this study, respectively.

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E. bieneusi infection rates in juveniles, post-weaned calves, pre-weaned calves, and adults of 4.5% (6/134), 7.7% (8/104), 10% (1/10), and 3.9% (13/332), respectively [23]. Similarly, da Fiuza et al. reported that pre-weaned calves (27.6%, 21/76) and post-weaned calves (28.8%, 44/153) showed a higher rate of prevalence of E. bieneusi compared with heifers (14.1%, 12/85) and adults (1.4%, 2/138) [4]. Meanwhile, Li et al. showed that calves aged < 3 months (29.3%, 127/434) and 3–12 months (23.9%, 63/264) had higher infection rates than juveniles and adults (13.3%, 24/181) [16]. In accordance with these results, it was supposed that age was negatively correlated with the prevalence of E. bieneusi in cattle, probably due to the underdeveloped immune systems of the young animals.

Here, we identified one novel genotype (HNC-I) and five known genotypes (EbpC, BEB4, J, I, and CHG5). The novel genotype showed high similarity to genotype EbpC (AF076042), with one base variation at position 237 (C → T). Out of the six genotypes, the most prevalent genotype was EbpC (14 specimens), which was found in four of the six locations, followed by BEB4 (12 specimens), but this genotype was only found in Wanning. Genotype J was found in two cattle from Chengmai. The remaining three genotypes I, CHG5, and HNC-I were found in a single specimen, with the former from Danzhou and the latter two from Wanning. These results differed from those reported from the other regions of China. For example, in Gansu, Guangdong, Henan, Ningxia, Jiangsu, Shaanxi, and Xinjiang provinces, genotypes J and I were reported to be the dominant genotypes, and in Heilongjiang, genotype O was dominant (Table 3). Meanwhile, region-specific difference in genotype constitutions of E. bieneusi can also be observed in cattle in some studies, such as genotype D in Iran [16]. Therefore, the genotype distributions of E. bieneusi in cattle differed by region, but the reason behind this phenomenon is unclear.

In the present study, human-pathogenic genotypes EbpC, BEB4, J and I were observed with high occurrence (93.5%, 29/31). Genotype EbpC has been detected in humans, such as in cancer patients in Iran [25], in immunocompetent patients in the Czech Republic [29], in children in Peru and China [3, 45], and in HIV-positive patients in Peru, China, Iran, Thailand, and Vietnam [7, 18, 21, 25, 35, 41]. It was also found in more than 15 animal species and water samples [20]. Likewise, genotypes BEB4, J, and I were also found in humans [28, 47], non-human primates [15, 46], and other animals [20], and they have been documented in cattle (Table 1). This suggests that cattle infected with genotypes EbpC, BEB4, J, and I may facilitate transmission to other animals and humans.

The remaining genotype CHG5 and the novel genotype HNC-I were first identified in cattle here. Genotype CHG5 has been reported in goats with a wide distribution in China [34, 53]. We also observed this genotype in the Asiatic brush-tailed porcupines in Hainan Province [52]. Thus, the detection of the same genotype (CHG5) in multiple species (cattle, goats, and rodents) in the same region (Hainan, China) suggests a vast host range along with the possibility of cross-species transmission among cattle, goats, and rodents.

The phylogenetic analysis revealed that EbpC and HNC-I, identified in this study, were divided into zoonotic Group 1, whereas genotypes BEB4, J, I, and CHG5 belong to Group 2 (Fig. 2). In total, 94.0% (79/84) of the genotypes identified in cattle clustered into Group 1 or 2 (except for genotypes CX1, CX2, TAR_fc3, CAM2, and S7) [20]. These findings suggest that E. bieneusi-infected cattle represent a potential threat to humans.

Conclusion

This study is the first evaluating the infection rates, genotype characteristics, and zoonotic potential of E. bieneusi in cattle from Hainan Province. Our results revealed a prevalence rate of 9.9% (31/314) for E. bieneusi within five of six cities in Hainan, China. We identified five known genotypes and a novel genotype. Genotype EbpC and novel genotype HNC-I were grouped into zoonotic Group 1, while genotypes BEB4, J, I and CHG5 were placed in Group 2. The observed high occurrence (93.5%, 29/31) of zoonotic genotypes (EbpC, BEB4, J, and I) emphasizes the possible role of cattle in the transmission of E. bieneusi to humans, which requires further investigations to reduce the threats posed by these animals to public health.

Acknowledgements. This work was supported by the Young Talents Science and Technology Innovation Project of the Hainan Association for Science and Technology (QCMX201802); Hainan major science and technology project (ZDKJ2016017-01); the Innovation Research Team Project of the Hainan Natural Science Foundation (2018CXTD340); the National Natural Science Foundation of China (No. 81672072 and No. 81760378), and the Graduate Student Innovation Foundation of colleges and universities of Hainan Province, 2019 (Hys2019-287). The funders had no role in the study design, data collection, data interpretation, or the decision to submit the work for publication.

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Cite this article as: Zheng X-L, Zhou H-H, Ren G, Ma T-M, Cao Z-X, Wei L-M, Liu Q-W, Wang F, Zhang Y, Liu H-L, Xing M-P, Huang L-I, Chao Z & Lu G. 2020. Genotyping and zoonotic potential of Enterocytozoon bieneusi in cattle farmed in Hainan Province, the southernmost region of China. Parasite 27, 65.