

Prevalence of *Cryptosporidium* spp. and *Enterocytozoon bieneusi* in beef cattle in the Hebei Province of China*

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Summary

Cryptosporidium spp. and *Enterocytozoon bieneusi* (*E. bieneusi*) are two common opportunistic pathogens that can infect humans and animals worldwide. The available data on the prevalence of these pathogens is insufficient in Hebei Province, China, which is located in the Bohai Sea region. In the present study, 718 fecal specimens of native beef cattle from eight farms in Hebei Province were examined for the presence of *Cryptosporidium* spp. and *E. bieneusi* using nested PCR targeting the small subunit (SSU) rRNA gene of *Cryptosporidium* spp. and the internal transcribed spacer (ITS) of *E. bieneusi*. The prevalence of *Cryptosporidium* spp. was 9.2% (66/718) and *E. bieneusi* was 8.6% (62/718). *C. andersoni* (n = 56) and *C. parvum* (n = 10) were identified in this study, and all *C. parvum*-positive specimens belonged to IIdA20G1 in the gp60 gene. The ITS sequence analysis obtained seven known *E. bieneusi* genotypes, including J (n = 48), BEB4 (n = 4), CHC8 (n = 3), EbpC (n = 2), I (n = 2), D (n = 2), and BEB6 (n = 1). Genotype D and EbpC belonged to the zoonotic Group 1, while the other genotype belonged to the host-adapted Group 2. This is the first report on the occurrence of *E. bieneusi* in beef cattle in Hebei Province. In this study, the presence of zoonotic *C. parvum* and two *E. bieneusi* genotypes suggest that cattle can be a potential zoonotic source for human or animal infection.

Keywords: beef cattle, *Cryptosporidium* spp., *Enterocytozoon bieneusi*, prevalence

Cryptosporidium spp. and *Enterocytozoon bieneusi* are important enteric zoonotic pathogens in humans and a wide variety of animals, causing acute or chronic diarrhea and other gastrointestinal symptoms (31). Humans can be infected via fecal-oral routes through contact with contaminated fomites or ingestion of polluted food or water, etc. (2). There are reportedly no useful and efficient treatments for the *Cryptosporidium* spp. and *E. bieneusi* (14).

Amongst the 45 valid *Cryptosporidium* species and > 120 genotypes, *C. parvum*, *C. bovis*, *C. ryanae*, and *C. andersoni* are the species most commonly respon-

sible for infections in cattle (3, 7, 8). Although a wide range of *Cryptosporidium* species has been identified in cattle throughout the world, *C. parvum*, the most important species causing zoonotic cryptosporidiosis, is responsible for watery diarrhea, lethargy, and sometimes death in calves, while *C. andersoni*, *C. bovis*, and *C. ryanae* are mostly identified in asymptomatic cattle (16, 19).

E. bieneusi is an obligate intracellular eukaryotic parasite, with > 500 genotypes identified to date based on sequence analysis of the ITS region of the rRNA gene (10, 18, 23). So far, *E. bieneusi* genotypes can be divided into 11 major groups, including the zoonotic Group 1 and potentially host-adapted Groups 2-11. According to growing evidence, some genotypes in Group 2, which were originally regarded as ruminant-specific, have been detected in human samples, suggesting these groups could also have zoonotic potential

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(18). Currently, more than 80 genotypes have been identified in cattle; among them at least 17 genotypes have also been identified in humans, and these data indicate the important role of cattle in the epidemiology of *E. bieneusi*, while cattle infected with *E. bieneusi* may pose a threat to public health (38).

In China, *Cryptosporidium* spp. and *E. bieneusi* infections have been discovered in dairy cattle and yaks in several areas (6, 35). However, there are only a few publications on the distribution of *Cryptosporidium* spp. and *E. bieneusi* in Chinese yellow cattle (*Bos taurus* domesticated cows), which are native beef cattle in China (27, 33, 34, 36).

Hebei Province is in the Bohai Sea region and around Beijing, China's political, economic, and cultural center. It is one of the main beef cattle producing provinces in northern China, with many beef cattle being sold to Beijing, Tianjin, and other coastal cities. To date, only two studies on *Cryptosporidium* spp. in cattle in Hebei Province have been published (35, 36) and there has been no report of *E. bieneusi* infections in cattle in Hebei Province. According to two studies, conducted in the Hebei Province, the IIdA20G1 of *C. parvum* were isolated from dairy cattle and neonatal calves (36, 37). Thus, the objective of the present study was to molecularly characterize and estimate the prevalence of these two pathogens in beef cattle in Hebei Province.

Material and methods

Study site. The research was conducted in Hebei Province (36°05'–42°40' N and 113°27'–119°50' E), which is located in northern China. This province is composed of the Hebei Plain in the east and south and the mountain range along the northern and western frontiers. Hebei has a temperate continental monsoon climate, with rain and heat over the same period, and four distinct seasons.

The annual average temperature of the whole province is between 4 and 13°C, –3 to 24°C in spring, 18 to

40°C in summer, 11 to 32°C in autumn, and –11 to 11°C in winter.

Specimen collection. A total of 718 fresh fecal specimens were randomly collected from native beef cattle (the local pure bred and improved Ji Nan beef cattle breed and Luxi cattle breed introduced from Shandong Province) on 8 farms scattered in Chengde, Qinhuangdao, Cangzhou, Shijiazhuang, Tangshan, and Langfang city in Hebei Province from March to December 2020, corresponding to spring, summer, autumn, and winter seasons in the northern hemisphere (Fig. 1). Considering the very small number of < one month old beef cattle, beef cattle in this study were divided into four age groups: pre-weaned (< 3 months old), post-weaned (3–12 months old) calves, juveniles (13–24 months old), and adults (> 24 months old) (Tab. 2). All farms were small, with fewer than two hundred animals, and the beef cattle are kept in an open-air cowshed. Except for the pre-weaned calves (less than 3 months), other cattle were feeding together in a common area in which the ground often has fecal accumulation. The pre-weaned calves are fed with breast milk or artificial milk, and other cattle fed freely on the forage poured into the trough three times a day. No obvious clinical signs including diarrhea were observed in the sampled animals. Approximately 30–40 g of fresh feces were collected immediately after its defecation onto the ground or from the rectum of beef cattle using a sterile disposable latex glove, respectively. To avoid fecal material that had contacted the ground, only the top layer of the feces was gathered and spread on the ground. The fresh samples were placed in a clean and sealed bag marked with relevant information, transported to the laboratory in coolers with ice packs, and stored at 4°C for further inspection.

DNA extraction and PCR analysis. Genomic DNA was extracted from 250 mg of each fecal sample using the Stool DNA Extraction Kit (Solarbio, Beijing, China), according to the manufacturer's instructions, and the obtained DNA was stored at –20°C before PCR analysis.

Cryptosporidium spp. and *E. bieneusi* were identified as described previously, based on the nested PCR amplification of the SSU rRNA gene and the ITS gene (22, 29). All *C. parvum*-positive specimens were subtyped by the 60-kD glycoprotein (gp60) gene (4). The secondary PCR products were electrophoresed by 1.5% agarose gel and visualized on a UV transilluminator after ethidium bromide staining.

Sequence analysis. The expected-size amplicons of the were sequenced bidirectionally with the corresponding secondary PCR primers. The sequences obtained were edited with BioEdit 7.1 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and aligned with reference sequences from GenBank via BLAST analysis (<http://blast.ncbi.nlm.nih.gov>) and using ClustalX 2.0.11 (<http://clustal.org/>) to identify the species/assemblage/genotype of the three pathogens under analysis. Phylogenetic analysis was conducted by using the neighbor-joining method executed with

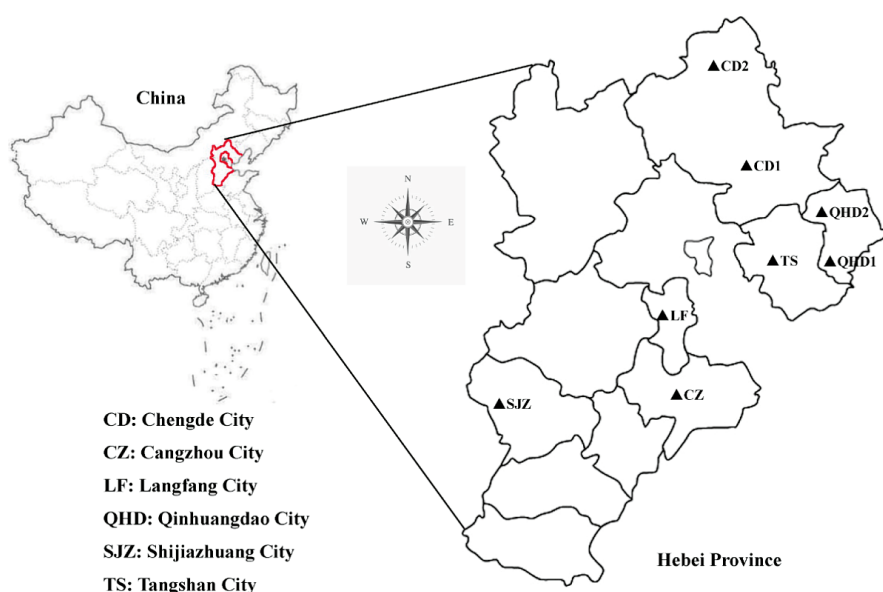


Fig. 1. Geographic map of the sampling locations (triangles) in Hebei Province, China

the Kimura 2-parameter model in MEGA 6.06 (<http://www.megasoftware.net/>), with 1,000 bootstrap replicates. The SSU rRNA gene of *Plasmodium cathemerium* (AY625607), and the dog-specific genotype of *E. bieneusi* (DQ885585) were used as the out groups for *Cryptosporidium* spp. and *E. bieneusi*, respectively. Representative nucleotide sequences generated in this study were deposited in GenBank under OK425867-OK425871 for *Cryptosporidium* spp., and OK393645-OK393650 for *E. bieneusi*.

Statistical analysis. The infection rates of 95% confidence intervals (CI) were calculated by the Wald method of SPSS for Windows (release 17.0 standard version, SPSS Inc., Chicago, IL, USA). The differences in corresponding infection rates among age groups and sampling seasons were determined by the Chi-square test, and differences were considered significant at $p < 0.05$.

Results and discussion

Prevalence of *Cryptosporidium* spp. and *E. bieneusi*. From the 718 beef cattle examined, sixty-six samples (9.2%) were positive for *Cryptosporidium* spp. Four farms were positive for *Cryptosporidium* spp., with infection rates ranging from 8.1% to 11.7%

(Tab. 1). The overall prevalence of *E. bieneusi* was 8.6% (62/718, 95% CI: 1.893-1.934), and the highest infection rate was observed in Chengde farm, followed by that in Qinhuangdao farm and Cangzhou farm. No mixed infections were detected in this study (Tab. 1). As shown in Table 2, the prevalence of *Cryptosporidium* spp. in summer was significantly higher than in other season groups ($p < 0.05$). There was no significant difference in season groups for *E. bieneusi* ($p > 0.05$). The *Cryptosporidium* spp. infection rate in pre-weaned calves was higher than post-weaned calves, juveniles and adults ($p < 0.05$; Tab. 2). The infection rates of *E. bieneusi* were 7.9%, 8.1%, 7.4% and 8.3% in pre-weaned calves, post-weaned calves, juveniles and adults, respectively ($p > 0.05$; Tab. 2).

***Cryptosporidium* species, and *C. parvum* subtype.** Based on sequence analysis of the SSU rRNA gene, two *Cryptosporidium* species were observed in this study, including *C. andersoni* ($n = 56$) and *C. parvum* ($n = 10$) (Tab. 1, and Fig. 2). All *C. andersoni* sequences obtained in this study shared 100% homology with cattle-derived isolate (KT884488), swan-derived

Tab. 1. Prevalence and species/genotype distribution of two enteric pathogens in beef cattle in Hebei Province

Area	Number of tests	<i>Cryptosporidium</i> spp.				<i>Enterocytozoon bieneusi</i>		
		positive number	infection rate %	species	<i>C. parvum</i> subtype (n)	positive number	infection rate %	genotype
Chengde	247	20	8.1	<i>C. andersoni</i> (15), <i>C. parvum</i> (5)	IIdA15G1(2), IIdA19G1(3)	22	8.9	J (8), BEB4 (3), CHC8 (3), I (2)
Qinhuangdao	223	26	11.7	<i>C. andersoni</i> (23), <i>C. parvum</i> (3)	IIdA15G1(3)	23	10.3	J (19), EbpC (2), D (2)
Gangzhou	179	19	10.6	<i>C. andersoni</i> (17), <i>C. parvum</i> (2)	IIdA15G1(2)	17	9.5	J (15), BEB4 (1), BEB6 (1)
Shijiazhuang	9	1	11.1	<i>C. andersoni</i> (1)		0	0.0	–
Tangshan	35	0	0.0	–	–	0	0.0	–
Langfang	31	0	0.0	–	–	0	0.0	–
Total	718	66	9.2	<i>C. andersoni</i> (56), <i>C. parvum</i> (10)	IIdA15G1(7), IIdA19G1(3)	62	8.6	J (48), BEB4 (4), CHC8 (3), EbpC (2), I (2), D (2), BEB6 (1)

Tab. 2. Prevalence of *Cryptosporidium* spp. and *E. bieneusi* in beef cattle in Hebei Province in different seasons and age groups

Factor	Sample size	<i>Cryptosporidium</i> spp.				<i>E. bieneusi</i>				
		no. of positives	positive rate %	p value	OR (95% CI)	no. of positives	positive rate %	p value	OR (95% CI)	
Seasons	spring	105	5	4.8		reference	5	4.8		reference
	summer	415	49	11.8	0.048	2.678 (1.039-6.898)	46	11.0	0.078	2.493 (0.965-6.440)
	autumn	165	12	7.3	0.568	1.569 (0.536-4.588)	11	6.8	0.703	1.429 (0.482-4.235)
	winter	33	0	0.0			0	0.0		
Age (months)	pre-weaned calves	76	12	15.8		reference	6	7.9		reference
	post-weaned calves	173	13	7.5	0.045	0.433 (0.188-1.000)	14	8.1	1.000	1.027 (0.379-2.784)
	juveniles	257	18	7.0	0.019	0.402 (0.184-0.877)	19	7.4	1.000	0.931 (0.358-2.422)
	adults	265	21	7.9	0.041	0.459 (0.214-0.982)	22	8.3	1.000	1.056 (0.412-2.707)
	unknown	23	0	0.0			1	4.3	0.907	0.530 (0.061-4.647)

Explanations: the four seasons from spring to winter were defined as January-March, April-June, July-September, and October-December, respectively

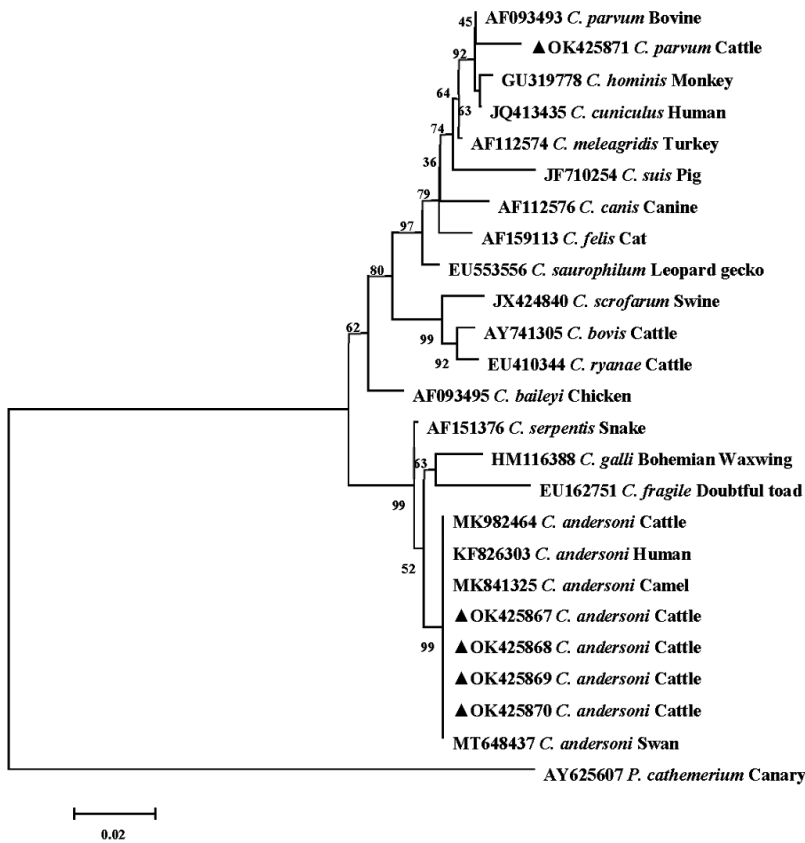


Fig. 2. Phylogeny of *Cryptosporidium* spp. based on the neighbor-joining analysis of the small subunit rRNA. The *Cryptosporidium*-positive specimen identified in this study is indicated by filled triangles

isolate (MT648437), camel-derived isolate (MK841325), and human-derived isolate (KF826303) in China. The ten *C. parvum* sequences obtained in this study shared 100% homology with a cattle-derived isolate in China (MT002720). By sequence analysis of the gp60 gene, 9 of the 10 *C. parvum*-positive specimens were efficaciously subtyped as IIdA20G1 (MK252647). *C. andersoni*, the dominant species in the present study, was identified in 5 farms, and *C. parvum* was isolated in 3 farms (Tab. 1). Although *C. andersoni* was detected in all age groups of cattle, all *C. parvum* were only identified in pre-weaned cattle (data not shown).

***E. bieneusi* genotypes.** Seven known ITS genotypes were detected among the 62 *E. bieneusi* specimens, including J (n = 48), BEB4 (n = 4), CHC8 (n = 3), EbpC (n = 2), I (n = 2), D (n = 2), and BEB6 (n = 1) (Tab. 1). Between them, genotype D and EbpC belonged to the zoonotic Group 1, while the other genotype belonged to the host-adapted Group 2 (Fig. 3). The dominant genotype J was detected in 4 farms. Genotype BEB4 was observed in 3 farms. Genotype CHC8 was seen in 2 farms. Genotype EbpC, I, D,

and BEB6 were detected in 4 farms, respectively (Tab. 1). Although genotype J was the most common genotype in all age groups of cattle, preweaned cattle had a higher diversity of genotypes, with five genotypes being found (data not shown).

In this investigation, the overall infection rate of *Cryptosporidium* spp. and *E. bieneusi* in beef cattle in Hebei Province was 9.2% and 8.6%. The prevalence of *Cryptosporidium* spp. was lower than that in Henan local beef cattle (26.5%), and Qinchuan cattle (20.2%), but higher than that in Longjiang Wagyu cattle (6.4%), Tibetan yellow cattle (0.3%), and Yunling cattle (0.8%) (13, 28, 30). The

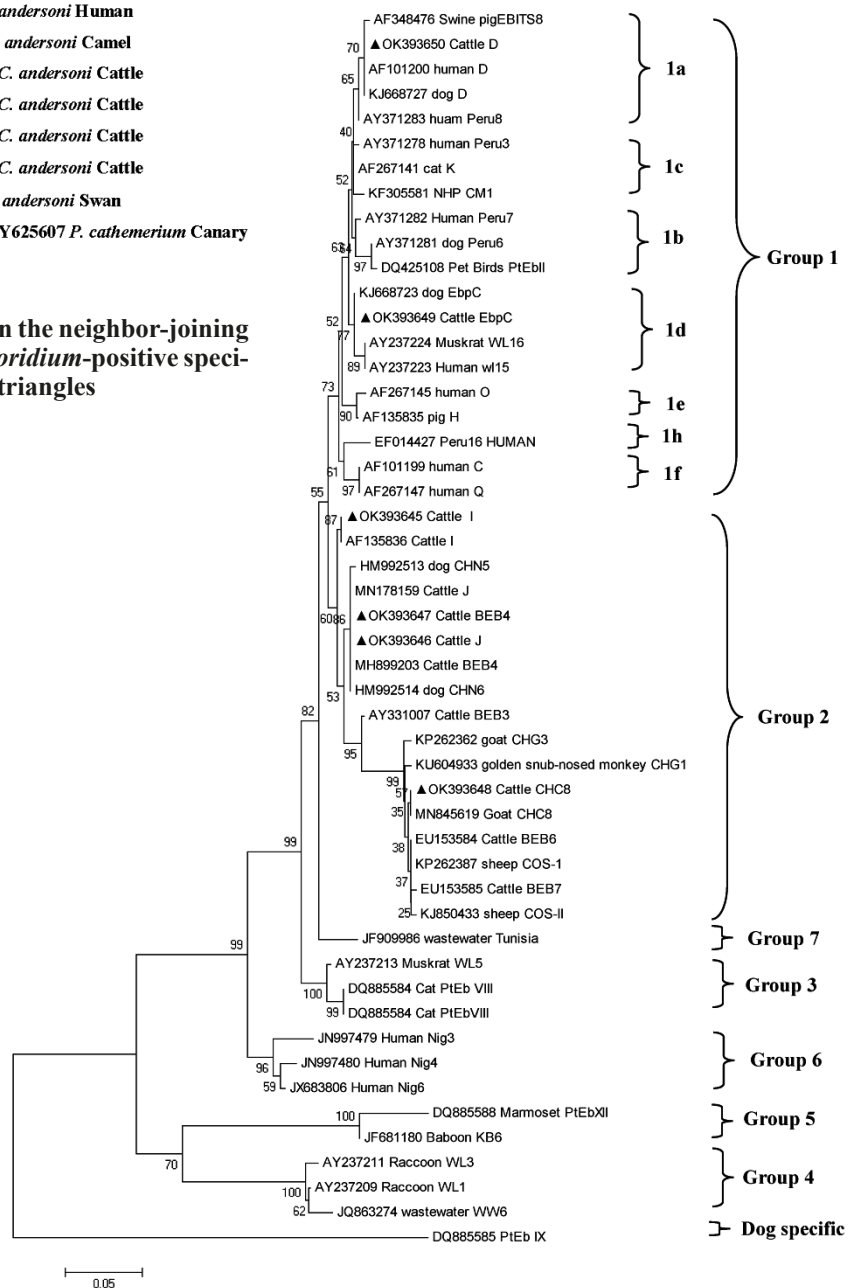


Fig. 3. Phylogenetic relationships based on neighbor-joining analysis of the *E. bieneusi* ITS genotypes identified in this study and other reported genotypes. Genotypes detected in this study are indicated by filled triangles

prevalence of *E. bienersi* was higher than that in Tibetan yellow cattle (5.5%) and Longjiang Wagyu cattle (7.1%) but was lower than in Qinchuan cattle (20.0%), and Henan local beef cattle (9.4%) (14, 24, 28, 30). Additionally, the prevalence of *Cryptosporidium* spp. in summer was significantly higher than in other season groups ($p < 0.05$). Some reports show that precipitation, temperature, and humidity in different seasons can also be related to the infection rates of *Cryptosporidium* spp. (6). Hence, higher precipitation, temperature, and humidity might have contributed to the higher prevalence of *Cryptosporidium* spp. detected in summer in this study. The highest infection rate of *Cryptosporidium* spp. was seen in pre-weaned calves, which agrees with the general observation in numerous previous studies in *Cryptosporidium* spp. (6, 25). There is no difference in the infection rates of *E. bienersi* between four age groups in this study, which accord with a previous study in China (14). Several previous studies showed that post-weaned calves were more susceptible to *E. bienersi* than pre-weaned calves (1, 20, 26). However, Jurankova et al. (9) reported that the infection rate of *E. bienersi* progressively decreased in cattle with increased age. Further epidemiological studies are needed to clarify the risk factors affecting the prevalence of these two pathogens.

So far, more than ten *Cryptosporidium* species, including *C. andersoni*, *C. bovis*, *C. parvum*, *C. ryanae*, and *C. xiaoi*, have been detected in cattle (5, 6). However, only two species, *C. andersoni* and *C. parvum*, were identified in the present study, which was not in agreement with the species reported previously in native beef cattle such as Qinchuan cattle, Longjiang Wagyu cattle, and Yunling cattle in China (13, 14, 28, 30, 33). But the *Cryptosporidium* species identified in this study is identical to the species reported previously in dairy cattle in Beijing (11). *C. andersoni* was the dominant species in this study, which agreed with the conclusion that *C. andersoni* was the most common species of *Cryptosporidium* in beef cattle in China. Some reports showed that *C. bovis* and *C. parvum* were the dominant *Cryptosporidium* species in pre-weaned cattle, and *C. andersoni* was the most abundant species in post-weaned cattle in China (6). The observation in this study is consistent with this conclusion.

The nine *C. parvum* isolates, successfully sequenced on the gp60 gene, were identified as subtype IIdA20G1, which was consistent with the results in neonatal calves in Hebei Province and Longjiang Wagyu and cattle dairy cattle in Heilongjiang Province (21, 36, 37). Currently, the IIdA20G1 subtype has been reported in livestock and humans in many countries, especially in some territories of the Middle East (37). Some reports showed that the IIdA20G1 subtype was associated with young age and diarrhea in cattle. Control measures including preventive hygiene measures and good management should be implemented to reduce the occurrence of outbreaks of *C. parvum*.

In this study, seven known ITS genotypes of *E. bienersi* were identified in beef cattle, with D and EbpC belonging to the zoonotic Group 1 and the other genotype belonging to the host-adapted Group 2. Genotype D, which is the most common genotype infecting humans, has been detected in more than 25 species of mammals and identified in two specimens in this study (15). The zoonotic genotype EbpC, which has previously been identified worldwide in humans, domestic and wild animals such as pigs, cattle, deer, sheep, dogs, horses, pandas, mice, foxes, beavers, and raccoons was also identified in two specimens in this study (27). Genotype J was the predominant genotype, and genotype I was only observed in two specimens, which is not consistent with that reported in beef cattle in Henan and Shaanxi, and dairy cattle in Hebei and Tianjin, whereas genotype I and J were the dominant genotypes (24, 37). Recently, genotype J and I have been discovered in human, non-human primates (NHPs) in China, as well as in other animals (12, 32). Genotype BEB4, previously identified in humans, pigs, and NHPs, and genotype BEB6, previously identified in humans, NHPs, and many other animals, have also been identified in cattle. Genotype CHC8 has also been previously identified in cattle and goats (17). The emergence of two zoonotic genotypes in Group 1, and the fact that four genotypes in Group 2 have been identified in humans in some reports indicate that beef cattle might have an important role in the epidemiology of *E. bienersi* as reservoir hosts for zoonotic infections (12, 17, 24, 32, 37).

In conclusion, we focused on the prevalence and genetic diversity of two intestinal pathogens from beef cattle in Hebei Province. The presence of zoonotic *C. parvum* and two *E. bienersi* genotypes in Group 1 and the identification of low zoonotic *C. andersoni* and four *E. bienersi* genotypes in Group 2, showed that beef cattle may act as biological disseminators in the transmission of these pathogens to humans. Therefore, further molecular epidemiological studies are required for a better understanding of the transmission dynamics and the significance for public health of these pathogens.

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