



Prevalence and molecular characterization of *Cryptosporidium* spp., *Enterocytozoon bieneusi*, and *Giardia duodenalis* in dairy cattle in Ningxia, northwestern China

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Abstract

Cryptosporidium spp., *Enterocytozoon bieneusi*, and *Giardia duodenalis* are common intestinal pathogens that infect humans and animals. To date, research regarding these three protozoa in the Ningxia Hui Autonomous Region (Ningxia) has mostly been limited to a single pathogen, and comprehensive data on mixed infections are unavailable. This study aimed to evaluate the zoonotic potential of these three protozoa. In this study, small subunit ribosomal RNA (SSU rRNA) and 60 kDa glycoprotein (*gp60*) genes of *Cryptosporidium*; internal transcribed spacer (ITS) gene of *E. bieneusi*; and SSU rRNA, glutamate dehydrogenase (*gdh*), triosephosphate isomerase (*tpi*), and beta-giardin (*bg*) genes of *G. duodenalis* were examined. DNA extraction, polymerase chain reaction, and sequence analysis were performed on fecal samples collected from 320 dairy cattle at three intensive dairy farms in Ningxia in 2021 to determine the prevalence and genetic characteristics of these three protozoa. The findings revealed that 61.56% (197/320) of the samples were infected with at least one protozoan. The overall prevalence of *Cryptosporidium* was 19.38% (62/320), *E. bieneusi* was 41.56% (133/320), and *G. duodenalis* was 29.38% (94/320). This study identified four *Cryptosporidium* species (*C. bovis*, *C. andersoni*, *C. ryanae*, and *C. parvum*) and the presence of mixed infections with two or three *Cryptosporidium* species. *C. bovis* was the dominant species in this study, while the dominant *C. parvum* subtypes were IIdA15G1 and IIdA20G1. The genotypes of *E. bieneusi* were J, BEB4, and I alongside the novel genotypes NX1–NX8, all belonging to group 2, with genotype J being dominant. *G. duodenalis* assemblages were identified as assemblages E, A, and B, and a mixed infection involving assemblages A + E was identified, with assemblage E being the dominant one. Concurrently, 11 isolates formed 10 different assemblage E multilocus genotypes (MLGs) and 1 assemblage A MLG and assemblage E MLGs formed 5 subgroups. To the best of our knowledge, this is the first report on mixed infection with two or three *Cryptosporidium* species in cattle in Ningxia and on the presence of the *C. parvum* subtype IIdA20G1 in this part of China. This study also discovered nine genotypes of *E. bieneusi* and novel features of *G. duodenalis* assemblages in Ningxia. This study indicates that dairy cattle in this region may play a significant role in the zoonotic transmission of *Cryptosporidium* spp., *E. bieneusi*, and *G. duodenalis*.

Keywords *Cryptosporidium* · *Enterocytozoon bieneusi* · *Giardia duodenalis* · Prevalence · China

Introduction

Cryptosporidium spp., *Enterocytozoon bieneusi*, and *Giardia duodenalis* are three important intestinal protozoa (Yang et al. 2022) that can cause clinical symptoms such as weight loss, malnutrition, and diarrhea in various hosts

(Wang et al. 2022a). The infections caused by these parasites are self-limiting in healthy individuals; however, the infection period may be prolonged or even life threatening in immunocompromised individuals (Yang et al. 2022). These pathogens spread primarily through the fecal–oral route (Fan et al. 2021).

To date, at least 45 valid *Cryptosporidium* species and approximately 120 genotypes have been reported worldwide (Wang et al. 2022a), among which, 19 *Cryptosporidium* species and 4 genotypes have been reported in humans (Ryan et al. 2021) and 12 *Cryptosporidium* species have been reported in

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cattle (Gong et al. 2017), with *C. parvum*, *C. bovis*, *C. ryanae*, and *C. andersoni* being the most dominant species (Feng et al. 2018; Gong et al. 2017; Hatam-Nahavandi et al. 2019; Santin 2020; Zahedi and Ryan 2020; Zhang et al. 2022). Zoonotic cryptosporidiosis is mainly caused by *C. parvum* (Feng et al. 2018; Guo et al. 2022; Zhang et al. 2022). In *E. bienersi*, > 685 distinct genotypes have been reported (Qin et al. 2022) and classified into 11 distinct groups (groups 1–11) via phylogenetic analysis, with > 90% of the genotypes belonging to group 1 or 2 (Li et al. 2019b; Wang et al. 2022a; Yang et al. 2022). Group 1 is a zoonotic evolutionary group (Lichtmannsperger et al. 2023; Wang et al. 2022b) in which the genotypes A, D, EbpC, and IV are the most common (Santín and Fayer 2011; Wang et al. 2022b). Some genotypes from groups 2, 5, and 6 can also infect humans (Dong et al. 2022). BEB4, BEB6, I, and J in group 2 constitute the main genotypes that infect humans and animals (Li et al. 2019b), whereas genotypes BEB4, I, and J are most commonly reported in cattle globally (Lichtmannsperger et al. 2023). *G. duodenalis* is currently classified into eight genetically distinct but morphologically identical lineages, namely assemblages A–H (Qi et al. 2016; Ryan et al. 2019; Xiao and Feng 2017). Assemblages A and B exhibit higher zoonotic risk (Kiani-Salmi et al. 2019; Taghipour et al. 2022b; Zhang et al. 2021), while assemblages C–F have also been identified in human patients (Kiani-Salmi et al. 2019). Assemblage E is the most common genotype in cattle globally (Cai et al. 2021; Feng and Xiao 2011; Minetti et al. 2014; Santin 2020; Taghipour et al. 2022b; Zahedi and Ryan 2020; Zhang et al. 2021), followed by assemblages A and B (Feng and Xiao 2011; Qi et al. 2016; Zhang et al. 2021). Assemblages C, D (Ryan and Cacciò 2013), and F have also been reported in cattle (Cardona et al. 2015; Ryan and Cacciò 2013).

Investigation of the three protozoa in cattle via a molecular method revealed that the global pooled prevalence of *Cryptosporidium* was 29.1% (Hatam-Nahavandi et al. 2019) and the Chinese pooled prevalence of *Cryptosporidium* was 17.0% (Cai et al. 2019). Furthermore, the global pooled prevalence of *E. bienersi* has been reported to be approximately 14% (Taghipour et al. 2022b), 12.0% (Qin et al. 2022), or 16.6% (Ruan et al. 2021), while its Chinese pooled prevalence has been reported to be 14.2% (Taghipour et al. 2022b), 11.2% (Qin et al. 2022), 20.0% (Qiu et al. 2019), or 14.0% (Wang et al. 2018). Furthermore, the global pooled prevalence of *G. duodenalis* was approximately 22% and its Chinese pooled prevalence was 14.1% (Taghipour et al. 2022b). In recent years, *Cryptosporidium* (Cui et al. 2014; Huang et al. 2014; Wang et al. 2023; Zhang et al. 2015), *E. bienersi* (Dong et al. 2022; Li et al. 2016), and *G. duodenalis* (Huang et al. 2014; Wang et al. 2023; Zhang et al. 2016) have been studied in cattle from Ningxia. However, most of these studies were limited to a single pathogen, and no comprehensive data regarding mixed infection are available.

Thus, to evaluate the zoonotic potential of *Cryptosporidium*, *E. bienersi*, and *G. duodenalis*, we investigated the molecular prevalence and genetic characteristics of these pathogens infecting dairy cattle in Ningxia, China.

Materials and methods

Study areas and sample collection

From April to May 2021, fresh fecal samples were randomly collected from 320 calves at three intensive dairy farms near Yinchuan and Zhongwei (105°46'E–106°60'E, 36°59'N–38°58'N) in northern Ningxia, China. The samples were collected from 70 preweaned calves (0–60 days), 70 postweaned calves (61–180 days), 70 young cattle (181–360 days), and 110 adult cattle (≥ 361 days). The fecal samples were collected through rectal sampling or from the inner top layer of fresh feces. Detailed information regarding whether the sampled animals had diarrhea was recorded, and the samples were stored at 4°C before being transported to the laboratory for DNA extraction.

DNA extraction and polymerase chain reaction (PCR)

DNA was extracted from the collected 320 fecal samples using E.Z.N.A.® Stool DNA Kit (Omega Biotek Inc., Norcross, GA, USA) according to the manufacturer's protocol and stored at –20 °C. Using the extracted DNA as a template, Premix Taq™ (TaKaRa Taq™ Version 2.0 plus dye) (TaKaRa, Beijing, China) was used to amplify the SSU rRNA gene of *Cryptosporidium* (Alves et al. 2003) and *G. duodenalis* (Appelbee et al. 2003). The ITS gene of *E. bienersi* (Buckholt et al. 2002) was amplified via nested PCR.

The sequencing of the SSU rRNA gene of *Cryptosporidium*-positive PCR products was outsourced to Sangon Biotech (Shanghai, China). The results of sequencing analysis were combined with those of the RFLP analysis of the SSU rRNA gene of *Cryptosporidium*-positive PCR products using restriction enzymes (*Ssp* I and *Mbo* II; TaKaRa, Beijing, China) (Feng et al. 2007). Upon confirming the presence of *C. parvum* DNA, the nested PCR of *gp60* (Alves et al. 2003) was subsequently performed, and *gp60* sequencing results were used for *C. parvum* subtype identification (Sulaiman et al. 2005). The SSU rRNA genes of *G. duodenalis*-positive DNA were further amplified via nested PCR for *bg* (Lalle et al. 2005), *gdh* (Cacciò et al. 2008), and *tpi* (Sulaiman et al. 2003). The ITS gene of *E. bienersi* and four genes of *G. duodenalis*-positive PCR products were also sequenced.

Sequence analysis

The sequences were aligned with reference sequences downloaded from GenBank (<http://www.ncbi.nlm.nih.gov>) using MEGA 7.0 software (<http://www.megasoftware.net/>). The sequencing results were analyzed using the BLAST online platform. To comprehensively investigate the relationship among different isolates, phylogenetic analyses were performed using a concatenated dataset of the ITS gene of *E. bieneusi* and the *bg*, *gdh*, and *tpi* sequences of *G. duodenalis*. Phylogenetic trees were constructed using the neighbor-joining algorithm based on a matrix of evolutionary distances calculated using the Kimura two-parameter model via MEGA 7.0 software. Bootstrap analysis was performed to assess the robustness of clusters using 1000 replicates.

Statistical analysis

Using chi-squared test in SPSS Statistics 21.0 (IBM Corp., New York, NY, USA) with a 95% confidence interval, the infection rates of *Cryptosporidium* spp., *E. bieneusi*, and *G. duodenalis* were compared among different farms, age groups, and groups with and without diarrhea. A two-tailed *p*-value of <0.05 was considered to indicate a statistically significant difference.

Results

Cryptosporidium, E. bieneusi, and G. duodenalis infection status

Among the 320 samples collected in this study, 197 (61.56%) were infected with at least one of the following pathogens: *Cryptosporidium*, *E. bieneusi*, and *G. duodenalis*. Furthermore, 10 samples (3.13%) were simultaneously infected with *Cryptosporidium*, *G. duodenalis*, and *E. bieneusi*. In addition, 26 samples (8.13%) exhibited mixed infection with *Cryptosporidium* and *E. bieneusi*, 39 samples (12.19%)

with *E. bieneusi* and *G. duodenalis*, and 7 samples (2.19%) with *Cryptosporidium* and *G. duodenalis* (Table 1).

Cryptosporidium infection status

Based on the identification of the SSU rRNA gene of *Cryptosporidium*, the overall prevalence rate of *Cryptosporidium* was 19.38% (62/320). The overall prevalence rates of *Cryptosporidium* in samples from animals with and without diarrhea were 16.13% (5/31) and 19.72% (57/289), respectively; the difference was not significant. The overall prevalence rates of *Cryptosporidium* in samples from the four age groups (in chronological order) were 24.29% (17/70), 28.57% (20/70), 22.86% (16/70), and 8.18% (9/110). The overall infection rates in the preweaned calves and young cattle were significantly higher than those in adult cattle. The overall infection rate in the postweaned calves was also significantly higher than that in adult cattle. The overall infection rates among samples from the three intensive dairy farms were 35.83% (43/120), 7% (7/100), and 12% (12/100), respectively. The overall infection rate at farm 1 was significantly higher than that at farm 2 (Table 2).

E. bieneusi infection status

Based on the PCR amplification of the ITS gene of *E. bieneusi*, infection was identified in 133 of the 320 samples (41.56%). The overall infection rates of *E. bieneusi* in samples collected from animals with and without diarrhea were 48.39% (15/31) and 40.83% (118/289), respectively. The overall infection rates of *E. bieneusi* in the four age groups (in chronological order) were 40% (28/70), 54.29% (38/70), 61.43% (43/70), and 21.82% (24/110), respectively. The overall infection rates in the postweaned calves and young cattle were significantly higher than those in adult cattle. The overall infection rate in the preweaned calves was significantly higher than that in adult cattle. The overall infection rates of *E. bieneusi* at the three intensive dairy farms were 52.5% (63/120), 38% (38/100), and 32% (32/100),

Table 1 Mixed infection with the three intestinal pathogens in dairy cattle in Ningxia, northwestern China

Farm	Samples size	Prevalence % (n)				
		At least one pathogen	Cryptosporidium spp. + Enterocytozoon bieneusi + Giardia duodenalis	Cryptosporidium spp. + Enterocytozoon bieneusi	Enterocytozoon bieneusi + Giardia duodenalis	Cryptosporidium spp. + Giardia duodenalis
1	120	70 (84)	5 (6)	19.17 (23)	8.33 (10)	0.83 (1)
2	100	58 (58)	2 (2)	2 (2)	17 (17)	2 (2)
3	100	55 (55)	2 (2)	1 (1)	12 (12)	4 (4)
Total	320	61.56 (197)	3.13 (10)	8.13 (26)	12.19 (39)	2.19 (7)

Table 2 Prevalence and species/assemblage/genotype distribution of *Cryptosporidium* spp., *Giardia duodenalis*, and *Enterocytozoon bienersi* in dairy cattle in Ningxia, northwestern China

Variable	Cryptosporidium spp.			Enterocytozoon bienersi							
	Samples size (Diarrhea/ Non-diarrhea)	Positive samples size (Diarrhea/ Non-diarrhea)	Overall prevalence (Prevalence of diarrhea/ non-diarrhea samples) (%)	Species (n)/C. parvum Subtypes (n)	P value	OR (95% CI)	Positive samples size (Diarrhea/ Non-diarrhea)	Overall prevalence (Prevalence of diarrhea/ non-diarrhea samples) (%)	Genotypes (n)	P value	OR (95% CI)
Location											
Farm 1	120 (10/110)	43 (5/38)	35.83 (50.00/34.55)	C. parvum (10), C. ryanae (5), C. bovis (15), C. andersoni (3), C. parvum + C. ryanae (1), C. bovis + C. ryanae (9)/ IIdA15G1 (9)	0.000	7.419 (3.158 – 17.428)	63 (6/57)	52.5 (60.00/51.82)	J (51), BEB4 (4), I (4), NX1 (1), NX2 (1), NX3 (1), NX4 (1)	0.002	2.349 (1.352 – 4.080)
Farm 2	100 (12/88)	7 (0/7)	7 (0/7.95)	C. bovis (2), C. andersoni (2), C. bovis + C. ryanae (2), C. parvum + C. bovis + C. andersoni (1)	–	–	38 (5/33)	38 (41.67/37.50)	J (19), BEB4 (19)	0.374	1.302 (0.727 – 2.333)
Farm 3	100 (9/91)	12 (0/12)	12 (0/13.19)	C. ryanae (3), C. bovis (7), C. parvum (1), C. bovis + C. ryanae (1)/ IIdA20G1 (1)	0.228	0.552 (0.208 – 1.466)	32 (4/28)	32 (44.44/30.77)	J (18), BEB4 (10), NX5 (1), NX6 (1), NX7 (1), NX8 (1)	–	–
Age											
Preweaned calves	70 (5/65)	17 (0/17)	24.29 (0/26.15)	C. parvum (11), C. ryanae (1), C. parvum + C. ryanae (1), C. bovis + C. ryanae (2), C. bovis (2)/ IIdA15G1 (9) + IIdA20G1 (1)	0.003	3.600 (1.503 – 8.624)	28 (5/23)	40 (100/35.38)	J (10), BEB4 (13), NX1 (1), NX2 (1), NX3 (1), NX5 (1), NX7 (1)	0.009	2.389 (1.237 – 4.614)

Table 2 (continued)

Variable	Cryptosporidium spp.				Enterocytozoon bienersi						
	Samples size (Diarrhea/ Non-diarrhea)	Positive samples size (Diarrhea/ Non-diarrhea)	Overall prevalence (Prevalence of diarrhea/ non-diarrhea samples) (%)	Species (n)/C. parvum Subtypes (n)	P value	OR (95% CI)	Positive samples size (Diarrhea/ Non-diarrhea)	Overall prevalence (Prevalence of diarrhea/ non-diarrhea samples) (%)	Genotypes (n)	P value	OR (95% CI)
Post-weaned calves	70 (11/59)	20 (4/16)	28.57 (36.36/27.12)	C. bovis (10), C. bovis + C. ryanae (8), C. ryanae (2)	0.000	4.489 (1.906 – 10.572)	38 (7/31)	54.29 (63.64/52.54)	J (33), BEB4 (1), I (2), NX6 (1), NX8 (1)	0.000	4.255 (2.216 – 8.172)
Young cattle	70 (0/70)	16 (0/16)	22.86 (0/22.86)	C. ryanae (4), C. bovis (8), C. bovis + C. ryanae (2), C. andersoni (2)	0.006	3.325 (1.378 – 8.024)	43 (0/43)	61.43 (0/61.43)	J (27), BEB4 (13), I (2), NX4 (1)	0.000	5.707 (2.948 – 11.048)
Adult cat-tle	110 (15/95)	9 (1/8)	8.18 (6.67/8.42)	C. andersoni (3), C. bovis (4), C. parvum + C. bovis + C. andersoni (1), C. ryanae (1)	–	–	24 (3/21)	21.82 (20/22.11)	J (18), BEB4 (6)	–	–
Symptom											
Diarrhea	31	5	16.13	C. bovis + C. ryanae (1), C. bovis (3), C. andersoni (1)	0.630	0.783 (0.288 – 2.128)	15	48.39	J (12), BEB4 (3)	0.417	1.359 (0.647 – 2.854)
Non-diar-rhea	289	57	19.72	C. parvum (11), C. ryanae (8), C. bovis (21), C. andersoni (4), C. parvum + C. ryanae (1), C. bovis + C. ryanae (11), C. parvum + C. bovis + C. andersoni (1)/ IIdA15G1 (9) + IIdA20G1 (1)			118	40.83	J (76), BEB4 (30), I (4), NX1 (1), NX2 (1), NX3 (1), NX4 (1), NX5 (1), NX6 (1), NX7 (1), NX8 (1)		

Table 2 (continued)

Variable	Cryptosporidium spp.		Enterocytozoon bienersi								
	Samples size (Diarrhea/Non-diarrhea)	Positive samples size (Diarrhea/Non-diarrhea)	Overall prevalence (Prevalence of diarrhea/non-diarrhea samples) (%)	Species (n)/C. parvum Subtypes (n)	P value	OR (95% CI)	Positive samples size (Diarrhea/Non-diarrhea)	Overall prevalence (Prevalence of diarrhea/non-diarrhea samples) (%)	Genotypes (n)	P value	OR (95% CI)
Total	320	62	19.38	C. parvum (11), C. ryanae (8), C. bovis (24), C. andersoni (5), C. parvum + C. ryanae (1), C. bovis + C. ryanae (12), C. parvum + C. bovis + C. andersoni (1)/IIdA15G1 (9)+IIdA20G1 (1)			133	41.56	J (88), BEB4 (33), I (4), NX1 (1), NX2 (1), NX3 (1), NX4 (1), NX5 (1), NX6 (1), NX7 (1), NX8 (1)		
Variable	Giardia duodenalis		Assemblages								
	Samples size (Diarrhea/Non-diarrhea)	Positive samples size (Diarrhea/Non-diarrhea)	Overall prevalence (Prevalence of diarrhea/non-diarrhea samples) (%)	SSU rRNA (n)	gdh (n)	tpi (n)	bg (n)	P value	OR (95% CI)		
Location											
Farm 1	120 (10/110)	24 (2/22)	20 (20.00/20.00)	E (12), A (10), B (1)	E (4), A (1)	E (4), A (4)	E (12), A (2)	–	–		
Farm 2	100 (12/88)	38 (5/33)	38 (41.67/37.50)	E (34), A (3), B (1)	E (10)	E (14)	E (14)	0.003	0.408 (0.223 – 0.745)		
Farm 3	100 (9/91)	32 (0/32)	32 (0/35.16)	E (29), A (2)	E (3)	E (3), A (1)	E (12)	0.042	0.531 (0.288 – 0.981)		
Age											
Preweaned calves	70 (5/65)	14 (4/10)	20 (80.00/15.38)	E (14)	E (9)	E (11)	E (11)	–	–		
Postweaned calves	70 (11/59)	20 (2/18)	28.57 (18.18/30.51)	E (10), A (9)	E (3), A (1)	E (5), A (5)	E (8), A (2)	0.237	0.625 (0.286 – 1.366)		

Table 2 (continued)

Variable	Samples size		Giardia duodenalis					P value	OR (95% CI)	
	Diarrhea/ Non-diarrhea	Positive samples size (Diarrhea/ Non-diarrhea)	Overall prevalence (Prevalence of diarrhea/ non-diarrhea samples) (%)	Assemblages						
				SSU rRNA (n)	gdh (n)	tpi (n)	bg (n)			
Young cattle	70 (0/70)	25 (0/25)	35.71 (0/35.71)	E (19), A (5), B (1)	E (1)	E (2)	E (6)	0.038	0.450 (0.210 – 0.965)	
Adult cattle	110 (15/95)	35 (1/34)	31.82 (6.67/35.79)	E (32), A (1), B (1)	E (4)	E (3)	E (13)	0.082	0.536 (0.263 – 1.090)	
Symptom										
Diarrhea	31	7	22.58	E (6), A (1)	E (5)	E (5), A (1)	E (5), A (1)	0.382	0.677 (0.281 – 1.630)	
Non-diarrhea	289	87	30.1	E (69), A (14), B (2)	E (12), A (1)	E (16), A (4)	E (33), A (1)			
Total	320	94	29.38	E (75), A (15), B (2)	E (17), A (1)	E (21), A (5)	E (38), A (2)			

respectively. The overall infection rate at farm 1 was significantly higher than that at farm 3 (Table 2).

G. duodenalis infection status

Based on the PCR amplification of the SSU rRNA gene of *G. duodenalis*, infection was identified in 94 of the 320 samples (29.38%). The overall infection rates of *G. duodenalis* in samples from animals with and without diarrhea were 22.58% (7/31) and 30.10% (87/289), respectively; the difference was not significant. The overall infection rates of *G. duodenalis* in the four age groups (in chronological order) were 20% (14/70), 28.57% (20/70), 35.71% (25/70), and 31.82% (35/110). A statistically significant difference in the overall infection rate was observed between young cattle and preweaned calves. The overall infection rates of *G. duodenalis* in samples from the three intensive dairy farms were 20% (24/120), 38% (38/100), and 32% (32/100), respectively. The overall infection rate at farm 2 was significantly higher than that at farm 1. In addition, the overall infection rate between farm 1 and farm 3 was statistically significantly different (Table 2).

Identification of species, genotypes and assemblages and sequence analysis of *Cryptosporidium*, *E. bieneusis*, and *G. duodenalis*

Species identification and sequence analysis of *Cryptosporidium*

Through RFLP analysis of the PCR amplification products from the 62 SSU rRNA genes and combining them with the 61 obtained sequences, 4 *Cryptosporidium* species, namely, *C. bovis* (59.68%, 37/62), *C. andersoni* (9.68%, 6/62), *C. ryanae* (33.87%, 21/62), and *C. parvum* (20.97%, 13/62), were identified. In addition, mixed infections with two or three *Cryptosporidium* species were identified (Table 2). Infections with these four *Cryptosporidium* species were identified in all three intensive dairy farms. *C. bovis* was the dominant species in this survey and was identified in all four age groups, primarily in the postweaned calves (48.65%, 18/37). The preweaned calves were mainly infected with *C. parvum* (12/17) and the postweaned calves were mainly infected with *C. bovis* (18/20). In young cattle, *C. bovis* infection (10/16) was the most common, whereas in adult cattle, *C. andersoni* (4/9) and *C. bovis* (5/9) infections were the most common. Three and four of the abovementioned *Cryptosporidium* spp. were detected in samples from animals with and without diarrhea, respectively. *C. bovis* was mainly found in the samples from the groups with and without diarrhea (4/5 and 34/57, respectively) (Table 2).

The 10 *gp60* sequences obtained in this study were analyzed, and the subtypes of the *C. parvum* family IId were successfully identified as IIdA15G1 (9) and IIdA20G1 (1) (Table 2).

Genotype identification and sequence analysis of *E. bienensis*

Based on the ITS gene of *E. bienensis*, 133 credible sequences were obtained, which were used for the sequence alignment of 243-bp ITS region and comparison with the reference sequences of different genotypes. Differences in no more than two bases were identified. The sequences identified in this study were classified into the genotypes J (66.17%, 88/133), BEB4 (24.81%, 33/133), and I (3.01%, 4/133) along with new genotypes NX1–NX8 (0.75%, 1/133) (Table 2).

Phylogenetic analysis classified the genotypes J, BEB4, I, and NX1–NX8 along with the multiple genotypes previously widely reported in humans and animals into group 2 (Fig. 1).

Assemblage identification and MLG analysis of *G. duodenalis*

Overall, 94 samples positive for the PCR products of the SSU rRNA gene were obtained, and 92 sequences were evaluated as reliable. Among these, 75 (81.52%, 75/92) were classified into assemblage E, 15 (16.30%, 15/92) into assemblage A, and 2 (2.17%, 2/92) into assemblage B. Assemblage E was identified in all four age groups of cattle, whereas only assemblage E was present in the preweaned calves (Table 2).

Overall, 94 positive samples were obtained via PCR and sequencing of *gdh*, *tpi*, and *bg* from *G. duodenalis*, yielding 18, 26, and 40 credible sequences, respectively. Overall, 18 isolates of *gdh* were identified, with 1 being identified as an assemblage A sequence (A1) and 17 identified as 11 assemblage E sequences with the following distribution: E1 ($n=5$), E2 ($n=2$), E3 ($n=1$), E4 ($n=1$), E5 ($n=2$), and E6–E11 (each $n=1$). Among the 26 isolates of *tpi*, 5 were identified as assemblage A sequences (A1–A5, each $n=1$) and 21 were identified as 20 assemblage E sequences (E1–E3 and E5–E20, each $n=1$, and E4 [$n=2$]). In addition, there were 40 isolates of *bg*, with 2 being identified as a single assemblage A sequence (A1, $n=2$) and 38 as 16 assemblage E sequences [E1 ($n=4$), E2 ($n=3$), E3 ($n=4$), E4 ($n=8$), E5 ($n=1$), E6 ($n=1$), E7 ($n=8$), and E8–E16 (each $n=1$)] (Table 2). Through the sequence alignment of the above four genes, the genotyping results of the two samples at different gene sites differed and mixed infection was identified (Assemblages A + E).

In a phylogenetic tree constructed based on the *gdh* (Fig. 2), *tpi* (Fig. 3), and *bg* (Fig. 4) sequences of *G. duodenalis*, these three genes were divided into two branches (assemblages A and E). The sequences of *gdh*, *tpi*, and *bg*



Fig. 1 Phylogenetic relationship among *Enterocytozoon bienensis* in a neighbor-joining tree based on the ITS gene. The isolates marked with black triangles (▲) and white triangles (△) represent *Enterocytozoon bienensis* identified as having genotypes J, BEB4, and I and novel genotypes from cattle in this study, respectively

were successfully obtained from 11 isolates. These three genes of the 11 isolates were combined for genotyping, resulting in 10 different assemblage E MLGs and 1 assemblage A MLG. Phylogenetic analysis revealed that the assemblage E MLGs formed five subgroups (Fig. 5).

Discussion

Cryptosporidium spp., *E. bienensis*, and *G. duodenalis* are three opportunistic pathogens that infect humans and animals (Wang et al. 2022b). To date, numerous studies worldwide have reported *Cryptosporidium* (Zhang et al. 2022), *E. bienensis* (Ruan et al. 2021; Taghipour et al. 2022a), and *G. duodenalis* (Taghipour et al. 2022b) infections in cattle. In China, high incidences of cryptosporidiosis, *E. bienensis* infection, and giardiasis have been reported in dairy cattle (Qiu et al. 2019; Ryan and Cacciò 2013; Santin 2020;



Fig. 2 Phylogenetic tree of *Giardia duodenalis* based on *gdh* sequences. The phylogenetic tree was inferred via neighbor-joining analysis of genetic distances calculated using the Kimura two-parameter model. Bootstrap values > 50% from 1000 replicates are shown to the left of the nodes. The isolates marked with black triangles (▲) and black squares (■) represent assemblages E and A identified from cattle in this study, respectively

Wang et al. 2017a, 2018). Cattle infected with these three protozoa are potential sources through which humans can acquire protoziasis (Dong et al. 2022; Kiani-Salmi et al. 2019; Ruan et al. 2021; Ryan and Cacciò 2013; Zhang et al. 2015). In the present study, a molecular epidemiological survey of these three protozoa was performed on 320 fecal samples from dairy cattle at three intensive dairy farms in Ningxia, China. The results reconfirmed that dairy cattle in Ningxia are infected with these three protozoa (Cui et al. 2014; Huang et al. 2014; Li et al. 2016; Wang et al. 2023; Zhang et al. 2015, 2016) and further identified new protozoon genotypes. Previously, in Ningxia, only mixed infections involving *Cryptosporidium* and *G. duodenalis* were reported (Wang et al. 2023). However, the present study also found mixed infections involving *Cryptosporidium*, *G. duodenalis*, and *E. bieneusis*; *Cryptosporidium* and *E. bieneusis*; *E. bieneusis* and *G. duodenalis*; and *Cryptosporidium* and *G. duodenalis* as well as mixed infections involving different *Cryptosporidium* species and *G. duodenalis* assemblages A and E. In addition, mixed infections involving pairs of taxa alongside *G. duodenalis* assemblage mixed infections have been reported in various animals in other regions, and it is not uncommon both within China and abroad (Jiang et al.

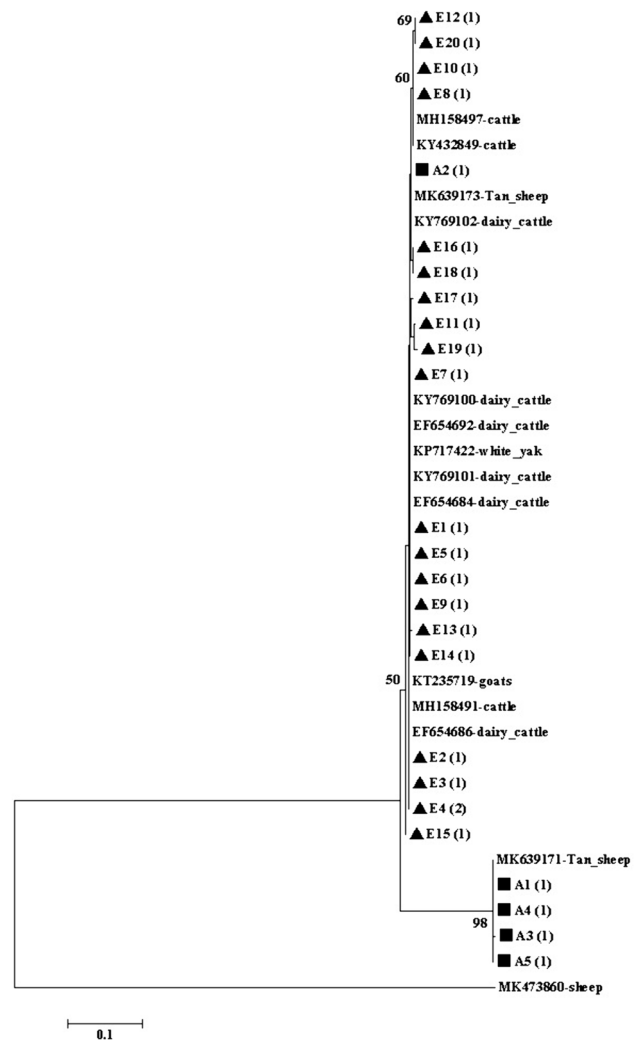


Fig. 3 Phylogenetic tree of *Giardia duodenalis* based on *tpi* sequences. The phylogenetic tree was inferred via neighbor-joining analysis of genetic distances calculated using the Kimura two-parameter model. Bootstrap values > 50% from 1000 replicates are shown to the left of the nodes. Assemblages E and A isolates identified from cattle in this study are marked with black triangles (▲) and black squares (■), respectively

2023; Wang et al. 2022a, 2022b; Yang et al. 2022). However, this phenomenon should be seriously considered and the mutual influence of the infectious agents and the impact of mixed infection on animals should be further studied.

Here, the overall infection rates of *Cryptosporidium*, *E. bieneusis*, and *G. duodenalis* in dairy cattle from Ningxia were 19.38% (62/320), 41.56% (133/320), and 29.38% (94/320), respectively. The infection rate of *Cryptosporidium* was lower than the previously reported global pooled infection rate (Hatam-Nahavandi et al. 2019) and higher than the Chinese pooled infection rate (Cai et al. 2019). Moreover, the infection rates of *E. bieneusis* (Qin et al. 2022; Qiu et al. 2019; Ruan et al. 2021; Taghipour et al. 2022a; Wang

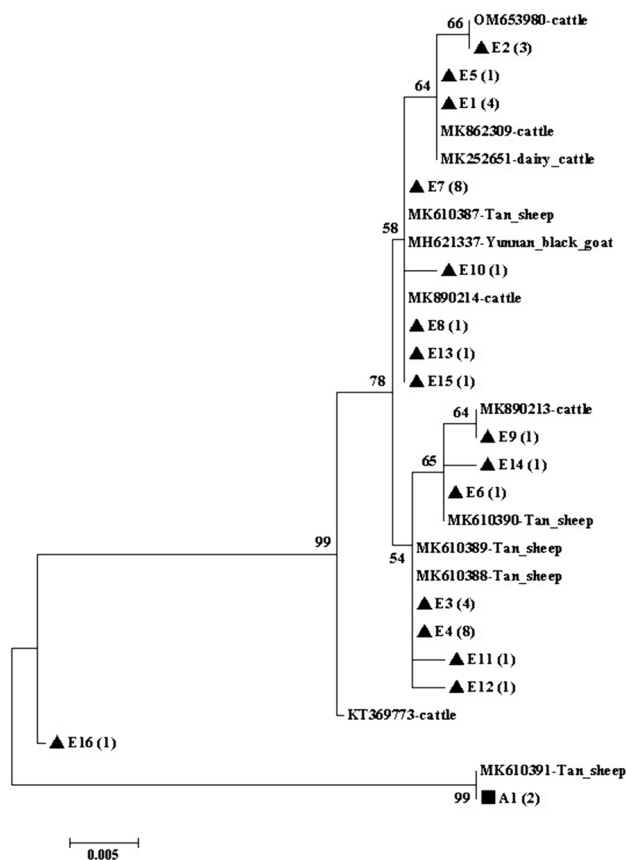


Fig. 4 Phylogenetic tree of *Giardia duodenalis* based on *bg* sequences. The phylogenetic tree was inferred via neighbor-joining analysis of genetic distances calculated via the Kimura two-parameter model. Bootstrap values > 50% from 1000 replicates are shown to the left of the nodes. The black triangles (▲) and black squares (■) represent assemblages E and A identified from cattle in this study, respectively

et al. 2018) and *G. duodenalis* (Taghipour et al. 2022b) were higher than the global and Chinese pooled infection rates. The infection rates of *Cryptosporidium* were higher than those previously mentioned in two reports on Ningxia dairy cattle, which were 5.45% (92/1688) (Zhang et al. 2015) and 1.68% (23/1366) (Huang et al. 2014); however, they were lower than those reported in two other studies on Ningxia cattle, which were 21% (53/252) (Cui et al. 2014) and 50.46% (111/220) (Wang et al. 2023). The infection rate of *E. bieneusi* was lower than that reported in a previous study on Ningxia dairy cattle, which was 46.8% (51/109) (Li et al. 2016). Furthermore, the infection rates of *G. duodenalis* were higher than those reported in three previous reports on Ningxia cattle, which were 2.12% (29/1366) (Huang et al. 2014), 4.38% (74/1688) (Zhang et al. 2016), and 13.64% (30/220) (Wang et al. 2023). However, it is difficult to compare the infection rate owing to the influence of various factors, including study design, diagnostic method, geographical conditions, climate, sanitation conditions,

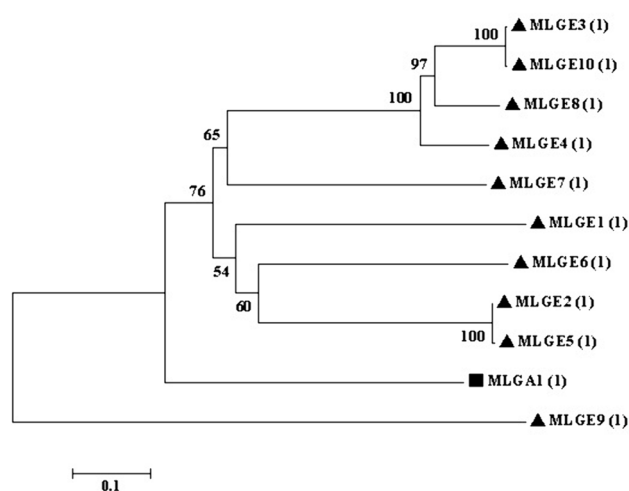


Fig. 5 Phylogenetic relationship among *Giardia duodenalis* assemblage E MLGs. The phylogenetic tree was constructed using a concatenated dataset of *bg*, *tpi*, and *gdh* sequences, and analysis via the neighbor-joining method resulted in identical topologies. Black triangles (▲) and black squares (■) represent assemblages MLGE and MLGA identified from cattle in this study, respectively

rearing conditions, total number of samples, age of animals, and sampling season (Gong et al. 2017; Qi et al. 2016; Hatam-Nahavandi et al. 2019). Thus, to prevent and control protozoa in Ningxia, there is an urgent need to clarify the infection rates among local animals.

Here, no significant differences were identified in the overall infection rates of the three protozoa between the groups with and without diarrhea. Moreover, all three protozoa-infected young cattle were present in the group without diarrhea and the *Cryptosporidium*-infected preweaned calves were found in the group without diarrhea. Infections with *Cryptosporidium* at farm 2 and *Cryptosporidium* and *G. duodenalis* at farm 3 also appeared exclusively in the group without diarrhea. The most common clinical manifestations of infection with these three intestinal protozoa include diarrhea and wasting (Qin et al. 2022; Wang et al. 2022a). In addition, some studies have reported a correlation among these three intestinal protozoa and cattle suffering from diarrhea in cattle (Cai et al. 2019; Qin et al. 2022; Taghipour et al. 2022b; Wang et al. 2017a). However, diarrhea is a common clinical symptom of various diseases, with causative agents including bacteria, viruses, and parasites, or other possible factors (Ichikawa-Seki et al. 2015); hence, it is difficult to evaluate the impact of the three protozoa on diarrhea.

In addition, the overall infection rate of *Cryptosporidium* and *E. bieneusi* was significantly higher in the preweaned calves, postweaned calves, and young cattle than in adult cattle. *G. duodenalis* exhibited the highest overall prevalence in young cattle, which differed significantly compared with that in the preweaned calves. The results of this study show that with increasing age, the infection rate of the three

protozoa first increased and then decreased, with the inflection point occurring at the postweaned calf or young cattle stage. Several studies have reported that the infection rate of the three protozoa decreases with increasing age (Qin et al. 2022; Wang et al. 2017a, 2018, 2019). Some studies have also reported a high infection rate in the postweaned calves (Fan et al. 2021; Gillhuber et al. 2014; Hamnes et al. 2006; Liu et al. 2009; Ma et al. 2021; Qi et al. 2015, 2016; Trout et al. 2004, 2005; Zhao et al. 2013); however, many reports have described a high infection rate in the preweaned calves (Feng et al. 2007; Geurden et al. 2012; Hamnes et al. 2006; Heyworth 2016; Jia et al. 2022; Ma et al. 2015; Mravcová et al. 2020; Shrivastava et al. 2017; Sulaiman et al. 2005; Trout et al. 2004, 2005; Wang et al. 2018; Yasouri et al. 2020; Zahedi et al. 2017; Zhao et al. 2013). Moreover, here, the three intensive dairy farms demonstrated different infection rates of the three protozoa. As mentioned earlier, several factors affect the infection rate, regional and age distribution, and occurrence of diarrhea in samples infected with the three different protozoa. Thus, there is a need for a more comprehensive understanding of such factors.

Herein, four *Cryptosporidium* species were identified at all three intensive dairy farms, consistent with the four dominant species in cattle reported within China and (Li et al. 2022a) abroad (Feng et al. 2018; Gong et al. 2017; Hatam-Nahavandi et al. 2019; Santin 2020; Wang et al. 2017a; Zahedi and Ryan 2020; Zhang et al. 2022). These four *Cryptosporidium* species were previously isolated from dairy cattle in Ningxia (Zhang et al. 2015). The dominant species in this study was *C. bovis*; however, this is inconsistent with the finding that *C. andersoni* was the most common species in China (Gong et al. 2017). In this study, *C. bovis* infection was primarily detected in postweaned calves; however, other reports have described that *C. bovis* infection is dominant in preweaned calves in China (Feng and Xiao 2017; Gong et al. 2017; Guo et al. 2022; Wang et al. 2017a). In addition, other previous reports have described that *C. bovis* infection is very common in postweaned calves (Li et al. 2019a). In the present study, the preweaned calves were mainly infected with *C. parvum*, consistent with previous findings (Gong et al. 2017; Li et al. 2022a). In addition, young cattle were most commonly infected with *C. bovis*, and the majority of adult cattle were infected with *C. andersoni* and *C. bovis*. This is inconsistent with the finding that *C. andersoni* was the dominant species among postweaned, juvenile, and adult cattle (Gong et al. 2017).

To the best of our knowledge, this study is the first to report mixed infection with two or three *Cryptosporidium* species in dairy cattle in Ningxia. The infection type is consistent with the appearance of *C. bovis* + *C. ryanae* in Guangdong (Liang et al. 2019), Shanghai (Cai et al. 2017), Xinjiang (Alves et al. 2003; Qi et al. 2020), and Henan (Wang et al. 2010) in China, as well as *C. parvum* + *C.*

ryanae in Xinjiang (Wu et al. 2020) and Henan (Wang et al. 2010), and also shows *C. parvum* + *C. bovis* + *C. andersoni*. Multiple mixed infections involving *Cryptosporidium* spp. have also been detected in cattle from other countries (Cai et al. 2019; Gong et al. 2017; Hatam-Nahavandi et al. 2019; Wang et al. 2017a), and humans have also been reported to exhibit mixed infection involving the protozoan species (Helmy et al. 2013). *C. parvum* is reportedly the chief causative agent of zoonotic cryptosporidiosis (Feng et al. 2018; Guo et al. 2022; Zhang et al. 2022), and IIdA and IIdC *C. parvum* gp60 subtype families are known to be zoonotically transmitted (Feng et al. 2018; Jia et al. 2022; Li et al. 2022a; Mravcová et al. 2020). Herein, the main *C. parvum* subtype was found to be IIdA15G1; the IIdA20G1 subtype was also identified. Reportedly, in China, *C. parvum* infections are entirely caused by IId subtypes in cattle, with IIdA15G1 and IIdA19G1 being the most common subtypes (Guo et al. 2022; Li et al. 2022a; Zahedi et al. 2017; Zhang et al. 2022). In addition, IIdA15G1 (Cui et al. 2014; Huang et al. 2014; Zhang et al. 2015) was previously reported in dairy cattle in Ningxia; however, IIdA20G1 is reported here for the first time in Ningxia to the best of our knowledge. This subtype was previously only reported in Xinjiang (Wu et al. 2020) and Heilongjiang (Tao et al. 2018) in China.

Regarding *E. bienensis*, the genotypes identified in this study were J (66.17%), BEB4 (24.81%), and I (3.01%) along with new genotypes NX1–NX8 (all 0.75%), which belong to *E. bienensis* group 2. Reportedly, the genotypes BEB4, J, and I are most common in *E. bienensis* globally (Lichtmannsperger et al. 2023; Taghipour et al. 2022b). In China, the most common genotypes of cattle are I and J (Wang et al. 2018). Furthermore, BEB4, BEB6, I, and J in group 2 were described as the main genotypes associated with infections in humans and animals (Qin et al. 2022; Wang et al. 2022b). In addition, *E. bienensis* infection has been reported in at least 42 countries globally (Qin et al. 2022; Ruan et al. 2021). Cattle are an important vector for the transmission of *E. bienensis* (Qin et al. 2022). A previous study reported the infection of dairy cattle with *E. bienensis* group 2 (genotypes J and I) and group 1 (genotypes EbpA, CM8, O, CHC5, CHC4, and CHC3) in Ningxia (Alves et al. 2003). Group 1 is a zoonotic evolutionary group (Li et al. 2022b; Lichtmannsperger et al. 2023; Wang et al. 2022b). Herein, BEB4 and NX1–NX8 were the genotypes that were newly discovered in Ningxia and have not been reported in other animals in this region (Dong et al. 2022; Peng et al. 2019, 2020; Yang et al. 2018; Zhang et al. 2020). The obtained findings reveal more and more genotypes of *E. bienensis* infecting cattle in Ningxia, which is associated with an increased risk of dairy cattle transmitting *E. bienensis* to humans in this region.

Through the SSU rRNA gene analysis, we found that dairy cattle were infected with *G. duodenalis* assemblages E (81.52%), A (16.30%), and B (2.17%) in Ningxia. As reported in the literature, assemblage E is the most common genotype in

dairy cattle worldwide (Cai et al. 2021; Feng and Xiao 2011; Minetti et al. 2014; Qi et al. 2016; Taghipour et al. 2022b; Zhang et al. 2021) followed by assemblages A and B (Feng and Xiao 2011; Qi et al. 2016; Zhang et al. 2021). Combined sequence analysis of the *gdh*, *tpi* and *bg* genes in *G. duodenalis* revealed the genetic diversity of these isolates and the presence of mixed infection with assemblages A + E, (Qi et al. 2016; Scheuerle et al. 2013).

Assemblages A and B are reportedly associated with a higher risk of zoonotic diseases (Kiani-Salmi et al. 2019; Taghipour et al. 2022b; Zhang et al. 2021), with assemblage E also being reported in human cases in Bangladesh (Li et al. 2023), Egypt (Abdel-Moein and Saeed 2016), Brazil (Fantinatti et al. 2016), and Australia (Zahedi et al. 2017). In the literature, there are reports regarding dairy cattle being infected with *G. duodenalis* assemblages A, B, and E in Ningxia (Huang et al. 2014; Zhang et al. 2016). This highlights the risk of *G. duodenalis* spreading to humans from dairy cattle in Ningxia. Indeed, dairy cattle are considered a significant reservoir of human giardiasis in China (Feng and Xiao 2017). Herein, assemblage E was identified in all four cattle age groups, while only assemblage E was present in the preweaned calves. This is inconsistent with the findings of previous studies, which reported that the infection rates of the calves with assemblages A and B were higher than those with assemblage E (Dixon et al. 2011; Geurden et al. 2008; Huang et al. 2014; Ng et al. 2011; Qi et al. 2016). However, our findings are consistent with those of a report from Sichuan, China, reporting that only assemblage E was detected in the preweaned calves (Zhong et al. 2018). The characteristics of the individual loci of *G. duodenalis* often lead to inconsistent genotyping results (Feng and Xiao 2011). Based on multilocus genotyping, the MLG model can be used to obtain a better understanding of the diversity of *G. duodenalis* in humans and animals in different geographic regions, which can help reveal the potential and dynamic transmission of zoonotic giardiasis (Huey et al. 2013). Herein, the *gdh*, *tpi*, and *bg* genes obtained from 11 isolates were combined to form 10 different assemblage E MLGs (5 subgroups) and 1 assemblage A MLG. Consistent with the findings of previous studies, *G. duodenalis* isolates classified into the same assemblage may be classified as distinct MLGs (Qi et al. 2016; Wang et al. 2017b; Zhang et al. 2016; Zhao et al. 2015).

Conclusions

This study reconfirmed that dairy cattle are infected with *Cryptosporidium*, *G. duodenalis* and *E. bieneusis*. Furthermore, to the best of our knowledge, this study is the first to report mixed infections involving two or three *Cryptosporidium* species in dairy cattle. The subtype IIdA20G1 of *C. parvum* was also reported here for the first time in Ningxia, and nine genotypes of *E. bieneusis* and new features of *G.*

duodenalis assemblages were also discovered. Zoonotic potential and diverse genotypes and assemblages of the three protozoa significantly elevate the risk of cryptosporidiosis, microsporidiosis, and giardiasis among local people and animals in Ningxia. Cattle are important reservoirs of these three protozoa, and in countries with a large population of cattle, more attention should be paid to preventing and controlling these pathogens.

Abbreviations *SSU rRNA*: Small subunit ribosomal RNA; *ITS*: Internal transcribed spacer; *gp60*: 60 KDa glycoprotein; *bg*: Beta-giardin; *gdh*: Glutamate dehydrogenase; *tpi*: Triosephosphate isomerase; *PCR*: Polymerase chain reaction; *RFLP*: Restriction fragmentlength polymorphism; *MLG*: Multilocus genotype

Author contributions MYW, SZ, ZSZ, LZ, and YHL conceived and designed the study and critically revised the manuscript. HLC, ZSZ, WXH, LZ, and YHL performed sample collection. MYW, SZ, ZSZ, XYQ, HLC, YW, WJF, CY, YLD and LZ conducted the laboratory experiments. All the authors read and approved the final manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Declaration of generative AI in scientific writing Not applicable.

Ethics approval Our study was performed in strict accordance with the international standards published in the Guide to the Feeding, Management and Use of Experimental Animals (8th Edition) and followed the Regulations on the Management of Experimental Animals and other relevant laws and regulations. The study was approved by the Biomedical Research Ethics Committee of Inner Mongolia Agricultural University (approval no. 2020 [081]). Additionally, permission was obtained from the farm owners prior to specimen collection, and all efforts were made to minimize animal suffering.

Competing interests The authors declare no competing interests.

Additional headings All authors consent to participate and consent to publish.

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