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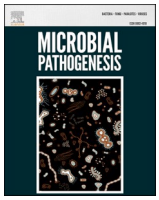

First report on occurrence and genotypes of *Enterocytozoon bieneusi*, *Cryptosporidium* spp. and *Cyclospora cayetanensis* from diarrheal outpatients in Ningbo, Southeast China

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ABSTRACT

Enterocytozoon bieneusi, *Cryptosporidium* spp. and *Cyclospora cayetanensis* are three important zoonotic pathogens which were a major cause of foodborne or waterborne intestinal diseases in humans and animals. However, very little data about occurrence and genotypes of the three parasites in Ningbo in the south wing of the Yangtze River Delta, China, which is important for a tourist city. In the present study, molecular characterization of *E. bieneusi*, *C. cayetanensis* and *Cryptosporidium* spp. in fecal samples from 489 diarrheal outpatients were carried out. As a result, a total of 35 (7.16%, 35/489) and three (0.61%, 3/489) samples were positive for *E. bieneusi* and *C. cayetanensis* respectively. No *Cryptosporidium*-positive sample or mixed-infections were detected. Four known *E. bieneusi* genotypes (Type IV, D, I and CHN4) and 8 novel genotypes (NBH1–NBH8) were identified with type IV was the dominant genotype (n = 14), followed by genotypes D (n = 5), NBH8 (n = 5) and NBH7 (n = 3). The remaining genotypes were found in one sample each, and these genotypes were belonged to the previously described high-potential zoonotic group 1. One novel sequence named NBC315, and the other two sequences (NBC30 and NBC370) identical with the reported sequence were detected. Therefore, the existence and importance of zoonotic potential of *E. bieneusi* and *C. cayetanensis* in diarrheal outpatients in Ningbo indicates the public health threats, and more investigations should be carried out in human populations, animals and other environmental sources from the One Health perspective.

1. Introduction

The emerging infectious diseases caused by intestinal protozoan including *Enterocytozoon bieneusi* (*E. bieneusi*), *Cryptosporidium* spp. and *Cyclospora cayetanensis* (*C. cayetanensis*) respectively are mainly characterized by severe or chronic diarrhea, vomiting, weight loss, malabsorption, and nausea [1–3]. These intestinal protozoan are among the major contributors to diarrheal diseases which are considered to be one of the primary cause of morbidity and mortality in young children in developing countries [4–6].

Microsporidia, also classified as fungi, are a diverse group of obligate intracellular pathogens which can infect both vertebrate and invertebrate hosts worldwide [7,8]. To date, there are over 200 microsporidian genera and nearly 1500 species, and *E. bieneus* is the most common of the known 17 species infecting in humans, accounting for 90% of overall microsporidia infections [1]. More than 500 *E. bieneusi* genotypes have been discovered based on the sequencing analysis of the internal transcriptional spacer (ITS) gene, and were systematically classified into 11 groups (Groups 1–11) with group 1 and 2 were the major zoonotic groups. Recently, *E. bieneus* has been reported to cause a foodborne outbreak with symptoms of diarrhea, abdominal pain, nausea, or vomiting from a workplace canteen in Denmark [9]. Moreover, *E. bieneusi* frequently cause gastrointestinal infections globally especially in immunocompromised individuals such as AIDS patients,

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cancer patients, organ transplant recipients and the elderly, respectively [10,11]. Meanwhile, immunocompetent people could also be suffered from this pathogen accompanied with asymptomatic infection or self-limiting diarrhea [12,13]. In addition, *E. bieneusi* can also be found in a variety of companion animals, livestock, wildlife and birds, and even in environmental water samples [14–16].

Cryptosporidium is a genus of protozoan parasites with a global distribution and a wide range of hosts, including humans, domesticated livestock and poultry, companion and wild animals [17,18]. Meanwhile, this parasite has been considered as a primary etiology of diarrhea in humans, and is closely associated with common causes of waterborne outbreaks of diarrheal illness globally, mainly through direct or indirect contact with the contamination of drinking and recreational water with the oocyst stage from host feces [19–22].

Many foodborne or waterborne cyclosporiasis outbreaks have been reported worldwide [20–22], and the presence of *C. cayetanensis* has been found in many mammalian and avian species such as dogs, cattles, monkeys and chickens. Humans were confirmed as the major reservoir of this parasite [23]. *C. cayetanensis* can prolong diarrhea accompanied by anorexia, malaise, nausea and cramping, among other symptoms. In our previous study, *C. cayetanensis* was detected in diarrheal patients in urban Shanghai [24].

Ningbo, a sub-provincial city, is situated in eastern part of Zhejiang province, in the low-lying coastal plain on the Yong River and in the south wing of the Yangtze River Delta, China. Altough the three parasites of interest in this study have been reported in humans, animals, and the environment in the Yangtze River Delta [25], there were few reports on investigation and characterization of these parasites in Zhejiang Province [26–28], especially in Ningbo, where *E. bieneusi* and *C. parvum* have been found in an investigation of pigs transported from north China [29], and *Cyclospora* has not been reported. Thus, it aims to investigate the occurrence and perform molecular characterization of these three intestinal parasites in diarrheal outpatients in Ningbo to assess the potential threat, and provide some evidence for prevention.

2. Materials and methods

2.1. Ethics statement

Ethical clearance for the collection and examination of human feces samples was obtained from the Ethics Committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (Approval No. 2019003). The objectives, procedures and potential risk were orally explained to all participants. Parents/guardians provided consent on behalf of all infant participants.

2.2. Samples

The fecal samples were collected from patients of local residents clinically diagnosed to be diarrhea with no less than three events of diarrhea per day in outpatient clinics in Ningbo, Zhejiang province, between January to December in 2019. A total of 489 samples were collected and the age and gender information of patients were recorded. Sufficient samples were collected for DNA extraction with fecal excretion heavier than 200 mg and were transported to the laboratory and stored at −20 °C until use.

2.3. DNA extraction and PCR amplification

The Genomic DNA was extracted and purified using the QIAamp DNA stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 200 μL of AE and stored at −30 °C until use for PCR amplification.

Nested PCR amplifications of a 830 bp *Cryptosporidium* SSU sRNA gene, a 500 bp partial *Cyclospora cayetanensis* 18S rRNA gene and a 390 bp nucleotide fragment of the *E. bieneusi* internal transcribed spacer (ITS) gene were performed to detect the presence of *Cryptosporidium* spp., *Cyclospora cayetanensis* and *E. bieneusi*, respectively. The primer sequences, PCR volume and reaction conditions were described in previous studies [11,12]. A positive and a negative control were added in each PCR amplification and the secondary PCR products were analyzed by 1.5% agarose gel electrophoresis using Gel-Red staining. Each sample was amplified at least twice.

2.4. Sequence and phylogenetic analyses

The PCR-positive products were sequenced twice in both directions using the secondary primers on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, USA) with a Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). ContigExpress was used to evaluate the wave peak and assemble the sequences to obtain the final sequences. All the nucleotide sequences obtained in the present study were subjected to the Basic Local Alignment Search Tool (BLAST), and then were aligned with reference sequences downloaded from GenBank and analyzed using Clustal X 1.83 (http://www.clustal.org/). A neighbor-joining phylogenetic tree based on 243 base pairs of the ITS gene sequences was constructed to describe the genetic relationship among the *E. bieneusi* obtained and reference genotypes published previously using 1000 bootstrap replicates in MEGA 7. *E. bieneusi* from the red kangaroo (GenBank: KY706128) were used as the outgroup for the phylogenetic analysis.

2.5. Statistical analysis

Data were analyzed using SPSS version 20.0 (SPSS, Chicago, IL, USA). The χ^2 test or Fisher's exact test were used for comparison of proportions and analysis of variance for comparison of means. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Infection of Cryptosporidium spp., Cyclospora cayetanensis and E. bieneusi

Of the 489 fecal samples examined in this study, 268 were males and 221 were females. A total of 38 samples were positive, and 35 (7.2%, 35/489) and three (0.6%, 3/489) samples were positive for *E. bieneusi* and *C. cayetanensis* respectively. No *Cryptosporidium*-positive sample and no mixed-infections were found (Table 1). Of the 35 *E. bieneusi*-positive samples, 24 were males and 11 were females with no sex-associated differences ($P = 0.13$; $\chi^2 = 2.3165$). Similarly, there is no gender differences in positivity of *C. cayetanensis* ($P = 1.0$; Fisher's exact test). Additionally, no age differences in positivity of *E. bieneusi* ($P = 0.57$; Fisher's exact test) and *C. cayetanensis* ($P = 0.18$; Fisher's exact test) were found in the present study.

Table 1
E. bieneusi, *Cryptosporidium* spp. and *C. cayetanensis* in fecal samples detected by PCR.

Genus	Number of positive specimens (%)			Genotype
	Male	Female	Total	
Enterocytozoon	24 (9.0)	11 (5.0)	35 (7.2)	Type IV, D, I, CHN4, NBH1–NBH8
Cyclospora	2 (0.75)	1 (0.45)	3(0.6)	
Cryptosporidium	0	0	0	–
Total patients	268	221	38 (7.8)	

3.2. Genotyping and phylogenetic analysis of E. bieneusi

Of the 35 *E. bieneusi*-positive samples, four known genotypes (Type IV, D, I and CHN4) and 8 novel genotypes (NBH1–NBH8) were identified. Type IV was the dominant genotype (n = 14), followed by genotypes D (n = 5), NBH8 (n = 5) and NBH7 (n = 3). The remaining genotypes were found in one sample each. The variations in ITS region sequence were showed in Table 2 based on sequence analysis, and there were two bases deletion in NBH1 and it was not found in other genotypes.

Of the 12 *E. bieneusi* ITS genotypes, type IV, D, CHN4 and all the novel genotypes and are belonged to the previously described high-potential zoonotic group 1 according to the phylogenetic analysis (Fig. 1). The type I was clustered into group 2.

3.3. DNA sequences of C. cayetanensis

Among the three *C. cayetanensis*-positive samples, one novel sequence named NBC315, and sequences of the other two (NBC30 and NBC370) were identical with the reported sequence (GenBank: KJ569533.1) from human samples. The further comparative analysis of the obtained sequences showed that there was a base deletion in NBC315 sequence and a single nucleotide polymorphism (SNP) at position 465 (NBC315-A; NBC30-G).

3.4. Nucleotide sequence accession numbers

Nucleotide sequences of the ITS gene of the five novel *E. bieneusi* genotypes obtained in this study were deposited in the GenBank database under accession numbers OM948686-OM948693. The accession number of new 18S rRNA gene sequence of *C.cayetanensis* was OP143736.

4. Discussion

E. bieneusi, *C. cayetanensis* and *Cryptosporidium* spp. are important zoonotic protozoa, and all the three pathogens can be transmitted directly by contact with infected humans or animals, or through the contaminated water or food indirectly [12,30,31]. In the present study, a total of 35 *E. bieneusi* and 3 *C. cayetanensis* positive samples were firstly detected in diarrheal outpatients in Ningbo, Zhejiang province, while no *Cryptosporidium*-positive sample was found. The overall positivity for *E. bieneusi* and *C. cayetanensis* were 7.2% (n = 35) and 0.6% (n = 3), respectively, with no gender or age differences in positivity of *E. bieneusi* and *C. cayetanensi*. No mixed infection was found and new zoonotic genotypes and new gene sequences were obtained.

In China, the infection rate of *E. bieneusi* in humans varied from many studies (Table 3), with the highest infection rate reached 39% in HIV-positive patients in Henan and the lowest infection rate was just 0.2% in diarrhea children in Wuhan [32,33]. The prevalence of *E. bieneusi* (7.2%) was similar with a recent identification and genotyping of *E. bieneusi* in humans in Myanmar [34], which was lower than that of the diarrheal patients [35] and higher than that of diarrheal children [36]. The infection rate was different due to the different regions and populations. The detection methods, the geographical locations and the specimens from individuals with different clinical features involved studies can also lead to different results [37].

In the present study, 11 genotypes were detected with three known genotypes (Type IV, D and CHN4) and 8 novel genotypes (NBH1–NBH8), which were all classified as group 1, while type I was clustered into group 2, and the two groups were both zoonotic. Inconsistent with the previous reports, zoonotic type IV was the dominant genotype with the proportion of 40% (14/35) in our study. In fact, type IV was also identified in HIV-positive patients and HIV-negative patients in Guangxi and Henan in China [11,32]. Genotype D was the second common genotype detected in this study which was in agreement with a

Table 2
The variations in ITS region sequence of the novel and known genotypes of *E. bieneusi* isolates from patients in this study.

Genotype	Nucleotide at position (ITS)																				Genbank no.
	127	131	134	151	152	156	172	178	181	193	213	217	229	231	233	237	238	245	247	258	260	265	276	297	315	327	
Novel																											
NBH-1	A	G	G	–	–	G	C	G	C	C	C	T	G	G	A	C	G	A	G	T	G	G	A	A	A	G	OM948686
NBH-2	A	G	G	G	T	A	C	G	C	T	C	G	G	G	G	C	G	A	G	T	G	G	A	C	A	G	OM948687
NBH-3	A	G	G	G	T	G	C	G	C	T	C	G	G	G	G	C	G	A	G	T	G	G	A	A	G	G	OM948688
NBH-4	G	G	G	G	T	G	C	G	C	T	C	G	G	G	G	C	G	A	G	T	A	G	A	A	A	G	OM948689
NBH-5	A	G	G	G	T	G	A	A	C	T	C	G	G	G	G	C	G	A	G	T	G	G	A	A	A	G	OM948690
NBH-6	A	G	G	G	T	G	C	G	C	T	C	G	G	G	G	C	G	A	G	T	G	G	A	A	A	A	OM948691
NBH-7	A	A	G	G	T	G	C	G	T	T	T	G	G	G	A	C	A	A	G	T	G	T	G	A	A	G	OM948692
NBH-8	A	A	G	G	T	G	C	G	T	T	T	T	G	A	G	C	A	A	G	T	G	T	G	A	A	G	OM948693
Known																											
I	A	G	A	G	T	G	C	G	T	T	T	G	A	C	G	T	G	A	A	A	G	G	A	A	A	G	MH899204
D	A	G	G	G	T	G	C	G	C	T	C	G	G	G	G	C	G	A	G	T	G	G	A	A	A	G	LC436479
Type-IV	A	G	G	G	T	G	C	G	C	T	C	G	G	G	G	C	G	A	G	T	G	G	A	A	A	G	AF267141
CHN4	A	G	G	G	–	G	C	G	C	T	C	G	G	G	G	C	G	A	G	T	G	G	A	A	A	G	HM992511

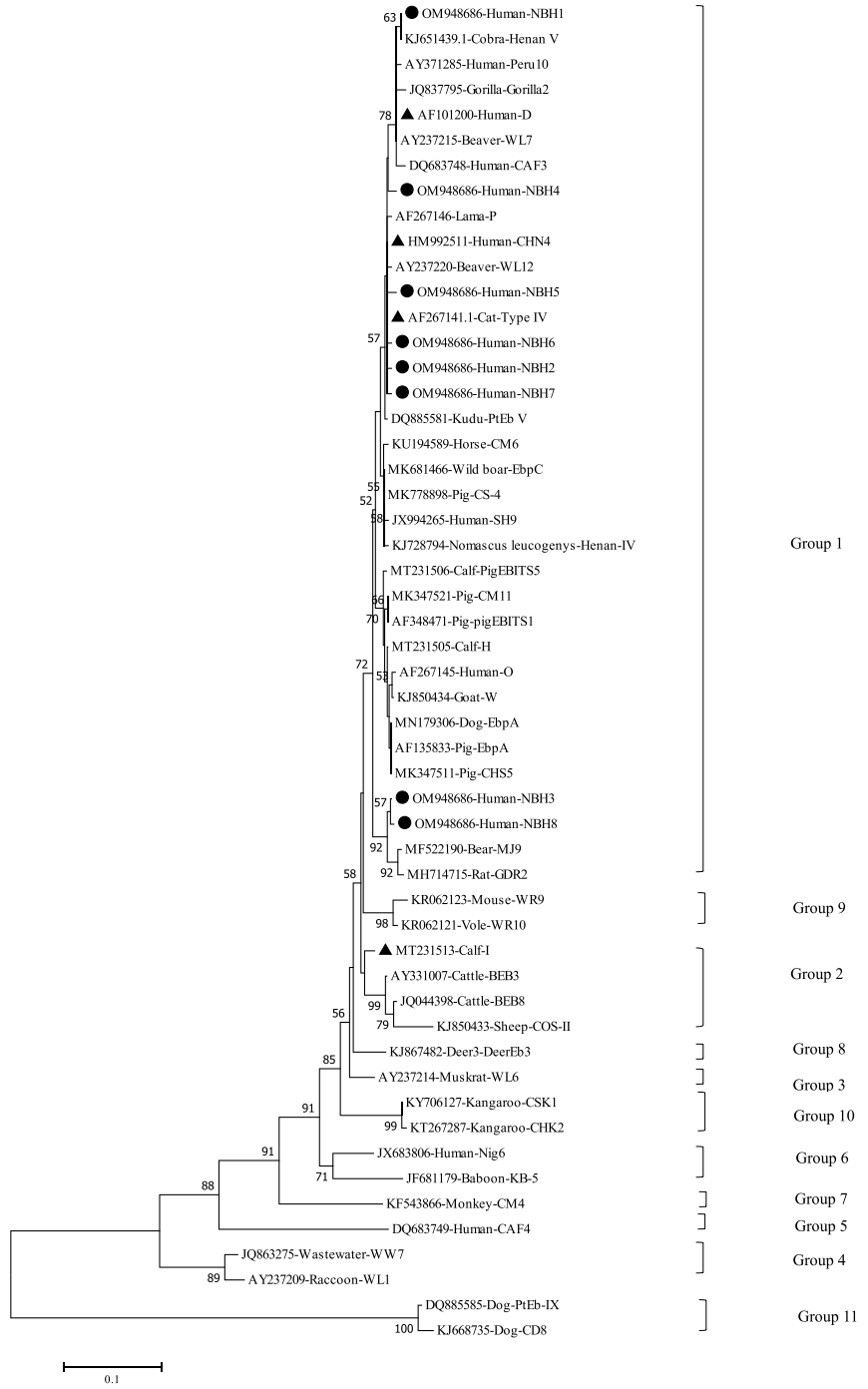

Fig. 1. Phylogenetic relationships of the 8 novel genotypes (NBH1 to NBH8) and four known genotypes (Type IV, D, I and CHN4) identified in this study of *Enterocytozoon bieneusi* inferred by a neighbor-joining analysis of ITS gene (internal transcribed spacer) sequences based on genetic distances calculated by the Kimura two-parameter model. Numbers on the branches are percent bootstrapping values from 1000 replicates. The circles and triangles filled in black indicate novel and known genotypes identified in this study, respectively.

study conducted children (age range: 2–6 years) at kindergartens in 11 counties in Southern Xinjiang [36]. In addition, genotype D was also frequently identified in children with diarrhea, HIV-positive patients, and HIV-negative patients in Shanghai, Henan, Hubei, and Guangxi [11,30,31,33]. Considering type IV and D being the frequent reported zoonotic genotypes, and may pose threats to public health, the *E. bieneusi* infections in diarrhea patients should be seen as a potential emerging diarrheal pathogen in this hospital. Therefore, the persistent investigation of patients and source tracking should be conducted in the future study.

In the present study, three cases of *C. cayetanensis* infection were identified. Sequence analysis of 18S rRNA gene indicated that the two isolates were identical with the human-derived sequence (GenBank: KJ569533.1) in previous study [24]. Gene sequence analysis of the other isolate displayed two single nucleotide polymorphisms (SNPs) among the three sequences. To the best of our knowledge, there were only 6 reports including this study about the investigation and molecular analysis of *C. cayetanensis* in China [22,23,37–39] (Table 4). The infection rate (0.6%) of diarrhea patients in our study was similar with that (0.7%) in Henan [41]. The increasing reports of *C. cayetanensis* in China are be of worthy of attention.

Clinically, the occurrence of diarrhea was usually thought to be caused by bacterial and viral agents, and parasites were always easily neglected especially in distressed area with poor hygiene and sanitation. However, parasitic diarrhea caused by the fecal–oral route by ingestion of food or water contaminated with intestinal parasites were common

Table 3
Occurrence and genotypes of *E. bieneusi* in humans in China.

Location	Patient group	Specimens	Positive (%)	Genotype (no.)[a]	References
Guangxi	HIV-positive patients	285	33 (11.6)	D (11), type IV/K (7), PigEBITS7 (7), EbpC (4), GX25 (1), GX456 (1), GX458 (1)	[11]
Shanghai	Diarrhea patients	252	34 (13.5)	–	[12]
Shanghai	Children patients	573	24 (4.2)	Peru11 (6), EbpA (2), SH2 (3), SH1 (1), SH3 (1), SH4 (1), EbpC (1), SH5–8 (1 each), D (1), SH9–12 (1 each)	[30]
Henan	HIV-positive patients	683	39 (5.7)	EbpC (18), D (7), type IV (6), PigEBITS7 (1), Peru8 (1), EbpD (1), Henan-I–V (1each)	[32]
Henan	HIV-negative patients	683	29 (4.2)	EbpC (21), D (5), type IV (1), Peru11 (1), Unknown (1)	[32]
Wuhan, Hubei	Diarrhea children	500	1 (0.2)	D (1)	[33]
Chongqing, Shandong, Hubei	Diarrheal patients	196	20(10.2)	D, CQH5, CQH6, CQH7, CQH8, CQH9, CQH10, CQH11	[35]
Xinjiang	Diarrhea children	609	36(5.9)	A (3), CHN6 (1), CXJH1 (1), CXJH 2 (1), CXJH 3 (1), D (6), EbpA (3), KB-1 (1), NIA1(19)	[36]
Changchun	Diarrhea children	40	9 (22.5)	CHN1 (5), CHN3 (4), CHN4 (3), I (3), J (3), CHN2 (2)	[46]
Harbin	Children patients or Non-diarrhea children	134	16 (11.9)	EbpC (9), CS-4 (3), Henan-IV (2), NEC1 (1), NEC2 (1), NEC3 (1), NEC4 (1)	[47]
Zhengzhou, Henan	Hospitalized children	2284	27(1.18)	D(n = 17), J(n = 2), PigEBITS7(n = 1), BEB6(n = 1), CM8 (n = 1)	[48]
Yunnan	Yao People (with diarrhea and without diarrhea)	289	8.3(24)	Peru6(21), YN104(1), YN241(1), YN249(1)	[49]
Ningbo, Zhejiang	Diarrheal patients	489	35(7.16)	Type IV(n = 14), D (n = 5), NBH8 (n = 5), NBH7 (n = 3), I(n = 1), CHN4(1), NBH1-6 (1each)	This study

[a] Not all the positive samples were sequenced successfully.

Table 4
Occurrence of *C. cayetanensis* in humans in China.

Location	Human group	Specimens	Positive (%)	Detection method	References
Shanghai	Diarrheal outpatients	291	5(1.72)	PCR	[24]
Yunnan	Diarrheal patients	378	15(3.97)	Microscopy	[38]
Wenzhou	Diarrheal patients	860	42(4.8)	Microscopy	[39]
Anhui	Diarrheal patients	610	14(2.3)	Microscopy	[40]
Henan	hospital patients	11 554	81(0.7)	PCR	[41]
Ningbo	Diarrheal outpatients	489	3(0.6)	PCR	This study

worldwide, even leading to death. For the three intestinal protozoa of interest in this study, the largest outbreak of cryptosporidiosis occurred in Milwaukee (USA) in the spring of 1993, resulting 403 000 people had watery diarrhea due to contamination of the intake source [42]. Also in the United States (USA), a possible cyclosporiasis outbreak with more than 600 cases of infection associated with fresh produce in 2013 [43]. In 2020, the second documented foodborne outbreak of *E. bieneusi* genotype C-associated diarrhea worldwide has been reported in Demark [9]. In fact, the human behaviour between the developed and developing countries is different in cultural, religious, ethnic and other related variables, which effects the epidemiology of parasitic diseases. For example, human's close association with pet animals provides more opportunities for transmission of *Cryptosporidium* spp. or other parasites such as *Giardia* spp [44]. However, with the continuous improvement of internationalization, the Chinese people's living habits have undergone great changes, such as eating lettuce, fresh food, drinking unboiled water or increasing the chance of human-animal contact, thereby increasing the risk of parasitic infection.

According to the previous reports, the three parasites have been widely and systematically identified in humans [12,24], animals [27,29] and water [45] in several cities in the Yangtze River Delta, such as Shanghai. The positive rates varied from different sources, and it was found that the infection and species/genotypes of *Cryptosporidium* spp., *E. bieneusi* and *C. cayetanensis* are more common and abundant in water and animals than in human sources, which means that more surveys on these parasites should be carried out in human populations, animals and other environmental sources from the One Health perspective in the future. Furthermore, relative health education should be enhanced to ensure more people know their importance of public health and the preventive knowledge.

5. Conclusion

In conclusion, the present study remarked that the presence of *E. bieneusi* and *C. cayetanensis* infection in diarrheal outpatients in Ningbo, while no *Cryptosporidium* spp. was found. The observation of the three known genotypes (Type IV, D and CHN4) and 8 novel genotypes (NBH1–NBH8), which were all classified as group 1, and type I was clustered into group 2, suggesting *E. bieneusi* infection posing a threat to local people. The occurrence of potentially zoonotic genotypes of the pathogens suggests that the real public health importance and further studies should be undertaken to understand their epidemiology and keep tracking the sources of human infections.

CRediT authorship contribution statement

Hua Liu: Writing – original draft, Methodology, Formal analysis, Data curation. **Hongxia Ni:** Resources, Investigation, Data curation. **Shike Liu:** Resources, Investigation. **Yujuan Shen:** Supervision, Funding acquisition. **Rong Wang:** Investigation. **Jianping Cao:** Supervision, Funding acquisition. **Jianhai Yin:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest associated with this work.

Data availability

Data will be made available on request.

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