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Molecular characterization of human pathogenic *Enterocytozoon bieneusi*, *Giardia duodenalis* and *Cyclospora cayetanensis* of diarrheal outpatients in Yangtze river delta region, China

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Abstract
Giardia duodenalis, *Enterocytozoon bieneusi* and *Cyclospora cayetanensis* are usually classified as Neglected Tropical Diseases, causing giardiasis, microsporidiosis and cyclosporiasis, respectively, which significantly contributed to food- and water-transmitted intestinal illnesses in both humans and animals. In this study, we conducted on the prevalence and molecular characterization of *Giardia*, *E. bieneusi* and *C. cayetanensis* of diarrheal outpatients from three areas in the Yangtze River Delta, China. A total of 2 720 fecal samples were collected and nested PCR was used to identify and describe the genetic characteristic of *Giardia*, *E. bieneusi* and *C. cayetanensis*. The overall prevalence of the three pathogens was 2.54% (69/2 720), with 1.03% for *E. bieneusi* (28/2 720), 0.8% for *G. duodenalis* (22/2 720), and 0.7% for *C. cayetanensis* (19/2 720), respectively. 9 *E. bieneusi* genotypes, together with two known genotypes A ($n=20$) and D ($n=1$), and 7 novel genotypes named JDFY-1 to JDFY-7 were identified. All the genotypes were clustered into zoonotic potential Group 1. In addition, 22 *G. intestinalis*-positive samples were genotyped as assemblages A ($n=4$), B ($n=17$) and E ($n=1$). 19 stool samples were identified to be *C. cayetanensis* and two new sequences were found. Therefore, our results underscored the existence and importance of *E. bieneusi*, *Giardia* and *C. cayetanensis* in diarrheal outpatients. Furthermore, the findings of *E. bieneusi* genotypes and new sequences of *Giardia* and *C. cayetanensis* indicates a high zoonotic potential of these pathogens in diarrheal outpatients. A broader range of investigations should be conducted on human, animal, and environmental sources to better understand the real transmission routes of the three enteric zoonotic protozoan.

Keywords *Giardia duodenalis*, *Enterocytozoon bieneusi*, *Cyclospora cayetanensis*, Diarrheal outpatients

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Background

Globally, diarrhoeal diseases caused by intestinal parasites have been gaining increasing interest and are associated with poverty [1]. Nearly 1.7 billion cases of childhood diarrhoeal disease were reported every year and contributed to most diarrhoeal disease load [2]. Diarrhea is a leading cause of mortality among immunocompromised individuals, especially children under 5 years old [3]. Protozoan parasites such as *Giardia duodenalis*, *Enterocytozoon bieneusi* and *Cyclospora cayetanensis* are responsible for giardiasis, microsporidiosis and cyclosporiasis, respectively, which are usually classified as Neglected Tropical Diseases [4, 5]. These opportunistic parasitosis could be transmitted through feces or contaminated water or food, or direct contact with infected humans or animals. *Giardia* and *C. cayetanensis* usually caused waterborne or foodborne outbreaks of enteric diseases. It has been reported that nearly 33% of people in developing countries had been infected with *Giardia* [6].

G. duodenalis is a species complex with broad host range and was classified into at least eight different assemblages (A–H). Assemblages A and B have been found in humans and various mammals and reported to high potential for zoonotic transmission. Assemblages C-H often have strong host specificities, C and D were usually seen in dogs, E in ruminants, F in cats, G in rodents, and H in marine mammals [7]. Globally, *E. bieneusi* is recognized as a widespread intestinal pathogen in humans, responsible for foodborne and waterborne outbreaks [8–10]. It is also usually found in diarrheal patients or immunocompromised/immunodeficient individuals, such as HIV patients [11, 12]. To date, more than 200 microsporidian genera and nearly 1500 species have now been identified [13]. Currently, *C. cayetanensis* is an increasingly important public health challenge worldwide. Humans usually were infected through ingesting food or water contaminated with sporulated oocysts [14]. *C. cayetanensis* is also a significant cause of traveler's diarrhea, particularly in industrialized areas [15].

Compared to microscopy, molecular methods such as PCR assays, show more sensitivity and specificity [16], and were widely used to trace the source of infection through amplifying the conserved genes. This study aims to delineate the prevalence and genotypes of *Giardia*, *E. bieneusi* and *Cyclospora* focusing on the diarrhea outpatients through molecular methods in Yangtze river delta from the One Health perspective. Our findings will provide new insights on the prevalence and zoonotic transmission and of these parasites.

Methods

Ethics statement

Individuals have already registered for the research process, completed the questionnaire (see supplementary file), and participated in the project with full knowledge of the study process. The written informed consent for participation were distributed to participants, and the potential risk were orally explained to all participants. All procedures of the collection and examination of human feces samples were approved by the Ethics Committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (Approval No. 2019003). Clinical trial number: not applicable.

Samples

A total of 2 720 fecal samples were collected from diarrheal patients of 5 hospitals in three different provinces/cities from 2019 to 2021. The information of age, gender and the diarrhea frequency of patients were recorded. More than 200 mg fecal excretion were collected for DNA extraction and were transported to the laboratory and stored at -80 ˚C until use.

DNA extraction and PCR amplification

Genomic DNA were extracted and purified of all the fecal samples using the QIAamp DNA stool Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. It should be noted that the lysis step was conducted at 95 °C to improve DNA yield and the final DNA was eluted in 150 µl of AE buffer. DNA were e stored at -30 ˚C until use for PCR amplification.

A 290 bp partial fragment of *Giardia duodenalis* SSU rRNA gene [17], a 390 bp nucleotide fragment of the *E. bieneusi* internal transcribed spacer (ITS) gene [18] and a 308 bp *Cyclospora cayetanensis* 18S rRNA gene [19] were amplified by nested PCR methods as previously described. Each sample was amplified with three times and the PCR products were detected by electrophoresis on 1.5% agarose gel stained with ethidium bromide.

Sequencing phylogenetic analyses

The positive PCR products were sequenced with both directions. ContigExpress and ClustalX 2.1 (http://www.clustal.org/) were used to aligne and assemble the sequences to obtain the final sequences. The species and genotypes were finally determined via sequence alignment with reference sequences downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Phylo genetic analysis was performed to describe the genetic relationship with the reference sequences published previously using 1,000 bootstrap replicates in MEGA 7.

Table 1 Detection rates of intestinal protozoa among patients with diarrhea from the Yangtze river delta in China

Variables		Intestinal protozoa	*G. duodenalis*	*E. bieneusi*	*C. cayetanensis*
		No. detection / No. examined (%) [95% CI]	No. detection / No. examined (%) [95% CI]	No. detection / No. examined (%) [95% CI]	No. detection / No. examined (%) [95% CI]
Regions	Shanghai	11/682 (1.61) [0.6–2.4]	1/682 (0.15) [-0.1-0.4]	4/682 (0.59) [-0.1-0.9]	5/682 (0.73) [0.1–1.4]
	Jiangsu	49/1 388 (3.53) [2.6–4.5]	21/1 388(1.51)[0.9–2.2]	22/1 388 (1.59) [0.9–2.2]	6/1 388 (0.43) [0.1–0.8]
	Zhejiang	11/650 (1.69) [0.8–2.9]	0	2/650 (0.31) [-0.1-0.7]	8/650 (1.23) [0.4–2.1]
	P value	0.009	0.240	0.012	0.135
Gender	Male	32/1 507 (2.12) [1.4–2.9]	8/1 507 (0.53) [0.2–0.9]	14/1 507 (0.93) [0.4–1.4]	9/1 507 (0.60) [0.2-1.0]
	Female	39/1 213 (3.21) [2.2–4.1]	14/1 213 (1.15) [0.6–1.8]	14/1 213 (1.15) [0.5–1.6]	10/1 213 (0.82) [0.3–1.3]
	P value	0.114	0.085	0.703	0.497
Ages	≤ 5	15/437 (3.43) [2.1–5.7]	3/437 (0.69) [0.3–2.5]	7/437 (1.38) [0.3–2.5]	5/437 (1.14) [0.1–2.1]
	6–17	2/139 (1.43) [-1.6-3.4]	2/139 (1.43) [-0.6-3.4]	0	0
	18–35	9/555 (1.62) [0.6–2.7]	3/555 (0.54) [0.1–1.2]	3/555 (0.54) [-0.1-1.2]	2/555 (0.36) [-0.1-0.9]
	36–59	27/752 (3.60) [2.3–4.9]	6/752 (0.80) [0.2–1.4]	12/752 (1.59) [0.7–2.5]	8/752 (1.06) [0.3–1.8]
	≥ 60	18/837 (2.15) [1.1-3.0]	8/837 (0.96) [0.2–1.5]	6/837 (0.72) [0.1–1.3]	4/837 (0.48) [0-0.9]
	P value	0.055	0.549	0.197	0.331
Diarrhea frequency/day	3–5	54/1 970 (2.74) [2.0-3.4]	15/1 970 (0.76) [0.4–1.1]	22/1 970 (1.07) [0.6–1.5]	16/1 970 (0.81) [0.4–1.2]
	6–10	16/543 (2.94) [1.5–4.4]	7/543 (1.29) [0.3–2.2]	6/543 (1.10) [0.2-2.0]	3/543 (0.55) [-0.1-1.2]
	≥ 10	1/207 (0.48) [-0.5-1.4]	0	0	0
	P value	0.103	0.215	0.380	0.574
Residence	Rural	60/2 092 (2.86) [2.1–3.5]	16/2 092 (0.76) [0.4–1.1]	28/2 092 (1.29) [0.8–1.8]	14/2 092 (0.67) [0.3-1.0]
	Urban	11/628 (1.75) [0.7–2.8]	6/628 (0.96) [0.2–1.7]	0	5/628 (0.80) [0.1–1.5]
	P value	0.152	0.615	0.002	0.785
	Total	69/2 720 (2.54) [2.0-3.2]	22/2 720 (0.80) [0.5–1.1]	28/2 720 (1.03)[0.6–1.4]	19/2 720 (0.70) [0.4-1.0]

Nucleotide sequence accession numbers
The new nucleotide sequences described here have been deposited in GenBank under accession numbers OQ240953 to OQ240959 (*E. bieneusi* ITS gene), OQ376968 to OQ376970 (*G. duodenalis* SSU rRNA gene), and OQ363213to OQ363214 (*C. cayetanensi* 18 S rRNA gene).

Statistical analysis
The differences in the detection rates of intestinal protozoa among diarrheal patients across different age groups, regions, and diarrhea frequencies were analyzed using Fisher's exact test and the Chi-square test in SAS 9.0 and SPSS 26.0 software, with the 95% confidence interval (95% CI) calculated. A two-tailed p-value < 0.05 was considered statistically significant.

Results
Occurrence of intestinal protozoa among patients
Totally, 2 720 fecal samples from 5 different hospitals in three areas were detected for the four intestinal protozoa using molecular techniques. Among these samples analyzed, 69 samples (2.54%) were identified to be one enteric pathogen, and 28 for *E. bieneusi* (1.03%), 22 for *G. duodenalis* (0.8%), and 19 for *C. cayetanensis* (0.7%) (Table 1). For *E. bieneusi*, the detection rates were different ($P = 0.012$) in three areas, with the highest (1.59%) in Jiangsu. In addition, all the *E. bieneus*-positive samples were from urban areas. There was no significant difference in the prevalence of each pathogen by gender, ages or diarrhea frequency/day in this study.

Table 2 Genotypes of *E. bieneusi* isolates from patients in this study

Region	Genotype (*n*)
Shanghai	A (3), D (1)
Jiangsu	A (15), **JDFY-1** (1), **JDFY-2** (1), **JD3FY-3** (1), **JDFY-4** (1), **JDFY-5** (1), **JDFY-6** (1), **JDFY-7** (1)
Zhejiang	A (2)
Total	A (20), D (1), **JDFY-1** (1), **JDFY-2** (1), **JD3FY-3** (1), **JDFY-4** (1), **JDFY-5** (1), **JDFY-6** (1), **JDFY-7** (1)

New genotypes are shown in bold

E. bieneusi genotypes
A total of 9 different *E. bieneusi* genotypes were identified based ITS nucleotide sequence analysis, including two known genotypes A ($n = 20$) and D ($n = 1$), and 7 novel genotypes named JDFY-1 to JDFY-7(Table 2). In phylogenetic analyses of the ITS sequences, all nine genotypes in this study belonged to the zoonotic potential Group 1 (Fig. 1).

Genetic identity of *G.duodenalis*
22 *G.intestinalis*-positive samples were genotyped as assemblages A (n=4), B (n=17) and E (n=1) based on SSU rRNA locus, with assemblage B being the dominant genotype. One new assemblage A and one new assemblage B sequences were identified in this study.

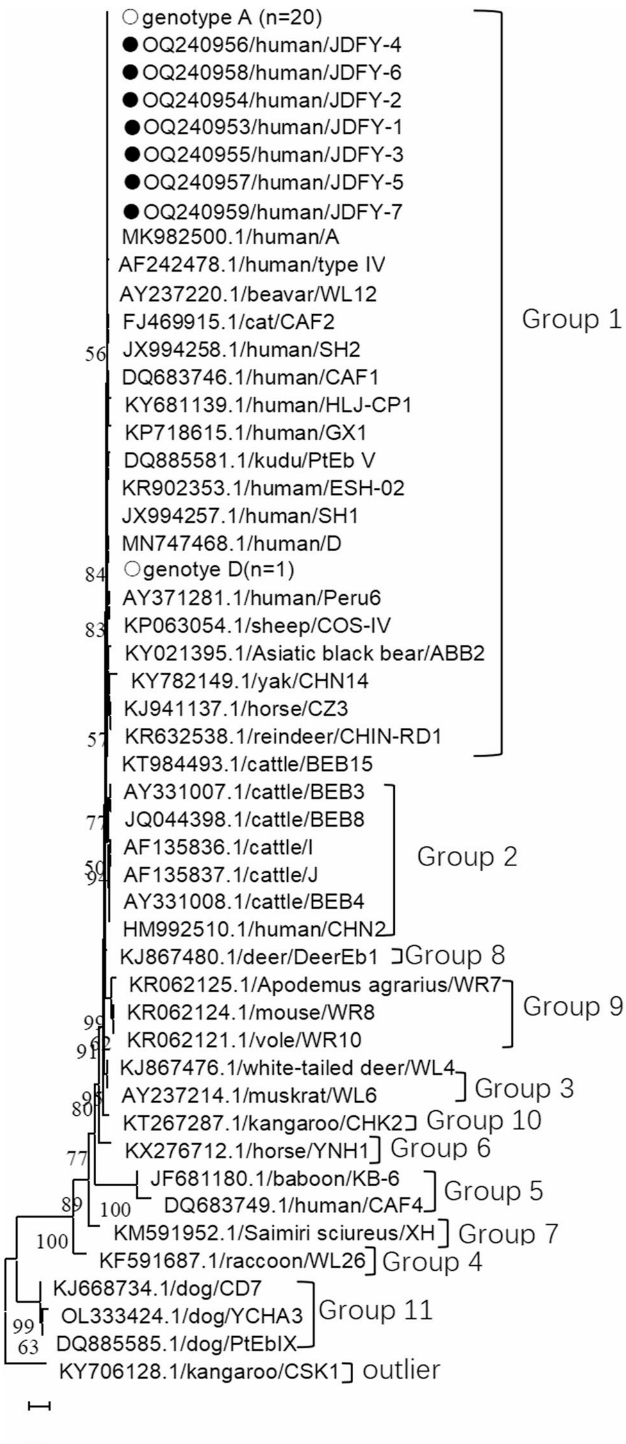

Fig. 1 Phylogenetic relationships of *E. bieneusi* genotypes: The phylogeny was inferred with a neighbor-joining analysis of the ITS sequences based on distances calculated with the Kimura two-parameter model. Bootstrap values > 50% from 1,000 replicates are shown on the nodes. The genotypes detected in this study are shown with circles; known genotypes observed in this study are marked with open circles (○) and novel genotypes are indicated by filled circles (●)

The NJ phylogenetic tree based on SSU rRNA locus were constructed, which also indicated all the nucleotide sequences were *G. intestinalis* and were clustered to be assemblage A, B and E (Fig. 2). Interestingly, no *G.duodenalis* were found in Zhejiang province in the present study.

Phylogenetic analysis of *C. cayetanensis*

19 stool samples were identified to be *C. cayetanensis* based on 18S rRNA gene, and the highest detection rate was in Jiangsu (1.23%, 8/650), with 0.73% (5/682) and 0.43% (6/1 388) in Shanghai and Zhejiang respectively. Two new sequences were found which have 99.6% nucleotide identity with the human-derived sequence (GenBank No: MN893885.1) from Guangxi, while the other sequences were the same with the human-derived sequences from Guangxi (GenBank No: MN893885.1) and Henan (GenBank No: KY770755.1 and GQ292781.1). The phylogenetic relationships of *C. cayetanensis* 18S rRNA locus indicated that these sequences obtained in this study were clustered together, and phylogenetically distinct from *Eimeria* spp (Fig. 3). Additionally, two samples mixed infection with both *E. bieneusi* genotype A and *C. cayetanensis* were from Shanghai and Zhejiang, respectively.

Discussion

In the present study, a cross-sectional survey of 2 720 fecal samples from 5 different hospitals were conducted in diarrhea outpatients from three areas in Yangtze river delta in China. The detection rate was 1.03% for *E. bieneusi* (28/2780), 0.8% for *G. duodenalis* (22/2780), and 19 for *C. cayetanensis* (19/2780), respectively, which were higher than those in our previous reports [20], which indicated that a larger sample size might give a higher infection rate.

Overall, the detection rate of *E. bieneusi* was consistent with previous reports of the prevalence ranging from 0.2% to 22.5% in China [21]. The detection rates of *E. bieneusi* were different in three areas , with the highest (1.59%) in Jiangsu which was close to that of Henan (1.18%) conducted on hospitalized children [22]. Interestingly, all the *E. bieneus*-positive samples were from urban areas and the potential reasons as follows: Firstly, the sample size of urban residents (2,092) was larger than that of rural residents (628). Secondly, with the economic and sanitary conditions, and the widespread availability of the internet in the countryside improving, rural residents now have access to extensive sources of health education regarding parasitic infections, leading to increased awareness and preventive measures. Moreover, residents in the three areas in Yangtze river delta actually live in urban or town settings, differing from traditional rural populations.

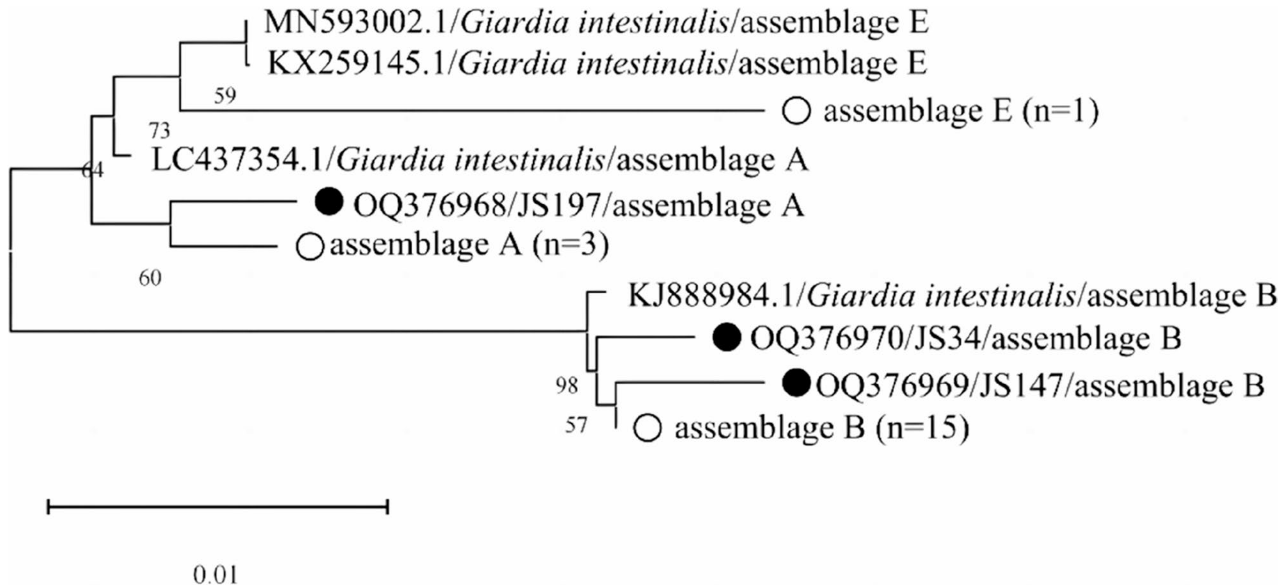

Fig. 2 Phylogenetic tree of *G. duodenalis* based on nucleotide sequences of the SSU rRNA gene: Trees were constructed using a neighbor-joining method based on genetic distance calculated by the Kimura 2-parameter model. Bootstrap values > 50% from 1,000 replicates are shown on the nodes. Isolates showed known and novel sequences obtained from this study are marked by open circles (●) and filled circles (●) respectively

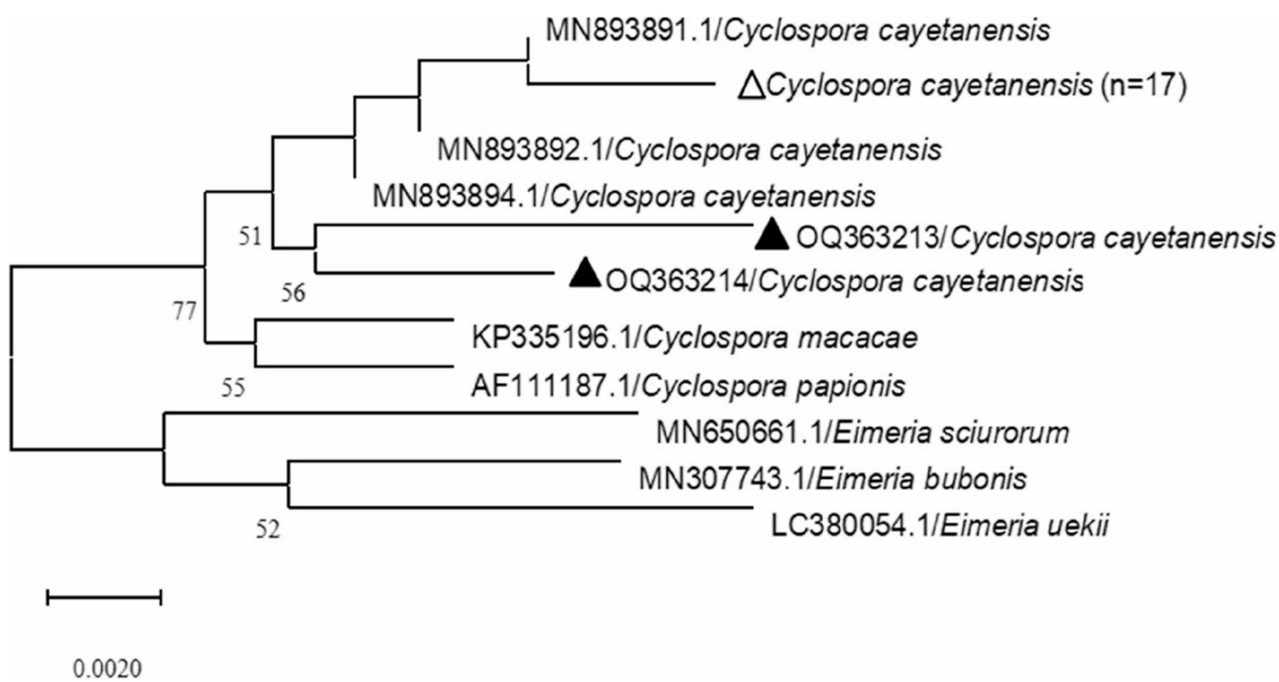

Fig. 3 Phylogenetic relationships of *C. cayetanensis* identified in this study: Phylogenetic relationships of *C. cayetanensis* identified in this study using a neighbor-joining analysis of 18S rRNA sequences. Only bootstrap values > 50% are shown in the phylogenetic tree. Isolates showed known and novel sequences obtained from this study are marked by open triangles (△) and filled triangles (▲)respectively

All the genotypes were clustered into Group 1 indicating the potential zoonotic transmission. Two known genotypes A ($n = 20$) and D ($n = 1$), 7 novel genotypes named JDFY-1 to JDFY-7 were identified, with genotype A being dominant. As far as we known, genotype A in humans has just been reported in 10 countries worldwide and most in HIV/AIDS patients, with the infection rates 1.3% in Peru [23], 0.88% (2/228) in Niger [24], 0.70% (1/142) in Congo [25], 0.47% (4/856) in Portugal [26], 4.75% (22/463) in Nigeria [27], 0.7% (2/279) of children [28] and 4.23% (94/2220) [29] of infants in Thailand, 0.9% (2/222) of HIV patients [30] and 22.72% (5/22) of kidney transplant recipient [31] in India. The genotype A was reported in Germany [32] and the Netherlands [33], but the infection rate was not mentioned. To the best of our knowledge, this is the first report of genotype A in outpatients with diarrhea worldwide. In addition, based on phylogenetic analysis of the ITS sequence, we identified the novel genotypes belonging to Group 1. Previous studies have defined the zoonotic potential transmission of the first group with a broad spectrum of hosts and more than 90% of *E. bieneusi* isolates were clustered into this group [13, 34]. In the future, more attends should be carried out in these areas to find the possible transmission route.

Globally, Giardiasis poses a major global health burden, ranking among the leading causes of waterborne and foodborne diarrheal diseases worldwide, with an estimated 280 million annual human cases [35]. To date, the highest reported infection rate of *G. duodenalis* infections in humans was 82.05% (32/39) in Turkey [36]. In United States, a report conducted on the survey about Giardiasis Outbreaks from 2012 to 2017 showed that it was closely associated with the contaminated water exposure, person-to-person contact and contaminated food ingestion [37]. In China, the infection rate in humans was from 0.03% (9/26886) in Xinjiang [38] to 4.12% (40/972) in Anhui [39]. In the present study, the prevalence in Jiangsu (1.51%, 21/1388) was consistent with that (1.4%, 7/500) of Wuhan [40], and the prevalence in Shanghai (0.15%, 1/682) which was consistent with that (0.21%, 24/11341) of Jiangsu [39]. Interestingly, no *G. duodenalis* being found in Zhejiang, which might be affected by many factors, including sample size, sampling time and different hospitals. In addition, assemblage B was mostly found among the three assemblages (A = 4, B = 17 and E = 1), with assemblage E being firstly reported in diarrheal children in China. In fact, assemblage E has been recently reported in a study conducted on the occurrence and molecular characteristics of *Cryptosporidium* species, *Giardia duodenalis*, and *Enterocytozoon bieneusi* among AIDS patients with severe immunodeficiency [41]. The potential risk of zoonotic transmission of assemblage E was also confirmed in a previous study on both children and calves with the finding that assemblage E being identified [42].

Cyclospora infections commonly caused diarrhea in children in developing countries whereas in developed countries, they are more often linked to traveler's diarrhea and food- or water-borne outbreaks and had become a global public health concern [43]. *C. cayetanensis* detection rate (0.73%, 5/682) in Shanghai in this present study was lower than that in 2013 (1.72%, 5/291) [44]. However, the detection rate in Zhejiang (0.43%, 6/1 388)) was similar with that (0.6%, 3/489) in 2019 [21]. The difference in detection rate could be due to the variations in sampling time/size/areas. Phylogenetic analyses of the study also confirmed its distinctiveness from *Eimeria* spp which was consistent with the previous study [45]. Furthermore, two co-infections of these pathogens were also found with both *E. bieneusi* genotype A and *C. cayetanensis* were from Shanghai and Zhejiang, respectively, which should be paid more attention.

While our study provided valuable insights into molecular analysis of three important parasites of diarrheal outpatients in Yangtze river delta, these findings should be interpreted in light of certain limitations. First, the reliance on a single stool sample per participant may lead to an underestimation of the prevalence, and future studies incorporating serial sample collections would be valuable to confirm the true infection rates. Second, additional methods such as microscopic examination for multi-layered verification could be included in future study. And finally, to enhance the representativeness of the findings, sample selection should be given more key epidemiological factors such as geographic distribution.

Conclusions

In conclusion, our study revealed the prevalence and genetic characteristic of *E. bieneusi*, *G. duodenalis* and *C. cayetanensis* derived from diarrheal outpatients in three areas in Yangtze river delta. An overall prevalence of 2.54% were reported of three parasites including *E. bieneusi* (1.03%), *G. duodenalis* (0.8%) and *C. cayetanensis* (0.7%). 9 *E. bieneusi* genotypes with two known genotypes A and D, and 7 novel genotypes were identified. Assemblages A, B and E were genotyped. Of 22 *G. intestinalis*-positive samples and two new *C. cayetanensis* sequences were found. The reports of new zoonotic genotypes, new sequences and mixed infections showed the importance of conducting the molecular epidemiology research on diarrheal patients. Since these parasites present a significant risk to public health, the importance of improved water and food safety measures in preventing enteric zoonotic protozoan transmission and reducing outbreaks is strongly supported by the reports.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-04655-4.

Supplementary Material 1.

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Authors' contributions

Y.S. designed the study. H.L., Y.Q. participated in the sample collection and methodology. H.L., Y.Q., Q.Z., L.H. and J.Z. contributed to data analysis. Y.S. and J.C. contributed reagents and materials. H.L. and Y.Q. wrote the manuscript. Y.S. revised the manuscript. All authors read and approved the final manuscript.

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Data availability
The new nucleotide sequences described here have been deposited in GenBank under accession numbers OQ240953 to OQ240959 (*E. bieneusi* ITS gene), OQ376968 to OQ376970 (*G. duodenalis* SSU rRNA gene), and OQ363213 to OQ363214 (*C. cayetanensi* 18 S rRNA gene).

Declarations

Ethics approval and consent to participate
This study was conducted in accordance with the ethical principles of the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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