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RESEARCH ARTICLE

Cyclospora cayetanensis infections among diarrheal outpatients in Shanghai: a retrospective case study

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Abstract ***Cyclospora cayetanensis*** **is a foodborne and waterborne pathogen that causes endemic and epidemic human diarrhea worldwide. A few epidemiological studies regarding *C. cayetanensis* infections in China have been conducted. During 2013, a total of 291 stool specimens were collected from patients with diarrhea at a hospital in urban Shanghai. *C. cayetanensis* was not detected in any of the stool specimens by traditional microscopy, whereas five stool specimens (1.72%, 5/291) were positive by PCR. These positive cases confirmed by molecular technology were all in the adult group (mean age 27.8 years; 2.94%, 5/170) with watery diarrhea. Marked infection occurred in the rainy season of May and July. Sequence and phylogenetic analyses of the partial 18S rRNA genes of *C. cayetanensis* isolated showed intra-species diversity of this parasite. This study showed, for the first time, that *C. cayetanensis* is a pathogen in outpatients with diarrhea in Shanghai, albeit at a low level. However, the transmission dynamics of this parasite in these patients remain uncertain.**

Keywords *Cyclospora cayetanensis*; outpatients with diarrhea; stool specimens; 18S rRNA gene

Introduction

Cyclospora cayetanensis is an apicomplexan parasite that infects the gastrointestinal tract and causes acute diarrheal disease in both immunocompromised and immunocompetent individuals [1,2]. In recent years, this parasite has led to several foodborne outbreaks in the United States [3], Mexico [4,5], Canada [6], and Turkey [7], mostly associated with imported produce and traveling. Besides these risk factors, the infection has been recognized as endemic in several areas of the developing world due to contact with animals and soil [8]. Water sanitary quality has been identified as a risk factor for infection in several areas, such as Spain [9] and Tunisia [10]. Variables associated with poverty have been associated with infection in Venezuela [11]. In China, cyclosporiasis infection rates range from 3.97% to 6.39% in humans with diarrhea [12–14]. Therefore, cyclosporiasis infection is receiving increasing attention worldwide.

Previous studies related to *Cyclospora* parasite detection mainly involved stool smear staining and morphological identification [15,16]. However, PCR-based detection methods allow us to distinguish human pathogenic *Cyclospora* parasites from those that do not infect humans and improve detection sensitivity and accuracy [9,17]. Current biological and epidemiological understanding indicates that *C. cayetanensis* is associated with age range [18], gender [19], season [20], and intraspecies variation of 18S rRNA gene sequences [21]. Although Shanghai is an international city, the extent and epidemiology of *C. cayetanensis* in Shanghai is unknown. Therefore, a retrospective case study was designed, involving outpatients from the infection department of a hospital over one year, to assess the prevalence and importance of this parasite in diarrheal patients.

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Materials and methods

Sample collection

In this study, we defined diarrhea as a 24-h period during which the patient was reported to have $\geqslant$ 3 liquid or semi-liquid stools [22]. The frequency of diarrhea was defined as the number of occurrences of diarrhea per day. From January 2013 to December 2013, a total of 291 stool samples were collected from outpatients with diarrhea in the Infectious Diseases Department of the Sixth People's Hospital of Shanghai affiliated to Shanghai Jiao Tong University, China.

The objectives and procedures were explained orally to all participants. Written informed consent was provided by all participants in this study, including signed consent by parents/guardians on behalf of all child participants. Patient details, including age, gender, occurrence, duration and frequency of diarrhea, and consistency of stools, were recorded.

Stool samples

Stool samples were sent to our laboratory within 24 h for detection of *C. cayetanensis* by two methods, namely, traditional microscopy and nested PCR. Each sample was divided into two parts, one for *Cyclospora* spp. detection with the acid-fast staining method described by Madico *et al*. [23] and the other for molecular confirmation [24].

DNA extraction and PCR amplification

Sufficient samples (200 mg of each stool sample) were used for DNA extraction and purification using a commercially available kit, the QIAamp DNA Mini Stool Kit (Qiagen, Valencia, CA). The extracted DNA samples were stored at –20 °C until PCR. The presence of *C. cayetanensis* in samples was detected by nested PCR amplification of an approximately 501-bp fragment of the 18S rRNA gene, as described previously [17]. Negative controls using DNase-free water and *C. cayetanensis*-free stool samples were included in each PCR run. A positive control, which had previously been confirmed by microcopy [25] and molecular methods, was used in each PCR run.

Sequence and phylogenetic analyses

PCR products were sequenced in both directions on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) by Shanghai Sunny Biotechnology Co. (Shanghai, China). The sequences obtained were blasted against the NCBI database and deposited in GenBank (accession numbers KJ569531 to KJ569535). Based on the reference AF111183 sequence (the *C. cayetanensis* 18S rRNA coding region), these sequences were edited using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned using MEGA5 software for construction of a phylogenetic tree [26].

Statistical analysis

Data were analyzed using SPSS version 11.0 (SPSS, Chicago, IL, USA). Chi-square tests were used for comparison of proportions and analysis of variance for comparison of means. The level of significance was $P < 0.05$.

Results

Occurrence of *C. cayetanensis* infection in patients with diarrhea

Stool samples from 291 patients with diarrhea were detected by microscopic examination in comparison with a confirmed *C. cayetanensis*-infected stool sample. However, no *Cyclospora* oocyst was detected in the stool samples of the 291 patients with diarrhea. By molecular detection, *C. cayetanensis* was identified in five samples (1.72%, 5/291; Table 1) using a modified nested-PCR method [27]. The five patients with *C. cayetanensis* infection had severe watery diarrhea, and the frequency of diarrhea ranged from 3.5 to 10 times per day (Table 2).

Characteristics of *C. cayetanensis* infection

The infection rates of *C. cayetanensis* ranged from 0% to 12.5% in the hospital in 2013 (Table 3). The greatest number of positive cases was observed in July (5.26%, 3/57), followed by May (12.5%, 2/16). *C. cayetanensis*

Table 1 Detection rate and distribution of *Cyclospora cayetanensis* by gender, age, and occupation of patients

Sample	Gender[a]		Age (year)			Occupation			Total
	Male	Female	14–17	18–44	⩾45	Worker	Service	Other	
No. examined	139	152	15	170	106	181	10	100	291
No. positive (%)	3 (2.16)	2 (1.32)	0 (0)	5 (2.94)	0 (0)	4 (2.21)	1 (10.00)	0	5 (1.72)[b]

All specimens were collected in 2013, and were analyzed by the small-subunit (SSU) rRNA gene of *C. cayetanensis*. [a]There was no significant difference in the detection rate of *C. cayetanensis* by gender ($\chi^2 = 0.3025$, $P = 0.5699$). [b]The detection rate of *C. cayetanensis* infection.

Table 2 Sequence analysis of variable 18S rRNA nucleotide sequences among *Cyclospora cayetanensis* isolates from patients with diarrhea in Shanghai and the associated clinical symptoms

Accession no. in GenBank	Nucleotide at position			Clinical symptoms			Same isolates found in other areas
	112	121	248	Frequency of diarrhea (times/d)	Fever	Vomiting	
AF111183	C	C	G				
KJ569531	.	Y	.	10	No	No	China (Henan), Mexico, Peru
KJ569532	.	.	.	3.5	No	No	Mexico
KJ569533	.	.	.	4	No	No	Mexico
KJ569534	T	.	.	4	No	Yes	China (Henan), Peru
KJ569535	.	.	C	4	Yes	No	China (Henan)

A dot (.) indicates an identical nucleotide compared with the reference sequence (AF111183).

Table 3 Monthly prevalence of *Cyclospora cayetanensis* in a hospital in Shanghai during the year 2013

Month	No. infected/No. patients	Infection rate (%)
January	0/8	0
February	0/2	0
March	0/12	0
April	0/19	0
May	2/16	12.50
June	0/15	0
July	3/57	5.26
August	0/31	0
September	0/48	0
October	0/32	0
November	0/21	0
December	0/30	0

infection showed greater occurrence in the rainfall season, although this observation was not statistically significant (P = 0.9861). The infection rates of male and female patients were 2.16% (3/139) and 1.32% (2/152); no significant difference was found in terms of gender (χ^2 = 0.3052, P = 0.5699; Table 1). The outpatients came from the Infectious Diseases Department of the hospital, and therefore excluded children under the age of 14 years. *C. cayetanensis* was only detected in adults (18–44 years; mean age 27.8 years; Table 1) and there was no significant difference (χ^2 = 1.53, P = 0.216) compared with other age groups. Infection rates in this age group by gender were 3.23% male (3/93) and 2.60% female (2/77). Four of the patients with *C. cayetanensis* infection were office workers from different companies (4/181, 2.21%; Table 1) and the other worked in a catering service preparing food (1/10, 10.0%; Table 1). They all had a habit of dining out, but no travel history within three months before the onset of diarrhea.

DNA sequences of *C. cayetanensis* at the SSU rRNA locus

Our five 18S rRNA gene sequences were blasted against 45 sequences, including a representative reference sequence of *C. cayetanensis* (AF111183) [28] and 44 other published sequences [17,25]. The five isolates from our hospital showed several intra-species variations at this locus (Table 2), including three polymorphic nucleotide positions (112, 121, and 245). However, these polymorphic sites showed no distinct geographic isolation patterns (Fig. 1).

Discussion

This report is one of the few studies to assess the prevalence of *C. cayetanensis* in endemic conditions and the first occurrence focused on outpatients with diarrhea from a hospital in Shanghai. Results indicate that *C. cayetanensis* is a pathogen in outpatients with diarrhea in Shanghai, albeit at a low level. Our study is limited by the relatively small positive sample size, which may have affected the statistical power and thus the ability to identify minor changes in risk factors for *C. cayetanensis* infection. However, our findings were supported by the results of an investigation from Henan Province, China, conducted with a relatively substantial number of individuals with diarrhea (2.97%; 12/404) [25]. The differences in cyclosporiasis infection rate in different studies may be related to socioeconomic conditions, local climatic factors, and eating habits, as well as immune status of hosts, sample structure or age, and infective doses of the parasite.

In the present study, we observed the same potential seasonal tendency toward *Cyclospora* infection (i.e., in May and July) as in previous work [29]. Interestingly, our findings were consistent with those observed in Henan Province [25]. Other studies have confirmed the seasonality of *Cyclospora* spp. infection in clinical samples from

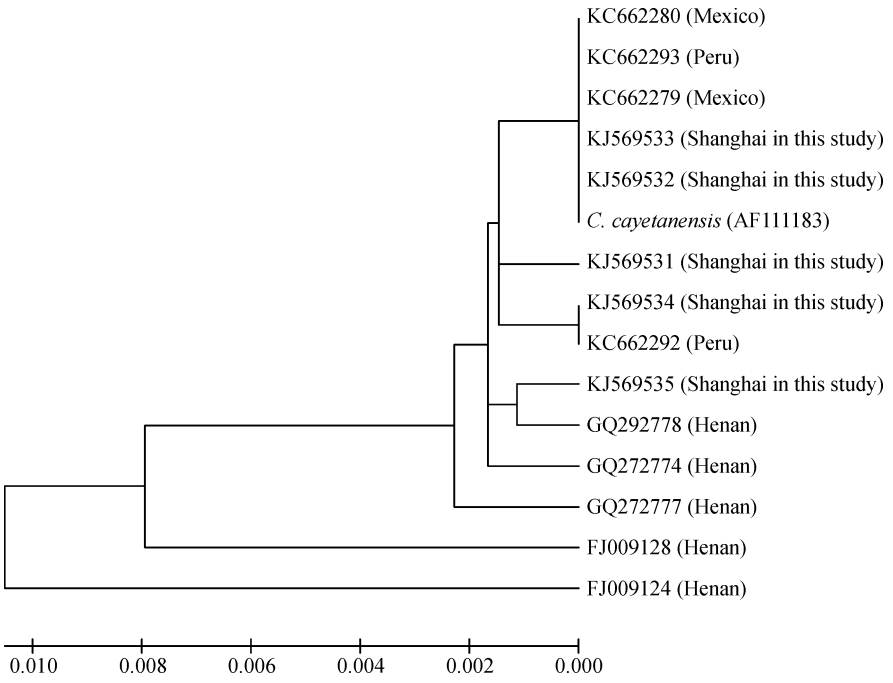

Fig. 1 Phylogenetic relationships of *C. cayetanensis* identified in this study by a neighbor-joining analysis of 18S rRNA sequences. Only bootstrap values > 50% are shown in the phylogenetic tree.

areas with humid and rainy seasons, such as Indonesia and Guatemala [30]. Consistent with these findings, we found no samples that were positive for *Cyclospora* spp. infection between October 2012 and March 2013 in Shanghai [27]. However, data about environmental conditions during the period in which the research was performed were lacking, thereby limiting our interpretation of results; we will further investigate the correlation between the infection pattern of *C. cayetanensis* and environmental conditions.

In a previous investigation, 63.4% of *Cyclospora*-infected patients were male and 36.6% were female (of 60 in Morelia, Mexico; $P = 0.042$) [20]; however, our study was limited by the low number of positive samples. In terms of age of infected patients, our findings correspond with other reports that included adults in Guatemala [31] and Nigeria [32]. Further investigations will increase our data pool and provide a more balanced view of the infection rate by gender and age.

We found four cases of *C. cayetanensis* infection in patients who worked for companies where eating out during working hours was necessary, and one in a patient who worked in a catering service. Thus, all five cases were possibly associated with eating contaminated food. This intriguing possibility is consistent with a report concerning cyclosporiasis and the eating of uncooked food items in Haiti [33]. Combined with the report of 2013 multistate outbreaks of cyclosporiasis during May to August in the United States, peaking in June and July, we suggest that the most likely vehicle of infection in our positive cases is that they ate onions in restaurants [34]. Nonetheless, studies involving patients with diarrhea should be conducted to obtain definitive answers about the food-borne transmission of this parasite based on traceback investigations.

Gene sequence analysis of the five specimens of *C. cayetanensis* showed slight intraspecies variation with one or two base variations; however, these single nucleotide polymorphisms did not reflect any clear geographic segregation. Our results were supported by examination of 18S rRNA gene sequences of isolates from China, revealing only minor sequence polymorphisms [25]. Sulaiman *et al.* [17] also showed only minor genetic diversity within the *C. cayetanensis* species, but significant, distinct genetic variation among members of *C. colobi*, *C. papionis*, and *C. cercophiteci*. However, a study that amplified 18S rRNA genes and used high-resolution melting curves of PCR-amplified DNAs extracted from the stool samples of 70 cyclosporiasis patients showed four categories of genotypic profile of *C. cayetanensis*, significantly relating genotypic profiles and gastrointestinal tract symptomatic status [21]. This finding is consistent with the existence of genetic diversity causing specific clinical forms of infection with *Leishmania* [35] and *Cryptosporidium andersoni* [36,37]. Therefore, more positive samples are needed to analyze variation of 18S rRNA gene sequences.

The new tool of multilocus sequence typing for *C. cayetanensis* may provide linkage between case and

infection/contamination source tracking [38]. Further exploration of this concept will require detection of more positive samples for analysis. A draft nuclear genome sequence from *C. cayetanensis* oocysts purified from a human stool sample [20] and the complete mitochondrial genome sequence [39,40] have been published; these data allows the investigation of *Cyclospora* outbreaks and exploration of clues associated with clinical symptom based on trace-back assays [41] by molecular detection.

Conclusions

We identified, for the first time, *C. cayetanensis* in patients with diarrhea in Shanghai and extended our knowledge of the prevalence of infection in certain months. Although there was no statistically significant association between gender, age or season and the molecular detection of *C. cayetanensis*, its detection in patients with diarrhea highlights a potential public health risk in Shanghai.

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Compliance with ethics guidelines

Yanyan Jiang, Zhongying Yuan, Guoqing Zang, Dan Li, Ying Wang, Yi Zhang, Hua Liu, Jianping Cao, and Yujuan Shen declare that there are no conflicts of interest. All procedures were performed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and the *Helsinki Declaration* of 1975, as revised in 2000. 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 (reference no. 2012-12). Informed consent was obtained from all patients for inclusion in the present study.

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