

Cyclospore cayetanensis in Anhui, China

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Abstract

AIM: To investigate the infection of *Cyclospore cayetanensis* in Anhui Province.

METHODS: Identification of *Cyclospore cayetanensis* was made microscopically by finding the oocysts of *Cyclospore cayetanensis* in fecal smears taken from the infants, pupils and adults with obstinate diarrhea, and immunocompromised individuals by using a auramine-phenol stain and modified acid-fast stain. Cellular immune function was detected with biotin-streptavidin (BSA), and the specific antibody against *Cyclospore cayetanensis* was detected with method of ELISA.

RESULTS: (1) The positive rates of *Cyclospore cayetanensis* infection in infants, pupils, infants and adults with obstinate diarrhea and with immunocompromised individuals were significantly different ($P < 0.01$), with the rates of 0 %, 0.50 % (1/200), 5.62 % (10/178), and 9.38 % (3/32) respectively. (2) The infection rates of males and females were 2.61 % (10/383) and 1.44 % (4/227) respectively, with no significant difference ($P < 0.05$). (3) The positive rates of population with oocysts in urban and rural areas were 0.92 % (3/325) and 3.86 % (11/285) respectively. (4) The positive rates of CD₃⁺, CD₄⁺, CD₈⁺ and the ratio of CD₄⁺/CD₈⁺ of individuals with and without oocysts were significantly different ($P < 0.05$, $P < 0.01$), and their values were (64.28±6.55) %, (43.55±5.80) %, (28.23±4.32) %, 1.52±0.32 and (58.97±5.23) %, (39.26±4.93) %, (30.54±5.17) %, 1.26±0.21, respectively. (5) Specific IgG, IgM and IgG+IgM in serum of the patients with oocyst were significantly different ($P < 0.01$) with the positive rates of 63.41 % (9/14), 17.07 % (1/14) and 19.51 % (4/14) respectively.

CONCLUSION: *Cyclospore cayetanensis* infection is present in Anhui, China and it was confirmed to be a new pathogen associated with children diarrhea, adults obstinate diarrhea and diarrhea in immunocompromised individuals. Among all the infected individuals, adult obstinate diarrhea patients and immunocompromised individuals are common. Feces examination of oocysts and serological examination of the specific antibody will be of much help in the diagnosis of *Cyclospore cayetanensis* infection.

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INTRODUCTION

Cyclospore cayetanensis is an emerging protozoan pathogen associated with diarrhea and human immune function^[1-16]. It is a kind of enteric parasite that infests in the gastrointestinal tract of human causing severe diarrhea. Jejunal biopsies showed an altered mucosal architecture with shortening and widening of the intestinal villi due to diffuse edema and infiltration by a mixed inflammatory cell infiltrate^[17,18]. There was reactive hyperemia with vascular dilatation and congestion of villous capillaries. Cyclosporiasis occurs in persons of all ages and in either immunocompetent or immunocompromised hosts^[19-30]. Human being is the only natural host for this parasite^[31]. Clinical symptoms of Cyclosporiasis are characterized by watery diarrhea (approximately 6 stools/day), nausea, anorexia, abdominal cramping, fatigue, and weight loss, and they are not so specific and can often be misdiagnosed^[32-37]. So cyclospora oocysts detection is needed by phase contrast microscopy, modified acid-fast staining, autofluorescence, and amplification by the polymerase chain reaction^[38-48].

In order to investigate the infection of *Cyclospore cayetanensis* in Anhui Province, we took an investigation of *Cyclospore cayetanensis* in rural and urban areas of Anhui Province. The results of our investigation showed that *Cyclospore cayetanensis* infection was present in Anhui Province and it was confirmed to be a new pathogen associated with children diarrhea and adult obstinate diarrhea.

MATERIALS AND METHODS

Materials

A total of 610 individuals from 11 cities, including Huainan, Hefei, Bengbu, were involved in this investigation. All of them aged from 1 to 55 years, 383 male and 227 female. And they were divided into 2 groups (control group and experimental group).

Screening population (Control group) 400 individuals (infants and pupils) were included in the control group, 217 male and 183 female.

Diarrhea patients (Experimental group) 178 individuals with undefined diarrhea from outpatients (infants and adult with obstinate diarrhea) as well as 32 immunocompromised individuals with obstinate diarrhea were involved in the experimental group, 166 male and 44 female. And 210 feces specimens from the experimental group were collected. Those who had any 2 of the following 5 criteria were considered as immunocompromised individuals: (1) weakened proliferation reaction in lymphocyte transformation test, reduction or lopsided development in T-cell subsets; (2) lower expression of one or more immune globulin (IgG, IgM, IgA, IgD and IgE); (3) deficiency in cytokine, such as IL-2, IFN-g as well as loss of expression in IL-1R and in IL-2R; (4) weakened function of phagocyte and (5) frequent occurrence of infection such as opportunistic pathogen, virus, fungi and parasites.

Methods

Inquiry of diarrhea history Histories of present illness, anamnesis, symptoms, personal hygiene habits, living

environmental hygiene of the objects and so on were inquired. **Feces examination** 2-3 g fresh feces per person was collected with disposable boxes and smeared into translucent layers. Then the smears were microscopically examined after auramine-phenol stain and modified acid-fast stain. It was described as positive when oocysts were found with apparent internal structure in red, but other non-specific granules revealed black-blue, which was significantly different from the oocysts.

Serological examination Oocysts of *Cyclospore cayetanensis* were taken as antigen from artificially infected guinea pigs and embraced on polystyrene board. Serological examination was performed with ELISA to detect the specific antibody of IgG and IgM in individuals with oocysts. Each time, values of OD of 3 holes (empty, positive, and negative respectively) were read from enzyme labeling meter, and it was judged to be positive when it was 2.1 times as much as the negative.

Detection of T lymphocyte cell subsets Cellular immune function of the individuals with oocysts was detected. The percentage of CD3+, CD4+ and CD8+, and the ratio of CD4+/CD8+ were detected with biotin-streptavidin (BSA) method. Mixed with heparin, peripheral blood was taken and diluted with an equivalence of liquid free of Ca2+ and Mg2+, then mononuclear cells were separated with lymphocytes separating medium and were made into cell suspension with concentration of (1-3)×106 cells per ml after washing and adjustment. 10 µl of the cell suspension was smeared in acid-proof varnish rings on the surface of slides each time, and when it dried naturally, McAb of anti-CD3+, anti-CD4+ and anti-CD8+, IgG of sheep against guinea pig, and SA-HRP were added. After development with DAB, the smears were observed under microscope. Cytoplasm in brown was regarded as positive, and non-pigmenting as negative. Each time a total of 200 cells were counted for statistics of the positive percentage of cells.

Statistical analysis

Data were expressed as mean ± standard deviation. And multiple comparison tests were performed with χ2 test and t test.

RESULTS

Feces examination

The positive rates of *Cyclospore cayetanensis* from feces taken from the control group and the experimental group were 0.25 % (1/400) and 6.19 % (13/210) respectively. To be exact, the positive rates of the diarrheal and immunocompromised individuals were 5.62 % (10/178) and 9.38 % (3/32) respectively. And the positive rates of the two groups were significantly different (P<0.01). The results are shown in Table 1.

Table 1 Feces examination of *Cyclospore cayetanensis* in different groups (n, %)

Group	n	Auramine-phenol stain+Modified acid-fast stain	
		Positive number	Positive rate
Control group ^b	400	1	0.25
Infants	200	0	0.00
Pupils	200	1	0.50
Experimental group ^b	210	13	6.19
Diarrheal patients	178	10	5.62
Immunocompromised individuals	32	3	9.38
Total	610	14	2.30

^bP<0.01, χ2=21.6684 vs: control group

Common clinical symptoms

Among individuals with *C.cayetanensis*, some had no or slight symptoms in early stage. In our survey, healthy pupils and immunocompromised individuals had no self-conscious symptoms. However, others had clinical symptoms mainly in lower digestive tract (60 %), such as abdominal distention, abdominal pain, loose stool and watery stool. After retrospective studies on 14 cases of *Cyclospore cayetanensis* infection, it was found that 10 of them (71.43 %) were misdiagnosed as bacterial enteritis (n=5), viral enteritis (n=3), non-specific enteritis (n=2), parasitic infection (n=2) and abdominal discomfort (n=2). The results are shown in Table 2.

Table 2 Clinical symptoms caused by *Cyclospore cayetanensis* (n,%)

Group	Case number	Case Percentage
Non-self-conscious symptom	4	28.57
Self-conscious symptom	10	71.43
General symptom	2	14.29
Upper digestive tract symptom	1	7.14
Lower digestive tract symptom	4	28.57
Upper and lower digestive tract symptom	1	7.14
General and upper digestive tract symptom	1	7.14
General and upper, lower digestive tract symptom	1	7.14
Total	14	100

General symptom: acratia and fever;
Upper digestive tract symptom: anorexia, nausea and vomiting;
Lower digestive tract symptom: abdominal distention, abdominal pain, loose stool and watery stool.

Relationship between infection of Cyclospore cayetanensis and gender

Total 610 individuals from different population groups were involved in this investigation on oocysts of *C.cayetanensis* in stools. It was shown that the total positive rate of oocysts was 2.30 % (14/610). In detail, the positive rates were 2.61 % (10/383) in male and 1.44 % (4/227) in female, and there was no significant difference between them (P>0.05). The results are shown in Table 3.

Table 3 Feces examination of *Cyclospore cayetanensis* of different gender

Group	n	Auramine-phenol stain+Modified anti-acid stain	
		Positive number	Positive rate (%)
Male	383	10	2.61
Female	227	4	1.44
Total	610	14	2.30

P>0.05, χ2=0.4581

Relationship between infection of Cyclospore cayetanensis and living environmental condition as well as personal hygiene habits

It was shown that the positive rate of oocysts in rural areas was significantly higher than that in urban areas (P<0.05). The

survey showed that there were much difference in living environmental condition and personal hygiene habits between the individuals in rural areas and in urban. In rural areas, plenty of simple toilets, deficiency of sanitary facilities and diffusing feces contamination were commonly seen, and most people were unaware of health knowledge and good hygiene habits. Thus, higher infection rate occurred in rural areas. The results are shown in Table 4.

Table 4 Feces examination of *Cyclospore cayetanensi* in different areas

Group	n	Auramine-phenol stain+Modified acid-fast stain	
		Positive number	Positive rate (%)
Urban area	325	3	0.92
Rural area	285	11	3.86
Total	610	14	2.30

$P < 0.05$, $\chi^2 = 5.8419$

Detection of specific antibody

It was shown that specific antibody of IgG was frequently positive in oocysts-positive individuals, and those of both IgG and IgM were positive in some cases. The results are shown in Table 5.

Table 5 Detection of specific antibody of individuals with *Cyclospore cayetanensis* (n, %)

Group	n	IgG ^b		IgM ^b		IgG+IgM ^b	
		Number	Rate	Number	Rate	Number	Rate
Oocysts positive	14	9	63.41	1	17.07	4	19.51
Oocysts negative	20	0	0.00	0	0.00	0	0.00

^b $P < 0.01$, $\chi^2 = 10.4962$ vs: oocyst negative

Detection of cellular immune function

It was found that the levels of CD₃⁺, CD₄⁺ and CD₄⁺/CD₈⁺ decreased compared with those of the oocysts-negative individuals ($P < 0.05$, $P < 0.01$). And the expression of CD₈⁺ seemed to be stable. The results are shown in Table 6.

Table 6 Detection of T lymphocyte cell subsets of individuals with *Cyclospore cayetanensis* (n, $\bar{x} \pm s$, %)

Group	n	CD ₃ ⁺	CD ₄ ⁺	CD ₈ ⁺	CD ₄ ⁺ /CD ₈ ⁺
Oocysts positive	14	64.28±6.55 ^a	43.55±5.80 ^c	28.23±4.32	1.52±0.32 ^b
Oocysts negative	20	58.97±5.23	39.26±4.93	30.54±5.17	1.26±0.21

^a $P < 0.05$, $t = 2.6213$; ^c $P < 0.05$, $t = 2.3226$ and ^b $P < 0.01$, $t = 2.8666$ vs: negative group

DISCUSSION

Cyclospore cayetanensis is a new human coccidian parasite associated with human prolonged diarrhea. Twenty years ago, the first known human cases of cyclosporiasis were reported in medical literature^[11,18,32]. And prevalence or case reports about it were presented only in about 20 countries^[49-58]. Its

characteristics of pathogenic immune had not been clarified until now. In 1986, Soave separated a kind of "coccidian-like body" for the first time, later more and more people found similar organisms in stools of diarrheic patients. Accordingly, a kind of round granular coccidian-like body was found in stools of patients with diarrhea in Peru in 1992, then it was termed *Cyclospore cayetanensis* and confirmed to be a new kind of pathogen that caused human coccidiosis enteritis^[59,60]. Later, Chinese scholars Chen and Han *et al* verified the presence of infection caused by *C. cayetanensis* in China after comparing the feces specimens between those collected from different areas of China and those from America by Cterling. In survey of cryptosporidium in 2001, we isolated red and round granular coccidian-like bodies from feces of patients with diarrhea, and considered 14 cases of them to be *Cyclospore cayetanensis* infection.

Cyclospore cayetanensis has a worldwide distribution. Patients with this prolonged diarrheal illness are described in North, Central and South America, the Caribbean, Africa, Bangladesh, Southeast Asia, Australia, England, and Eastern Europe^[49,58]. Routes of transmission are still unknown, although the fecal-oral route, either directly or via water, is probably the major one. And it was considered that there were two sources of infection: contaminated drinking water and fruits^[61-68].

This investigation showed that the infection rate of *Cyclospore cayetanensis* was 0.25 % in normal population including infants and pupils, but 5.62 % in patients with diarrhea and 9.32 % in immunocompromised individuals, which was significantly different from normal population. The results showed that the positive rates of oocysts had no significantly different between males and females, but was significantly different between individuals in rural and urban areas, which was 0.92 % and 3.86 % respectively. So infection of *Cyclospore cayetanensis* is present in both urban and rural areas in Anhui Province, and it seemed to be related to personal hygiene habits and immune functions. When ingesting *Cyclospore cayetanensis* oocysts contaminated food and becoming immunocompromised, one could be infected. Individuals only infected by *Cyclospore cayetanensis* were fewer, and they were chiefly non-apparently infected with slight or no symptoms. On the other hand, multi-infection was very common. *Cyclospore cayetanensis* usually co-infected with bacteria and caused non-specific symptoms, including inertia, anorexia, abdominal distention, abdominal pain, loose and watery stool, which could easily be confused with other common intestinal diseases^[69]. So it should be considered as *Cyclospore cayetanensis* infection if individuals experienced prolonged diarrhea.

Detection of cellular immune function in this study showed that the level of CD₃⁺, CD₄⁺ and CD₄⁺/CD₈⁺ in the individuals with oocysts decreased, and the expression of CD₈⁺ seemed to be stable. It indicated that *Cyclospore cayetanensis* infection was related to cellular immune function of individuals, and it appeared with the decrease of CD₃⁺ and CD₄⁺ cells, which was associated with the decrease of immune defense, immune clearance and immune reaction to invading *Cyclospore cayetanensis*. CD₄⁺/CD₈⁺ is the hub of immunoregulation network. The decrease of CD₄⁺ cells indicated that auxiliary T lymphocyte cell took part in the pathogenesis process of diarrhea, and that both humoral immunity of *Cyclospore cayetanensis* antibody and cellular immunity response to *Cyclospore cayetanensis* infection were limited. So it made prolonged diarrhea and led to weak immunological killer effect. Thus we concluded that cellular immune took part in pathogenic process of diarrhea caused by *Cyclospore cayetanensis*.

The specific antibody detection showed that after *Cyclospore cayetanensis* infection, the specific antibody was mainly of IgG, sometimes both IgG and IgM, in rare cases of IgM only. It indicated that *Cyclospore cayetanensis* infection was a kind of chronic infection, and present infection should be considered even if there was IgG (+) only. ELISA usually served in the specific antibody detection, and it was more simple, practical, specific and sensitive. Furthermore, it was convenient to perform and capable of being widely used. Serological examination of specific antibody performed among patients with prolonged diarrhea would be more effective at early diagnosis and treatment.

Cyclospore cayetanensis discussed in the present study was encountered in our survey of *Cryptosporidium* in 2001. We found that auramine-phenol stain could be used in the detection of *Cyclospore cayetanensis* as well. More attention should be paid to the distinction between *Cyclospore cayetanensis* and *Cryptosporidium*: (1) The average diameter of the former (7.953×8.253 mm) was larger than that of the latter (about 5mm); (2) The internal structure of the former was not too clear to be seen any feature of the oocysts of the latter; (3) The density and quantity of the former was less than the latter and (4) The brightness of fluorescence under cryptoscope of the oocysts after auramine-phenol stain of the former was weaker than that of the latter. It remained to be further confirmed that the wall of the oocysts and its structure of *Cyclospore cayetanensis* might vary in different stages of the development of the protozoa. *Cyclospore cayetanensis* is differentiated from other coccidians by its sporulation characteristics and autofluorescence. Because of the autofluorescent properties of the oocysts, particular attention should be drawn to the role of fluorescent microscopy in providing a rapid, inexpensive, and sensitive technique for diagnosis of Cyclospora infections in stool samples^[50].

We've tried to deal with the disease caused by *Cyclospore cayetanensis* with spiramycin, and it seemed to be effective. It was reported that oral trimethoprim-sulfamethoxazole was a better choice in the treatment for Cyclospora infection^[70-76]. Sometimes, it can also be healed with non-antibacterial therapy. In immunocompetent individuals, diarrhea is usually self-limited but might last for several weeks. In immunocompromised hosts it is prolonged, severe and can be associated with biliary tract involvement.

In conclusion, *Cyclospore cayetanensis* infection may not be so rare in China, and further studies should be carried out on its prevalence areas, prevalence principle, life history, mode of transmission and prevention.

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