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Recovery of *Cyclospora cayetanensis* among asymptomatic rural Thai schoolchildren

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ABSTRACT

Objective: To obtain the prevalence with clinical symptoms of *Cyclospora cayetanensis* (*C. cayetanensis*), a coccidian protozoan parasite, in Thailand which is the cause of an intestinal infection characterized by sporadic-to-frequent explosive diarrhea. **Methods:** In a field survey conducted by the Faculty of Tropical Medicine, Mahidol University, as part of the existing parasite-control program, a total of 2 540 faecal samples from villagers in Nan Province, Thailand, were collected and examined to determine the prevalence and clinical characteristics of parasitic infections. **Results:** Twelve cases of *C. cayetanensis* infection were found during faecal examination of schoolchildren aged 5–12 years. None exhibited obvious clinical symptoms, especially evidence of diarrhea; 5 of 12 had loose faeces, one reported frequent symptoms of abdominal discomfort, and another had pale conjunctiva with low hematocrit. The children were generally asymptomatic. **Conclusions:** This finding confirms a public-health issue with potentially serious consequences whereby children can be exposed to an environment contaminated with food-and water-borne transmitted oocysts, and can hence become infected with *C. cayetanensis*.

1. Introduction

Cyclospora cayetanensis (*C. cayetanensis*) is an emerging coccidian parasite, first described by Ashford from three Papua New Guinea patients[1]. It has recently been recognized as a new cause of prolonged diarrheal illness worldwide, but with greater prevalence in tropical and subtropical areas[2,3]. Its mode of person-to-person transmission is via oral-faecal route by oocysts in contaminated water or food[3,4]. *C. cayetanensis* appeared to be associated mainly with prolonged self-limiting diarrhea (typically 7–9 weeks) in both immunocompetent and

immunocompromised patients[5–11] and also as a cause of travelers' diarrhea confined to adult foreigners infected while visiting endemic regions[3,12,13]. Patients normally report symptoms of nausea, vomiting, anorexia leading to weight loss, abdominal cramping and pain with increased gas, flatulence, fatigue and watery diarrhea[14,15]. In developing countries, especially among populations where sanitation is poor and insufficient, this protozoa is also associated with diarrhea among children. However, asymptomatic excretors have also been reported[4].

Few *Cyclospora* infected Thai cases have been reported recently in the literature[16–21]. Most reports dealt with immunocompromised hosts. The limited number of reported cyclosporiasis cases in Thailand is probably due to a lack of experience and awareness among laboratory workers. It is hoped that our findings will stimulate microscopists to be alert of potential *Cyclospora* infection in Thailand even

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when faecal samples are not collected from HIV/AIDS–infected patients or patients with prolonged diarrhea. The normal or immunocompetent hosts can probably serve as carriers of the disease without any obvious clinical symptoms or symptoms that might probably occur later. More research is required to monitor *Cyclospora* organisms in immunocompetent hosts and the environment. This should yield further data to help clarify the real incidence and prevalence of cyclosporiasis in Thailand.

2. Materials and methods

2.1. Study area and study group

This study was approved by the Ethics Committees of the Faculty of Tropical Medicine, Mahidol University, Thailand. The hill–tribe people of Thailand mostly inhabit the North provinces such as Mae Hong Son, Chiang Rai, Chiang Mai and Nan Provinces. They principally earn their livelihoods through agriculture. Mountainous areas in tropical zones, such as Nan Province, are favourable for the development of the parasite and transmission of the disease, due to the traditional lifestyle and poor hygiene of hill–tribe people, who may harbor several different kinds of parasites. Epidemiological survey results for intestinal helminthiasis have been reported in details elsewhere[22]. Among the total 2 540 faecal samples, 497 were identified to have protozoan infections by direct smear technique. Most of the protozoa found were *Blastocystis hominis* (*B. hominis*) (22.2%) whilst the others were *Giardia intestinalis* (*G. intestinalis*) (1.9%), *Sarcocystis hominis* (*S. hominis*) (0.7%), *Entamoeba histolytica* (*E. histolytica*) (0.7%) and *amoeba trophozoite* (0.7%). Some non–pathogenic protozoa were also found, such as *Entamoeba coli* (*E. coli*) (5.5%), *Endolimax nana* (*E. nana*) (5.5%) and *Trichomonas hominis* (*T. hominis*) (0.1%). In addition, human migration is common in the area, therefore disease transmission from place to place is possible. In view of this, the hill–tribe people in Nan Province were selected as the target group to investigate the prevalence of hidden *C. cayetanensis* infection. The prevalence of *C. cayetanensis* in faecal specimens was evaluated among the hill–tribe people in all ages in both sexes, male and female, who were willing to join the project, in Thung Chang and Chalerm Phrakiat Districts, Nan Province, northern Thailand, on the Thai–Lao borders.

2.2. Faecal examination

Faecal samples were collected from residents of Thung

Chang and Chalerm Prakiet Districts, Nan Province, northern Thailand. A plastic container was provided to each participant for faecal specimen collection. Faecal examination was examined for both protozoan and helminthic infections. For protozoan infections, samples were examined by direct–smear technique, in both normal saline solution and 1% iodine solution[23]. The modified cellophane thick–smear method was used for helminthic infections[24]. Subjects whose faecal samples were found by direct wet mount to contain *Cryptosporidium*–like organisms were selected for further laboratory investigation with rapid DMSO modified acid–fast stain to identify for *C. cayetanensis* infection[25].

2.3. Physical examination and clinical laboratory investigation

The students/villagers were approached at the selected schools/communities to participate in the study for those who were positive for *C. cayetanensis* and treated. The participants were asked to have an interview according to the questionnaires was taken in a private area. Physical examination was performed by medical doctors for all participants corresponding with the positive results of *C. cayetanensis*–positive results and the signs and symptoms of each patient were recorded. About 1 mL of blood was drawn for evaluation of CBC only in the Thung Chang Community Hospital where was nearby the study area. The follow up of the treatment was performed by faecal examination after a month to evaluate the effectiveness of medicine.

2.4. Molecular study

DNA was extracted and analyzed by PCR, using a PSP Spin Stool DNA kit (Stratek, Germany), according to the manufacturer’s instructions. Nested PCR, targeting small subunit rRNA, was performed. Primary primers CYCF1 (5′–ATT ACC CAA TGA AAA CAG TTT–3′) and CYCR2 (5′–TGC AGG AGA AGC CAA GGT AGG–3′) were used for the first PCR. Secondary primers CYCF3 (5′–GCC TTC CGC GCT TCG CTG CGT–3′) and CYCR4 (5′–TCG TCT TCA AAC CCC CTA CTG–3′) were used for the second PCR. Amplification with these primers generated a 294–bp fragment from the *C. cayetanensis* small subunit rRNA coding region[26]. Each 25 μ L PCR mixture contained 1 \times PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 2.5 U *Taq* polymerase (Fermentus, USA), and 1 μ M of each primer. For primary and secondary PCR, 30 cycles of 94 $^{\circ}$ C for 1 min, 60 $^{\circ}$ C for 1 min and 72 $^{\circ}$ C for 1 min

were used. Of the PCR results, 10 μ L were separated by agarose gel electrophoresis (1.5% agarose) and visualized after staining in a 1 μ g/mL ethidium bromide solution for 10 minutes.

3. Results

Among the 2 540 participants, 2 001 were adults and 539 were children. Twelve children (7 males and 5 females, gender ratio 1.4: 1.0) were found to have *C. cayetanensis* infection. The positive cases were all primary-schoolchildren from Ban Pang Kae, Thung Chang District and Ban Huai Kon, Chalerm Phrakiat District, Nan Province. By age group, prevalence ranged between 5–12 years, the children had no experience of prolonged fever, vomiting or weight loss, but some had episodes of upper respiratory tract infections.

C. cayetanensis-positive faecal samples can be detected by direct smear with normal saline solution. Numerous spherical organisms, containing 8–12 refractile globular granules; turned brown and revealed a distinct, thick wall in 1% iodine solution. These are identified as *C. cayetanensis* oocysts (Figure 1). The sizes of the oocysts varied from 8–10 μ m in diameter. With modified acid-fast stain, these round bodies remain unstained, but are tinged glassy-green, with a pink background. Some sporulated oocysts are confirmed by the observation of two characteristic sporocysts within each oocyst after modified acid-fast staining (Figure 2).

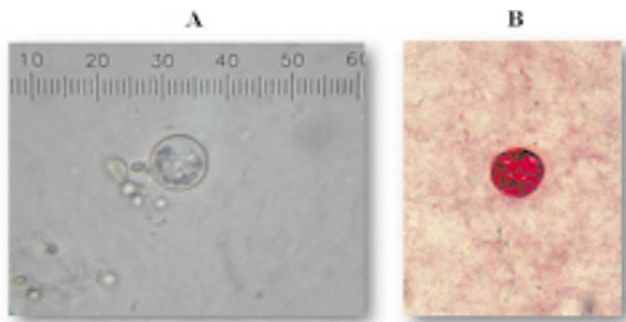


Figure 1. Organisms identified as *C. cayetanensis*.

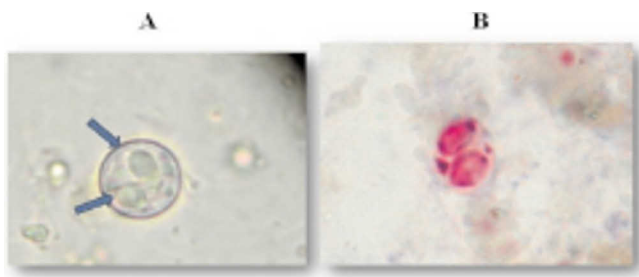


Figure 2. Modified acid-fast staining revealed sporulated oocysts.

The findings from physical examinations were mostly unremarkable. Infected schoolchildren showed no obvious clinical features or diarrhea except for somewhat loose faeces (41.7%), abdominal pain (8.3%), and pale conjunctiva with low hematocrit (8.3%) and no eosinophilia. Some (66.7%) had mixed infections of hookworms with the intensity ranged between 23–897 EPG and non-pathologic protozoa, such as *E. coli*, *E. nana*, and *T. hominis*. In the molecular study, samples were found positive for *C. cayetanensis* by nested PCR. The infected schoolchildren were all treated with trimethoprim-sulfamethoxazole (TMP-SMZ) (160 mg/800 mg) 1 tablet/day for a week for *C. cayetanensis* infection and albendazole (200 mg) 2 tablets single dose for hookworm infection. Faecal sample were collected and examined repeatedly as treatment follow-up, however no parasites were found.

4. Discussion

Cyclosporiasis is generally associated with diarrhea among children in developing countries while travelers' diarrhea and food- and water-borne disease outbreaks, usually occur in developed countries[27]. This corresponds with the finding of *C. cayetanensis* among hill-tribe children in rural areas of northern Thailand, where sanitation and personal hygiene are poor. Cases of cyclosporiasis are frequently missed, despite the increase in data on this parasite, mainly because the parasite can be difficult to detect in human faecal samples[7].

Diagnosis in many cases is made by microscopic faecal examination and appropriate staining techniques, such as modified acid-fast stain, which yields greater sensitivity in the diagnosis of *Cyclospora* organisms. They are generally refractile, and 8–10 μ m in diameter under wet-mount, which can be difficult for those unfamiliar with them. Hence, rapid DMSO modified acid-fast stain is recommended since the organisms uptake the stain and appear red or pink with variable intensity, with 10- to 20-dot granular inclusions. The method is more suitable for routine investigation in remote area than other established methods for positive diagnosis of *C. cayetanensis*, including the recovery of oocysts in intestinal fluid, small bowel biopsy or even amplification by polymerase chain reaction, which required special equipments, trained personnel, labour-intensive, and higher cost. The environmentally resistant form of the organisms, the oocyst, is shed in faeces, and subsequently sporulates into the infectious form after 5–13 days' incubation at ambient temperature[2,10,11]. Repeated

examinations of the same faeces over consecutive days with special stains should be considered to increase the detection of *Cyclospora* sporocysts, which possess a more typical morphology, and easier to recognize than unsporulated oocysts. Regarding molecular studies, *Cyclospora* DNA has been successfully amplified from food samples using nested PCR. In the regimen, primary PCR products, 639–bp fragments, are recommended for DNA sequencing[26]. In the present study, the primers were used for stool samples under modified conditions. The results showed *C. cayetanensis*–positive amplified–product bands at 294 bp of secondary PCR products. But few bands were seen at 639 bp from primary PCR products. Therefore, the amounts of primary PCR products were inadequate for sequencing. Low sensitivity for this nested PCR techniques with stool samples has been reported elsewhere[28]. It is hoped that more research will be conducted and published on molecular diagnostic techniques in stool samples to elucidate this organism further.

This study also described the clinical features of the infected schoolchildren who appeared normal. This increase concerns about indirect human–to–human spread of the parasite via oocysts. To the best of our knowledge, this is the first report of infection among asymptomatic children who were undertaking their normal activities and daily lives in Thailand. Information about cyclosporiasis remains scanty in this country. There was no evidence of diarrhea in any of the *C. cayetanensis*–positive cases in this study, nor of other main symptoms noted in previous reports (*eg.*, nausea, fatigue, abdominal cramp, fever, headache)[29]. However, some clinical features were presented in some cases *eg.* loose faecal excretion with mucous in 5 of 12 cases (41.7%) even most of them (4 out of 5 cyclosporiasis cases) were mixed–infected with non–pathogenic protozoa such as *E. coli* and *E. nana*. Meanwhile, only one case each of sporadic abdominal pain corresponding with meal times, and pale conjunctiva with low hematocrit, were found. These presentation were probably not caused by *Cyclospora* infection, particularly one with mixed *Cyclospora*–hookworm infection (897 EPG), which was classified as a moderate infection by the number of eggs found (range 100–999) in an entire microscopic fields[30,31]. This could have caused the anemia in addition to the child’s malnutrition[32]. The other parasites in mixed infections; *E. coli*, *E. nana*, and *T. hominis*, were non–pathogenic and asymptomatic.

In conclusion, this study appears that the clinical features of the infected schoolchildren of *C. cayetanensis* gave no obvious signs of which might possibly serve as disease carriers whereby children can be exposed to an

environment contaminated with food–and water–borne transmitted oocysts, and can hence become infected with *C. cayetanensis*.

Conflict of interest statement

We declare that we have no conflict of interest.

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