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RESEARCH ARTICLE

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Cyclospora cayetanensis and other intestinal parasites associated with diarrhea in a rural area of Jordan

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Abstract *Cryptosporidium* spp. and *Cyclospora cayetanensis* have emerged as important causes of epidemic and endemic diarrhea in immunocompetent and immunocompromised hosts. The exact modes of transmission in certain rural areas are still unclear. Reports of water-borne and food-borne outbreaks suggest that fecally contaminated water or food acts as a vehicle of transmission. Two hundred stool samples of patients with gastroenteritis from four health centers in a rural area of Jordan were examined using formalin-ethyl acetate concentration, wet preparation, and modified acid-fast staining methods. Oocysts of *C. cayetanensis* and *Cryptosporidium* spp. were found in 6% and 8% of the samples respectively, mainly those of children. Parasites such as *Entamoeba histolytica*, *Giardia lamblia*, and other enteropathogens were also observed. The results reflect the seasonality of natural cyclosporiasis and cryptosporidiosis, being higher in the spring. The risk factors that were found by the Fisher test to be significant and might be associated with illness are the source of drinking water, contact with animals, and eating unwashed vegetables ($p < 0.028$, $p < 0.0005$, $p < 0.00005$ respectively).

Keywords *Cryptosporidium* spp. · *Cyclospora cayetanensis* · Food-borne · Coccidian parasites · Jordan rural areas

Introduction

Cyclospora cayetanensis has emerged as an important human coccidian parasite that causes protracted, relapsing gastroenteritis in both immunocompromised and immunocompetent hosts [10]. It was first described in 1979 [1] and classified in 1994 [26]. The life cycle, reservoir hosts, and prevalence among the human population have not been systematically studied [25]. Humans are the only host identified to date, although a closely related *Cyclospora* species has been isolated from baboons [16]. No natural infection of wild or domestic animals with this parasite has been documented [5, 27], except in one study, which described the presence of oocysts morphologically compatible with *Cyclospora* in poultry [8]. The only stage seen in a fresh stool specimen is the unsporulated oocysts, which are noninfectious, and could easily be overlooked or misidentified [1, 26]. The oocysts are thought to require from days to weeks outside the host, under favorable environmental conditions, to sporulate and become infectious [25]. Persons with no previous immunity as well as very young children in developing countries are likely to exhibit symptoms. Limited data suggest that in disease-endemic countries, frequent exposure may predispose to asymptomatic infection in children and absence of infection in adults [18]. Symptoms include protracted diarrhea, weight loss, and fatigue, which can last from 1–5 weeks [2, 12]. Gastroenteritis caused by *Cyclospora* responds to antibiotic treatment with trimethoprim-sulfamethoxazole [15]. *Cyclospora* infections have been confirmed in North, Central, and South America, the Caribbean, England, Eastern Europe, Africa, the Indian subcontinent, Southeast Asia, and Australia [29]. Several water-borne and food-borne outbreaks caused by *C. cayetanensis* were reported in the 1990s in the United States and Canada, and were associated with the consumption of imported raspberries [2, 3, 9].

Cryptosporidium parvum is a leading cause of persistent diarrhea in developing countries. This coccidian parasite causes a self-limiting diarrheal infection in immunocompetent individuals, and full recovery is expected with proper oral hydration therapy [4]. The organism can cause illnesses lasting longer than 1 or 2 weeks in previously healthy persons or indefinitely in

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immunocompromised patients; furthermore, in young children in developing countries, cryptosporidiosis predisposes to substantially increased diarrheal illnesses. Although *Cryptosporidium* is not new and has been widely studied, evidence suggests that it is spread in day-care centers and possibly in widely distributed water supplies, public pools, and institutions such as hospitals and extended care facilities for the elderly [19]. Data about this parasite are of interest and are provided for comparison with those of *Cyclospora*, which is a less-known coccidian parasite. In one study in West Africa, *Cryptosporidium parvum* was identified to be the most significant pathogen in infections characterized by acute and persistent diarrhea [30].

There are ten valid species of *Cryptosporidium* and perhaps other cryptic species hidden under the umbrella of *C. parvum*. The oocyst stage is of primary importance for the dispersal, survival, and infectivity of the parasite and is of major importance for detection and identification [7]. The resistance of oocysts to the chlorine level used in water treatment contributes greatly to the survival and spread of the organism via drinking water [17]. Volunteer studies have demonstrated that as few as ten oocysts can cause infection in otherwise healthy adults and that isolates from geographically diverse regions differ in infectivity and, perhaps, virulence. To date, no specific virulence factors have been unequivocally shown to individually cause direct or indirect damage to host tissues nor have mutant strains been produced that could prove that particular deletions result in less virulent strains [23]. The lack of effective drug therapy is of major concern in immunocompromised individuals, in whom the parasite's unique self-perpetuating life cycle can cause long-duration diarrhea resulting in major fluid loss [4].

Since the various *Cryptosporidium* species and *C. parvum* genotypes infectious to humans differ in their host ranges, their epidemiology is also likely to differ. The relative importance of foreign travel, consumption of food or water, person-to-person transmission, and infected animals in disease transmission remain to be ascertained [7, 10]. This study reports on the prevalence of the coccidian parasites, *C. cayetanensis*, *Cryptosporidium* spp. and other intestinal parasites in a rural population in Jordan. The isolation of the two coccidia from feces of animals and water sources in that area might implicate them as a source of infection. Other epidemiological factors leading to their transmission are also discussed.

Materials and methods

Patients

Two hundred stool specimens from patients with gastroenteritis were collected between September 1999 and September 2001 from the Jordan Badia, a rural area located in the northeast of Jordan where inhabitants are recently settled Bedouins. All patients had diarrhea, defined as reported by the patient as at least three unformed stools within an 8-h period and accompanied by nausea, vomiting, abdominal pain, cramps, and other symptoms, including anorexia, weight loss, bloating, stomach cramps, muscle aches, low-grade fever, and fatigue lasting 2–14 days. A questionnaire filled out for each patient included age, sex, occupation, education, symptoms, source of drinking water, contact with animals, and other relevant information. A second stool sample was collected from 70 subjects. Seventy-eight samples were collected from children including malnourished children with moderate or severe malnutrition, as determined by weight-for-height percentiles. Informed and voluntary consent was obtained from all patients and from the parent or adult responsible for each child.

Parasites

Fecal samples were divided into three portions, one was examined fresh, a second was put in merthiolate iodine formalin (MIF), and a third was put in 10% buffered formalin (BF) for the preservation of parasite eggs and protozoa. Wet mounts were prepared from the fresh and the preserved samples and viewed under the high power of a microscope. Formalin-ether concentration techniques and special stains, such as trichrome, and modified acid-fast stains (for coccidian parasites) were included in the procedures. All specimens were processed by the Sheather's flotation and formalin-ethyl acetate concentration techniques. Wet mounts were prepared for protozoa and/or helminthes, and the remaining pellet was observed under the microscope. A smear was stained by a modified acid-fast staining method for *C. cayetanensis*, *Cryptosporidium* and other coccidia.

Control samples

Stool samples from healthy individuals from the same area who did not have diarrhea were examined and artificially inoculated with oocysts of *Cryptosporidium* spp. isolated from five cows. These samples were used as a positive control to check on the sensitivity and specificity of the acid-fast stain.

Bacterial cultures

Stool specimens were cultured on differential and selective media for members of *Enterobacteriaceae* such as, e.g., MacConkey, S-S agar, Selenite F broth, and *Yersinia* selective agar (Oxoid, Hampshire, England).

Data analysis

The Fisher test was used to determine the effect of epidemiological factors such as source of drinking water, contact with animals, and eating unwashed vegetables. Data are presented as percentages calculated for different parasites; age range and means are provided for adults and children.

Results

The results of the examination of 200 samples are shown in Table 1. *C. cayetanensis* oocysts were observed in stools of 12 (6.0%) patients, distributed as follows: seven in children less than 14-years-old (four of whom were malnourished children as determined by weight-for-height percentiles), and five adults. *Cryptosporidium* spp. oocysts were observed in samples of 16 (8%) patients, distributed as follows: ten in children less than 14-years-old (four of whom were malnourished), and six adults.

Both parasites were observed in the stool samples with no other pathogens.

For the 200 subjects, based on iodine wet preparation, trichrome and modified acid-fast stains for coccidia, the observed parasites were *Blastocystis hominis* (24%), *Giardia lamblia* (16%), *Entamoeba histolytica* (8%), *Entamoeba coli* (10%), *Dientamoeba fragilis* (2%), *Endolimax nana* (7%), *Iodomoeba butschlii* (4%), *Chilomastix mesnelli* (4%), *Isospora belli* (1.3%), *Hymenolepis nana* (5%), and *Ascaris lumbricoides* (3%). Bacterial enteropathogens (32%) included *Salmonella* spp., *Shigella* spp. and *Escherichia coli*. Co-infections with second enteropathogens were observed in 68 (34%) of the samples.

The source of drinking water, unwashed vegetables, and contact with animals were all suspected to have been a source of infection for *Cryptosporidium* and *Cyclospora*. Data collected in the questionnaire showed that the source of drinking water was municipal water in 140 (70%) of the patients, wells or storage tanks in 60 (30%) of the subjects, and both municipal and well water in 60 (30%) of the subjects. Structures morphologically consistent with *Cyclospora* and *Cryptosporidium* oocysts were detected in the sediment of water in the storage tanks at the homes of six patients who had *Cyclospora* or *Cryptosporidium* but not from other patients. One hundred and twenty (60%) of the subjects had eaten unwashed fresh vegetables such as tomato, cucumber, cauliflower, which are products of the farms in that area. The source of irrigation water was from reservoirs in the Badia region. One hundred and forty-eight (74%) of the subjects had had contact with animals (chicken, sheep, goats, cows or dogs) raised in the vicinity of their homes or during their work on a farm.

Cryptosporidium oocysts were identified in four fecal samples from animals (one dog, one goat, and two cows) raised by some patients who were positive for these parasites. *Cyclospora* oocysts were observed in two fecal samples, one from a goat and another from a chicken. Identification of the oocysts was based on their morphology, size, and acid-fast stain. No oocysts were observed in animals raised by patients who were negative for these two parasites.

Nine of 12 (75%) *Cyclospora* and ten of 16 (62.5%) *Cryptosporidium* cases were diagnosed in the spring (April and May), possibly reflecting the high seasonality of natural cyclosporiasis and cryptosporidiosis. This possibility could have been confirmed only if routine surveillance for these parasites had been implemented for at least two consecutive years. No oocysts of the two parasites were observed in any of the control samples. All the artificially inoculated stool samples with the *Cryptosporidium* oocysts were positive by acid-fast stain, indicating the sensitivity of the method. The risk factors that were found by the Fisher test to be significant and highly significant, and might be associated with illness were the source of drinking water, contact with animals, and eating unwashed vegetables ($p < 0.028$, $p < 0.0005$, $p < 0.00005$ respectively).

Discussion

The most common causes of travelers' diarrhea in adults in developing countries include infection with *Cryptosporidium* and *Cyclospora* spp. [14]. Since *Cryptosporidium* has been widely studied, the data obtained for this parasite are of interest for comparison with those of *Cyclospora*, which is a less known coccidian parasite. Although *Cyclospora* infections have been reported in several countries [29], they appear to be most common in tropical and subtropical areas. In various laboratory surveys conducted during non-outbreak periods in North America and the United Kingdom, the proportion of stool specimens positive for *Cyclospora* was lower than 0.5% [6], which suggests that the prevalence of infection in the general population is very low. Although occasional unexplained cases of cyclosporiasis in

Table 1 Prevalence of *Cyclospora cayetanensis,* and other intestinal parasites observed in stools of 200 patients with diarrhea

Parasites	Adults (%) 15–87 years (mean 51 years)	Children (%) 1–14 years (mean 7.5 years)	Total (%)
Cyclospora cayetanensis	5 (41.7)	7 (58.3)	12 (6.0)
Cryptosporidium spp.	6 (37.5)	10 (62.5)	16 (8.0)
Isospora belli	1 (50)	1 (50)	2 (1.3)
Blastocystis hominis[b]	22 (45.8)	26 (54.2)	48 (24)
Giardia lamblia	17 (53.1)	15 (46.9)	32 (16)
Entamoeba histolytica	10 (62.5)	6 (37.5)	16 (8.0)
Entamoeba coli[a]	11 (55)	9 (45)	20 (10.0)
Dientamoeba fragilis	3 (75)	1 (25)	4 (2.0)
Endolimax nana[a]	8 (57.1)	6 (42.9)	14 (7.0)
Iodomoeba butschlii[a]	5 (62.5)	3 (37.5)	8 (4.0)
Chilomastix mesnelli[a]	5 (62.5)	3 (37.5)	8 (4.0)
Hymenolepis nana[a]	2 (20)	8 (80)	10 (5.0)
Ascaris lumbricoides[a]	2 (33.3)	4 (66.7)	6 (3.0)
Bacterial pathogens	30 (46.9)	34 (53.1)	64 (32.0)
More than one pathogen	24 (35.3)	44 (64.7)	68 (34.0)

[a]These parasites are not associated with the diarrhea reported in the cases
[b]Not all cases were associated with diarrhea

developed countries have been reported, infection typically has been associated with international travel or consumption of imported products.

This study reports an infection rate of 6% and 8% for *Cyclospora* and *Cryptosporidium* spp., respectively. The number of cases of *Cryptosporidium* and other intestinal parasites in children younger than 14 years in this rural area is higher than those previously reported in a urban area [20, 21, 22]. In a 2-year, cross-sectional, community-based study in Peru, the prevalence of *C. cayetanensis* infection was highest among children age 2–4 years and was 0% among persons older than 11 years, which suggests that immunity develops with repeated exposure [18]. The cases of gastroenteritis in children as well as immune adults in this study show that exposure and infection with *Cyclospora* might be recent to the area. Other studies have reported that infected children in developing countries are often asymptomatic or have relatively mild symptoms [18, 25]. Illness typically is more severe and lasts longer among infected non-immunized adults (e.g., foreigners traveling to endemic areas such as in Nepal, and in outbreaks in the United States) [9, 18, 27].

The main source of drinking water in the study area is chlorinated municipal water, which supplies 70% of the houses. The finding of structures morphologically consistent with *Cyclospora* and *Cryptosporidium* oocysts in the sediment of water storage tanks of four houses suggests that water could be one of the sources of infection. Also, the presence of other parasites in the stool specimens of other patients such as *Entamoeba histolytica* and *Entamoeba coli*, known to be transmitted by water, supports the possibility of water-borne infection. However, the use of more than one source of untreated water stored in tanks and/or in wells in some houses makes it hard to reach a firm conclusion. Further studies using molecular methods for the detection and identification of these two parasites will be conducted to investigate this possibility. The transmission of *Cyclospora* through water depends on the probability that the source of drinking water becomes contaminated and that water treatment kills or removes oocysts. *Cyclospora* oocysts, probably like *Cryptosporidium* oocysts, are highly chlorine-resistant, but they should be more easily removed by conventional filtration because they are about twice the size of *Cryptosporidium* oocysts. Consumption of untreated water was identified as a risk factor for cyclosporiasis during two outbreaks of *Cyclospora* in Nepal that were also linked to water-borne transmission [12, 28]. Structures morphologically consistent with *Cyclospora* oocysts were found in the source of drinking water in both studies. In the United States, several isolated cases of cyclosporiasis, possibly associated with exposure to drinking or recreational water or to sewage, have been reported [10]. The number of reported food-borne outbreaks and cases of cyclosporiasis are inexact because of under-recognition and under-reporting of cases. Several types of fresh products, such as raspberries, basil, and a mixture of young salad greens, have been vehicles of food-borne outbreaks of cyclosporiasis [10].

Cyclospora oocysts are easily overlooked, not only because they are relatively nondescript when unsporulated, but also because they are typically shed in relatively low numbers, even by non immunized ill persons. Research to explore possible modes of contamination has been constrained by the limited numbers of samples (e.g., water, soil, fresh products) that have been available for testing. A concentration method might need to be applied to stool specimens to maximize detection of oocysts. It is important to realize that diagnostic tests for *Cyclospora* infection in stool specimens are not routinely made in stool examined for ova and parasites unless such testing is specifically requested. Even testing for *Cryptosporidium*, which is also not necessarily routine, does not always allow for detection of *Cyclospora*. *Cyclospora* is not detected when EIA or fluorescent antibody testing is done for *Cryptosporidium*, whereas both organisms are detectable in modified acid-fast-stained slides. The diagnosis of *Cyclospora* infection can also be confirmed by detecting the parasite DNA with PCR technique [24]. PCR assays using primers specific for *C. cayetanensis* have the potential to be more sensitive than microscopy, but this technique cannot distinguish between sporulated and unsporulated oocysts.

Certain ranges of temperature, humidity, and other environmental factors allow or facilitate sporulation and survival of oocysts. This might partially explain the marked seasonality of the *Cyclospora* infection noticed in this study, and which might vary in different settings [10, 11]. The possibility of reservoir hosts has been considered, but confirmed natural infection in animals other than humans has not yet been documented [5, 27] except in a study that described the presence of oocysts morphologically compatible with *Cyclospora* in poultry [8]. Preliminary data in one region of the United States have linked *Cyclospora* infection to gardening and working with soil [13]. Higher infection rates than observed in the study area are expected because *Cyclospora* oocysts can be excreted intermittently and in small numbers. Thus, a single negative stool specimen does not rule out the diagnosis; three or more specimens at 2- or 3-day intervals may be required. Although concentration techniques were used to maximize recovery of the oocysts from stool specimens, the use of molecular techniques would be more sensitive and is recommended for the detection of these two parasites in stool samples and suspected sources such as water and animals.

The reporting of newly emerging coccidia in this rural area increases awareness about these parasites, and they should be included in the differential diagnosis of protracted gastroenteritis. It is recommended that documented cases be reported to health officials [3]. This is a preliminary study based on the epidemiology of the diarrheic cases in the Jordan Badia region, and further data will be obtained by continuing studies of the pathogens found. However, the findings of this study do emphasize the need for further longitudinal epidemiological studies in which the issue of indigenous infections should be investigated. Seasonality of infections is an important parameter to be evaluated in order to properly establish the prevalence of these parasites in rural areas, as well as the natural sources of the parasite and routes of transmission.

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