Cyclospora cayetanensis in Nepal: A Study of Microbiological and Epidemiological Aspects

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Abstract

Introduction	Prior to 1995, the coccidian parasite *Cyclospora cayetanensis* was reported as a cause of gastroenteritis among children living in poor sanitary condition and adults from industrialized countries who lived or traveled in developing countries. In 1999 and 2001 (Sherchand & Cross), *Cyclospora* infection in Nepal was linked to the consumption of undercooked, contaminated green leafy vegetables and exposure to contaminated water. Some domestic animals were also found passing of *Cyclospora* like oocyst. Although *C. cayetanensis* is reported from all areas of the world, still the biology of the organism and the means of transmission remains an enigma.
Objective	The study aimed to extend our understanding of the Cyclosporiasis, and to assess risk factors for the disease as well as possible sources of infections in Nepal.
Method	The study were carried out between September 2002 to August 30, 2003 from different areas of health care institutions and community vegetable farmers, as well as case control study, to assess possible risk factors for the Cyclosporiasis in Nepal. In addition, animals' specimen, water, vegetables and fruits were also collected and examined to determine the possible sources of infections.
Results	A total of 2138 surveillance specimens tested, 137 (6.4%) were positive for *Cyclospora* with prevalence peaked in June 36/137 (26.3%). Infection was most common in children 2-9 years of age. Among 176 vegetable farmers and family members monitored from June 25 to August 30, 18 had *Cyclospora* infection. In the case control analysis, 61(95.3%) of 64 persons with *Cyclospora* infection reported drinking unsafe water within 12 days before illness, compared with 64 (74.4%) of 86 controls [Odds ratio (OR) 7.0, at 95% confidence intervals (CI)].
Conclusion	The risk factors included water sources, type of sewage drainage, domestic animals or chickens in the household, and contact with soil.
Keywords	*Cyclospora cayetanensis*, Epidemiology, Nepal

Introduction

Cyclospora cayetanensis, a coccidia parasite of human pathogen[1,2] causes explosive watery diarrhea that can persists for several weeks[3,4]. The mechanisms of transmission of *Cyclospora* have not been determined, but a marked seasonality has been observed in endemic areas around the world including Nepal [5, 6].

Although *C. cayetanensis* is reported from all areas of the world, still the biology of the organism and the means of transmission remains an enigma. Susceptible humans are suspected to be infected by ingesting sporulated oocysts. Water is probably an important vehicle, either drinking parasite contaminated water directly or indirectly when water is used to grow plant foods. Water has been implicated in outbreaks in the United States[7], and in Nepal[5,6,8]. Food borne transmission is also suspected with reports of finding oocysts in

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washing of leafy vegetables in Peru[9] and Nepal[4,10]. Patients suffer from a chronic watery diarrhea, fatigue, nausea, vomiting, abdominal cramps, anorexia, weight loss and myalgia. However, it is not known what is responsible for the pathogenesis.

Our study between 1995 and 2002 in Nepal, has identified that the possible sources of infection with *Cyclospora* is due to the consumption of raw or undercooked, contaminated green leafy vegetables and exposure to contaminated water. Some domestic animals such as chicken, dog, monkey and rats were also found passing of Cyclospora-like oocyst[6,10]. To extent our knowledge of the parasitosis the present studies were carried out from September 2, 2002 to August 30, 2003 to determine the prevalence of *Cyclospora* infection from different areas of health care institutions and community vegetable farmers, as well as case control study, to assess possible risk factors for the Cyclosporiasis in Nepal. In addition, animals' specimen, water, vegetables and fruits were also collected and examined to determine the possible sources of infections.

Methods and Materials

Surveillance Healthcare Institution

Four outpatient clinics and hospitals of Kathmandu, Kavre and Rupandehi districts were included for this study. Stool specimens were collected from those patients whose health care providers had ordered stool for parasitic investigation and bacteriological examination. In each center, we screened stool sample and the information was recorded about age, sex, and current status of diarrhea (defined as 3 or more loose or liquid stools in 24 hours) or other gastrointestinal and other clinical symptoms.

Vegetable Farmers Study

On the basis of verbal consent, 176 vegetable farmers and their family members were included in the study between June 25 to August 30, 2003. Before collection of stool specimens, the aim of study was described in detail to the participants. Early morning specimens were collected every week and each time a participant reported clinical symptoms of gastroenteritis were recorded. Participants found to have *Cyclospora* infection were treated with trimethoprim-sulfamethaxole and they were monitored until their stool specimens were negative. If the participants were infected with helminthic parasites they were given a single dose albendazole.

Case Control Study

From June 25 to August 30, 2003, we recruited participants from health-care institution for the case control study. All persons with symptomatic *Cyclospora* infection detected through the surveillance were sought in their homes; however, some could not be located because of incorrect addresses. The aim of the visit was to distribute antibiotic treatment; at the same time, we solicited participation in the case-control study. We also requested stool specimens from family members of case-patients. For controls, we included those persons who had a stool specimen screened in the surveillance health care institutions during the period in which we were enrolling cases. Participants were eligible to be controls if they reported no gastrointestinal symptoms and had negative stool results.

A questionnaire was administered to focus on potential risk factors for *Cyclospora*, including drinking water source and handling, household sanitation, presence of animals, socioeconomic variables and consumption of vegetables and local fruits.

Laboratory Methods

Stool specimens were processed by using a standard formalin-ethyl acetate concentration method and examined by three methods: direct light-microscopy examination, stool smear stained with modified acid-fast stain and UV epifluorescence examination of a wet mount. To confirm the identity of the parasite as *Cyclospora cayetanensis*, the positive samples were stored in 2.5% dichromate solution at room temperature (22°C to 25°C) and were examined at regular intervals over a 2-week period, starting from the time the sample was collected. We observed microscopically the characteristic of sporulation from all positive samples.

Samples of Water and Green Leafy Vegetable

During sample collections in the different areas, samples from irrigation canal, sewage water, public drinking water of different sources and green-leafy vegetables were collected to determine the contamination of *Cyclospora* oocyst and to investigate the possible sources of infection. The leaves were washed in distilled water, the washings and the samples of water were centrifuged and the sediments were examined microscopically. The excess amounts of sediments were resuspended in 2.5 % potassium dichromate solution, and the recovery of sporulation was noted.

Bacteriological Study

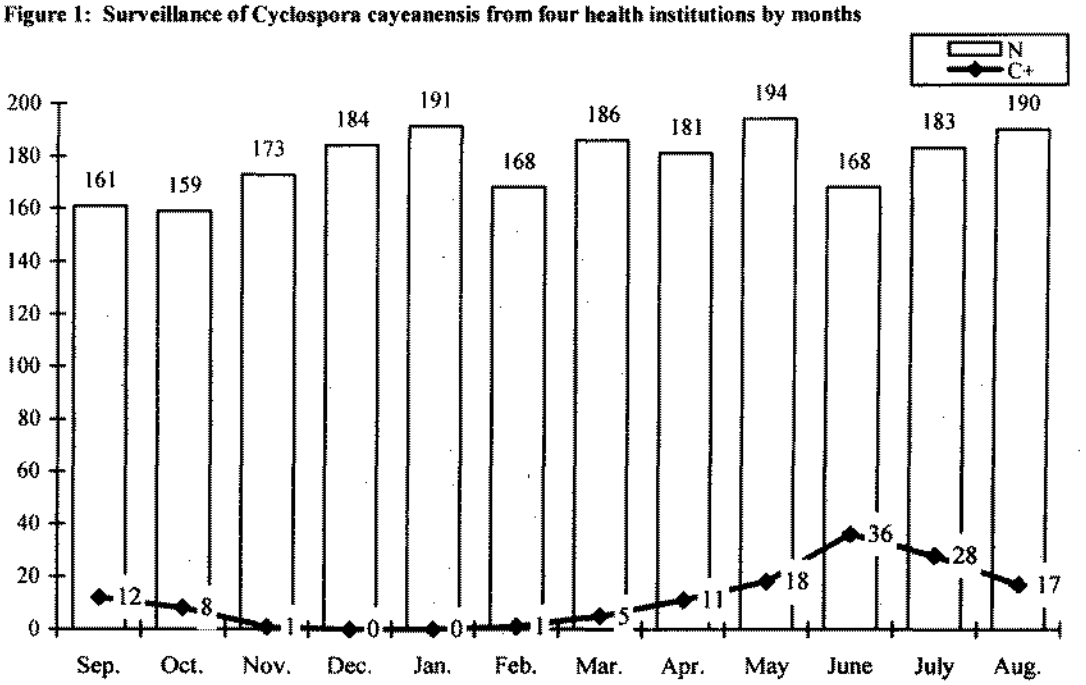
Additional diagnostic tests for bacterial enteropathogens were performed in 152 patients with diarrhea between 3-7 days and fever. Fresh stool samples were cultured for *Escherichia coli, Shigella, Salmonella* species and *Vibrio cholerae*, by standard microbiological method as described earlier[10] and commercially available antisera.

Results

Health Cares Institution Surveillance

A total of 2138 stool samples examined during a period between September 2, 2002 to August 30, 2003 from different areas of healthcare institution of Nepal. Oocysts of *Cyclospora cayetanensis* were found in 137 (6.4%) specimens, and the prevalence rate peaked in June 26.3% (Figure 1). In the winter season of December, January and February, *Cyclospora* infection was detected in less than 1% of specimens. *Cyclospora* infection was more frequent among children and among persons with gastroenteritis (Table 1).

Figure 1: Surveillance of Cyclospora cayeanensis from four health institutions by months



Age and Sex-wise Distribution of *Cyclospora* Infection

A total of 2138 stool samples were collected from 974 males and 1164 females ranging in age from 2 years to 70 years of age (Table 1). Of the 137 positive patients, 59 (43.1%) were male and 78 (56.9%) were female.

Although we did not collect data concerning TB and HIV status, one clinic submitted 42 specimens from HIV- infected patients, 6 (14.3%) were positive for *Cyclospora.* Among 68 TB infected patients 5 (7.3%) were *Cyclospora* positive.

Table 1: Prevalence of *Cyclospora* in specimens from outpatients attending four health care institutions (September 2, 2002 to August 30, 2003)

Prevalence by age group		Prevalence by symptoms	
Age (yrs)	**n+ve/NS (%)**	**n+ve/NS (%) gastroenteritis**	**n+ve/NS (%) no gastroenteritis**
2-5	44/428 (10.3)	37/305 (12.1)	7/123(5.7)
6-9	42/532 (7.9)	31/411 (7.5)	11/121(9.1)
10-13	21/490 (4.3)	16/325 (5.0)	5/165(3.0)
14-17	17/402 (4.2)	12/306 (4.0)	5/96(5.2)
>18	13/286 (4.5)	11/215 (5.1)	2/71(2.8)
All ages	137/2138 (6.4)	107/1456 (7.3)	30/682(4.4)

Vegetable Farmers Cohort Study

The vegetable farm cohort study consisted of 176 farmers and family members who submitted stool specimens from June 25 to August 30, 2003. Eighteen (10.2%) were *Cyclospora* positive in which 5 occurred in family members, ages 2 to 10 years; all had diarrhea and gastroenteritis. Three farmers were asymptomatic and other 9 had abdominal pain and discomfort where as one farmer had blood in his stool.

Other common intestinal parasites were found in these farmers and their family members; 41.5% were positive for *Hookworm*, 35% for *Ascaris lumbricoides*, 55% for *Trichuris trichiura*, 40% for *Giardia lamblia*, 12.6% for *Entamoeba histolytica*, 1.5% for *Cryptosporidium*. More than 40% had nonpathogenic protozoa such as *Entamoeba coli*, *Iodamoeba butschlii*. No organisms were detected in only 8% of the samples. Of the 18 *Cyclospora*-positive stool samples, 32.5% were co-infected with *Giardia*; 5% were co-infected with *Hymenolepis nana* and 25% had more than one parasitic co-infection.

Case-Control Study

During the case control study *Cyclospora* infections were examined among 64 cases who had gastrointestinal symptoms and 86 controls who had no gastrointestinal symptoms and had negative stool results. Of 64 cases, 38 (59.3%) were between the ages of 2-9 years and 31 (48.4%) were males. Among 86 controls, 48 (55.8%) were 2-9 years of age and 51 (59.3%) were male ($P > 0.05$).

In addition to sample collections from the case control study, we screened stool specimens from 169 family members belonging to 52 families of case patients. Of these, 38 persons from 34 families tested positive for *Cyclospora*. The detection rate was highest among children 2 to 9 years {21 (28.4%) of 74}.

According to available data on characteristic of illness in 64 patients in the case control study (Table 3) the median duration of illness before diagnosis was 12 days in those < 9 years of age and 10 days in those > 9 years of age. Young children had a higher number of stools per day than older patients and were significantly more likely to have fever and mucoid stools. Of the 64 patients, 37(57.8%) complained watery or liquid stools, but only 5 (7.8%) reported normal (soft) defecation. Of the 64 for whom information were available, 27(42.2%) had been treated with an antibiotic, most often metronidazole, cotrimoxazole, anti-helminthic drugs such as mebendazole or albendazole and occasionally herbal medicine, before *Cyclospora* was diagnosed.

Table 2: Clinical characteristics of 64 persons with *Cyclospora* infection who participated in the case control study

Characteristic of persons	Age group of participants		P value
	2-9 years (n= 38)	> 9 years (n= 26)	
Days of illness	12(3-65)	10 (2-75)	> 0.05
Stool per day	6(1-10)	4 (1-15)	> 0.05
Stool consistency	N (%)	N (%)	
Liquid/ watery	25(65.8)	12 (46.2)	
Semisolid	12(31.6)	10 (38.5)	
Soft	1(2.6)	4 (15.4)	
Blood in stool	11(29.0)	2 (7.7)	> 0.05
Mucus in stool	28(73.7)	8 (30.8)	< 0.005
Fever	25(65.8)	7 (27.0)	< 0.005
Vomiting	15(39.5)	6 (23.1)	
Abdominal pain	12(31.6)	11 (42.3)	
Anorexia	9(23.7)	7 (27.0)	
Headache	3(8.0)	5 (19.2)	
Had any antibiotics	13(34.2)	14 (53.8)	
Metronidazole	8(21.05)	12 (46.1)	
Cotrimoxazole	3 (8.0)	5 (19.2)	
Anti-helminthes	8(21.05)	12 (46.2)	
Herbal medicine	3(8.0)	6 (23.1)	

Risk Factors Associated with *Cyclospora* Infection

Risk factors associated with *Cyclospora* infection from case control analyzed data were related to sources of water, sanitation, keeping domestic animals in the household (Table 3). Persons with *Cyclospora* infection **were significantly more likely** than controls to report having drunk untreated water within the 12 days before illness, having obtained drinking water from a unsafe water source or drunk water from a river or spring. In addition, having a septic tank rather than municipal sewage drainage and having had direct contact with soil were associated with an increased risk for infection. Persons with *Cyclospora* infection were twice as likely as control to own dogs, chickens and other animals such as cats and pigs were not associated with increased risk for infection. We asked about eating different fresh uncooked produce in the 12 days before illness, including lettuce, carrot, cabbage, radishes, mint and bush-berries (Aaiselu); none was associated with an elevated risk for *Cyclospora* infection. However, examination of washed solution of green leafy vegetables such as cabbage, lettuce, spinach and mustard leaves found to be contaminated with *Cyclospora* (Table 5).

Table 3: Characteristic of risk factors associated with *Cyclospora* infection among 64 cases and 86 controls

Characteristic	Proportion with characteristic{n/ N (%)}		OR (95% CI)
	Cases	Control	
Unsafe water source*	16/64 (25)	11/86 (12.8)	2.1
Drank untreated water**	61/64 (95.3)	64/86 (74.4)	7.0
Drank river or spring water	13/64 (20.3)	6/86 (7.0)	3.4
Contact with soil/ field	48/64 (75.0)	52/86 (60.5)	1.9
Dogs	29/64 (45.3)	28/86 (32.6)	1.7
Chickens	37/64 (57.8)	42/86 (48.8)	1.4
Any animals in household	44/64 (68.7)	54/86 (62.8)	1.3
Uncooked vegetables	23/64 (36.0)	26/86 (30.2)	1.2

n = number expose individuals with specific risk factor
N = number of specimens
* Unsafe water source: public standpipe, well, spring, water truck. Little safe water as municipal water piped into house or commercial bottled water.
** Drank untreated water = not commercially bottled and had not been boiled, chlorinated, or filtered before drinking.

Examination of *Cyclospora* in Vegetables

Green vegetables collected from study areas and local vegetable markets consisted of cabbage, lettuce, cauliflower, spinach, green onions, radishes, green leafy vegetables, mustard leaves and carrot, in which cabbage, lettuce, spinach and mustard leaves were found to be contaminated with *Cyclospora* (Table 4). *Cyclospora* was further confirmed by the development of 2 sporocysts, after 2 weeks incubation period in potassium dichromate solution.

Table 4: Study on oocysts of *Cyclospora* contamination in vegetables (Months: June to August 2003)

Samples	June N/ +ve (%)	July N/+ve (%)	August N/+ve (%)
Cabbage	47/2(4.2)	31/ 0	34/ 0
Lettuce	40/ 0	67/ 3	44/ 0
Cauliflower	21/ 0	(4.5)	41/ 0
Green onions	44/ 0	30/ 0	36/ 0
Radishes	42/ 0	41/ 0	42/ 0
Spinach	38/3(7.9)	38/ 0	41/ 0
Mustard	54/ 0	42/ 0	55/ 0
leaves	45/ 0	56/2(3.6)	43/ 0
Carrots		41/ 0	

Note:
N = Number of different vegetables samples.
+ve =Positive for *Cyclospora;* (%) = Percentages

Examination of Water Samples

Water samples were collected from different areas and studied for *Cyclospora* as shown in Table 5. *Cyclospora* contamination was found in June, July in irrigation canal and sewage water. Several other parasites: *Giardia lamblia, amoeba,* unidentified trophozoites, ova of *Ascaris lumbricoides*, larvae of helminthes and many small insects and worms were detected. In 3 sources of water samples (Pond, sewage and irrigation canal) *Cryptosporidium parvum* oocysts were also identified.

Table 5: Parasitological study of *Cyclospora* from different sources of water between June to August 2003

Sources of water	June +ve/N (%)	July +ve/N (%)	August +ve/N (%)
Tap water	0/ 31	0/ 22	0/ 30
Tube-well	0/ 13	0/ 14	0/ 15
Pond	0/ 15	0/ 16	0/ 15
Sewage	2/ 12 (16.7)	3/ 14 (21.4)	0/ 12
Irrigation canal	0/ 11	1/ 11 (9.0)	0/ 10
River or spring water	0/ 3	0/ 4	0/4

Examination of Stool Samples from Different Animals

Of total 504 different animal samples examined, 2.2% animals were found to be positive for *Cyclospora* oocysts. Among these animals, 3 chicken (4.2%), monkeys 5.7%), dogs (5.1%) and rats (2.9%) were found positive as depicted in Table 6.

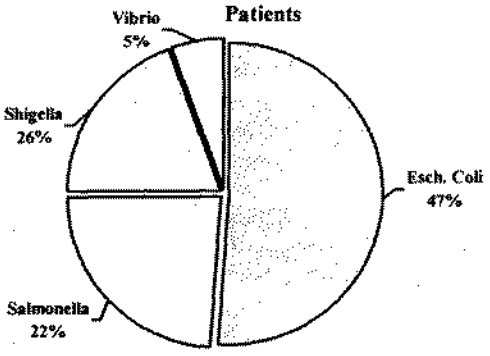
Table 6: *Cyclospora* oocysts examination in different animals (2003)

Animals	No. Examination	Cyclospora positive
Chicken	72	3 (4.2 %)
Pigs	30	0
Monkey	35	2 (5.7 %)
Dogs	78	4 (5.1 %)
Cats	33	0
Cows	51	0
Buffaloes	43	0
Goats	62	0
Rats	68	2 (2.9 %)
Pigeons	32	0
Total	**504**	**11 (2.2 %)**

Bacterial Pathogens

A total of 152 samples from Cyclosporiasis patients, *Esch. coli* (47%) followed by *Shigella* species 26%, *Salmonella* (22%), and *Vibrio cholerae* (5%) were found bacterial pathogens as depicted in Figure 2.

Figure 2. Bacterial Pathogens in Cyclosporiasis Patients



Discussion

Cyclospora cayetanensis is coccidian parasites that infect humans and causes prolonged diarrhoea in both immunocompetent and immunocompromised hosts. The mechanisms of pathogenesis and virulence factors of *Cyclospora cayetanensis* are yet to be defined, but tissue damage and jujunitis have been reported[9]. The present study advances our knowledge of the epidemiology of *Cyclospora cayetanensis* in Nepal where there are repeated outbreaks of the emerging diseases associated with gastroenteritis. Although *Cyclospora* infection has been reported from all parts of the world, most of the epidemiological information comes from studies in Nepal, Haiti, Peru and United States, where it is endemic[1,4,6,10,11,12]. Cyclosporiasis appears to be seasonal, with peak incidence during the rainy seasons from April to June in Peru and May to September in Nepal[1,6,10,13]. Although all age groups can acquire the disease, the highest attack rates occur among children older than 18 months[11], where as in our study the highest attack rates was found among children age between 2 to 5 years. There is no apparent immunity to infection, and reinfection can occur at all ages[14].

Cyclospora is an increasingly recognized cause of traveler's diarrhea, causing up to 11 % to 20 % of cases of diarrhea in studies of expatriates in Nepal[11,15]. In the United States, the outbreak of diarrheal disease associated with *Cyclospora* in 21 medical residents in 1990 was epidemiologically linked to a contaminated water supply[16,17]. Subsequently, more than 1000 confirmed cases in the US and Canada were reported[17]. In this study oocysts of *Cyclospora* were found in irrigation canal and sewage water in June, July during the high transmission period. *Cyclospora* infection occurs most commonly via contaminated water[7,18] and the oocysts are resistant to chlorination and not readily detected by methods that are currently used to assure the safety of drinking water. *Cyclospora* has been detected in water samples, however, the available method for detection in water has very low sensitivity[19]. Contaminated food has long been proposed as a possible route for transmission of *Cyclospora*[20]. Vegetables in particular are suspicious since they are often ingested raw or undercooked. Vegetables are easily contaminated and provide organisms with an optimal environment for survival prior to host ingestion. It is believed that *Cyclospora* must sporulate for at least 7-10 days in the environment to be infectious. Fertilization of plants with human waste or indirectly via contaminated water used for crop irrigation and to freshen produce could lead to contamination of vegetables with *Cyclospora*. In this study, cabbage, lettuce, spinach and mustard leaves were found to be contaminated with *Cyclospora*, which confirmed that food-borne

transmission is feasible. It still remains to be determined if recovered oocysts are infectious. The source of vegetable contamination with oocysts is still unknown, but it may be due to fecally contaminated water used on the vegetables from the irrigation water or directly from food handlers. Moreover, in Nepal, vegetables coming in the markets are dipped and rinsed into highly contaminated water of small ponds or rivers in order to wash and clean it, but actually it becomes to contaminated once again. There are thousands of such instances of how food is rendered unsafe due to unhygienic conditions, handling and practices and poor environment. In cities of Nepal, the water supply is contaminated through seepage into water pipes from sewage. In rural areas, the source of water itself (wells, ponds, rivers etc) is polluted from the contact of waste disposal deposits. One of the common food contamination problems is again from insects and rodents and as a result food becomes unfit for human consumption.

Additional study from the same diarrhoeal samples identifed other causative agents in the series: *Esch. Coli* (47%) followed by *Salmonella* (22%), *Shigella* species 26% and *Vibrio cholerae* (5%). Since these pathogenic micro-organisms are responsible for gastroenteritis and a major killer disease in Nepal[21]. This preliminary study of bacterial pathogen in relation to Cyclosporiasis patient need further work to determine the severity of infection and to assess mortality rate in the context of seasonal fatal diarrhoea in Nepal. Since annual death rate due to gastroenteritis in Nepal is 30-40 thousand[22,23]. Now it is necessary to know that among death due to diarrhoea with variety of causative agents how many of them are due to Cyclosporiasis.

Although one year of surveillance is insufficient to say with certainty what the pattern of *Cyclospora* infections will be from year to year, the data so far suggest that the seasonality of *Cyclospora* in Kathmandu City is similar to that in Guatemala, at approximately the same altitude (1200-1500m) above sea level[10,13]. In Nepal, the possible source of infection appears to be untreated water. That water truly is an important vehicle for *Cyclospora* in Nepal is underlined by the other water-related factors that were associated with infection. Our results support the findings in other studies that contaminated water is a likely source of infection[10,11]. However, multiple routes of transmission for *Cyclospora* in Nepal almost certainly exist. Among very young children, soil contact was a strong risk factor; an outbreak investigation in the Guatemala (1999) also raised the possibility that soil might be a potential source of infective oocysts[24]. Family members of patients had a rate of infection similar to that of persons in the same age group screened in our surveillance system at the same time of year, a finding consistent with the postulated lack of direct person-to-person transmission.

The fact that the presence of chickens or other domestic animals was a significant risk factor is intriguing but difficult to interpret. Scientists have failed to establish experimental *Cyclospora* infection in any bird; a limited survey of more than 100 birds captured in June/ July 2001 and 2002 in Nepal did not demonstrate natural infection, although other coccidian can be observed [25]. *Cyclospora* is a pathogen commonly associated with pediatric gastroenteritis in Nepal, especially from June through August. The seasonality of *Cyclospora* in Kathmandu follows a pattern similar to that seen in Guatemala. Our study on case-control analysis suggests those contaminated water and, for young children, soil, are likely vehicles of transmission.

Although more studies are needed to clarify the direct link between *Cyclospora* infection and these sources, the results suggests that sewage water sources of infection in Nepal.

To obtain more evidence on the source of infection, specimens from rodents, birds, insects and domestic animals are need for extensive study. Wider dissemination of skilled laboratory diagnosis is a prerequisite for a better understanding of the epidemiology of this infection and its association with disease. In vitro cultivation system for drug screening and controlled trials of drug therapy are needed. Better knowledge of the behavior of *Cyclospora* in AIDS patients along with other coccidia, *Cryptosporidium, Isospora* and *Toxoplasma* need to be studied in the context of Nepal.

In addition, more sensitive, inexpensive, reliable diagnostic tools are urgently needed to help establish how fresh produce becomes contaminated.

Acknowledgments

The authors thanks the following members for their co-operation: Dr. Dirgh Singh Bam, Dr. Chandra Shakya, Dr. Shushil Shakya, Ms. Sarala Sherchand, Mr. Govinda Gurung, Mr. Ashok Chaudhary and all Staffs of Tribhuvan University Institute of Medicine and Infectious and Tropical Disease Research and Prevention Centre/ Nepal.

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