EMERGING PATHOGEN *CYCLOSPORA CAYETANENSIS* INFECTION IN NEPAL

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Abstract. *Cyclospora cayetanensis,* an emerging parasitic pathogen of human is being increasingly recognized throughout the world, however the means of transmission and the possibility of a reservoir host remain an enigma. A longitudinal study on cyclosporiasis in different parts of Nepal was carried out from April, 1995 until November, 2000. Fecal specimens were collected from symptomatic and asymptomatic patients. The data shows a distinct seasonality with the highest infection rates occurring during the summer and rainy season of the year. Attempts have been made to determine the sources of infection and possible reservoir hosts. Stools were examined from nearly 700 animals such as chickens, pigs, buffalos, cows, dogs, cats, monkeys, rats, mice and pigeons. In addition, vegetable farms around the Kathmandu Valley were examined during the seasonal high and low periods of transmission. *C. cayetanensis*-like oocysts were found in sewage water and from vegetable washings on five occasions during June, July, August, October, and November. Similarly, *C. cayetanensis*-like oocysts were recovered from mice, rats, chickens, and dogs. These results suggest that these sources may be important in the transmission of this parasitosis. However, further studies will be required to obtain definitive answers on transmission.

INTRODUCTION

Cyclospora cayetanensis is a coccidian protozoan parasite that causes prolonged diarrhea in humans worldwide (Ortega, 1993). It was first reported from Papua New Guinea (Ashford, 1973) and years later in travelers returning to the United States or Britain from Haiti, Mexico, Puerto Rico, Morocco, Cambodia, Pakistan, India and the Solomon Islands (Wurtz, 1994). More recent reports have been from Guatemala (Pratdesaba *et al,* 1994), Italy (Caramello *et al,* 1995), Brazil (Schubach *et al,* 1997), Malaysia (Sinniah *et al,* 1994), Thailand (Wanachiwanawin *et al,* 1995), Indonesia (Fryauff *et al,* 1996) and China (Han *et al,* 1993). The most highly endemic areas, however, are in Peru (Ortega *et al,* 1993) and Nepal (Hoge *et al,* 1993; Cross and Sherchand, 1997).

Although *C. cayetanensis* is reported from all areas of the world, little is known about the biology of the organism and the means of transmission remains an enigma. Susceptible humans are 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, (Huang *et al,* 1995) and in Nepal (Rabold *et al,* 1994; Sherchand and Cross, 1997). Food borne transmission is also suspected with reports of finding oocysts in washing of leafy vegetables in Peru (Ortega *et al,* 1997) and Nepal (Sherchand *et al,* 1997). Patients suffer from a chronic watery diarrhea, fatigue, nausea, vomiting, abdominal cramps, anorexia, weight loss and myalgia. However, it is not known what causes the symptoms.

A number of studies have been carried out in Nepal among expatriates and tourists (Shlim *et al,* 1991; Hoge *et al,* 1993) but few studies have been carried out among indigenous people of Nepal. We therefore conducted this study to determine the prevalence of *Cyclospora* infection in patients with clinical symptoms and with diarrhea who were seen at a children's hospital, at rural health clinics at Tribhuvan University Teaching Hospital and Infectious and Tropical Disease Research Center. Additional studies were also done on sewage water, green-leafy vegetables collected from various areas of Kathmandu valley to determine the possible sources of infections. The issue of potential animal host for *Cyclospora* has not been completely resolved. *Cyclospora* like organisms have been recovered from ducks, chickens, dogs, and primates (Garcia-Lopez *et al,* 1996; Yai *et al,* 1997; Sherchand *et al,* 1998). Even though epidemiological investigations of *Cyclospora* have been thorough and convincing, they raise environmental and transmission issues which require further investigation.

MATERIALS AND METHODS

Stool sampling

The study was conducted between April 1995 and

November 2000. A total of 6,562 stools samples were collected from health care units: 3,017 samples from Kanti-Children's Hospital, 810 from a rural health clinic, 971 from Tribhuvan University Teaching Hospital, 1,405 from the Infectious and Tropical Diseases Research Center and 359 from Nepal Health Clinic who provides health service for expatriates. Stool samples collected were examined soon after passage by direct light microscopy at 400x and stools were also preserved in 2.5% potassium dichromate solution. During the study period, the patients were asked to record their clinical history on the study form.

Sewage water and green leafy vegetable sampling

Between May and November of 1997 and 2000 sewage water and green-leafy vegetables were collected every month from various areas of Kathmandu valley to determine the possible source of infection. The leaves were washed in distilled water, the washings and sewage water centrifuged and the sediment examined microscopically. The excess amount of sediments were resuspended in 2.5 % potassium dichromate solution, and the recovery of sporulation were noted.

Study of *Cyclospora* in various animals

A study of 698 domestic animals between 1999 and 2000 from different endemic areas was done and animals samples were collected and examined for *Cyclospora*.

Microscopic examination and standard staining application

Direct microscopic examination after concentration with formalin-ether and sucrose solution and phase contrast microscope were performed. All the positive samples from human and animals feces as well as sewage and green leafy vegetables were further confirmed by the following modified acid fast staining method: The samples were fixed with heat and stained with carbolfuchsin (Sigma, St Louis) for 30 minutes. Samples were decolorized for 1 minute with a 1% solution of HCl in 75% ethanol. *C.cayetanensis* oocysts were identified by their characteristic size (8-10 mm), round shape with red color.

The presence of other protozoa and helminth eggs was recorded. Diagnostic tests for bacteria or viral enteropathogens were not performed.

RESULTS

The examination of 6,562 stool specimens patients, 1,619 (24.6%) were positive for *C. cayetanensis*. Out of 6,562 patients, 3,640 were males and 2,922 were females with the age range of 2 months to 70 years (Table 1) and oocysts of the parasites were found in 876 (24.1%) males and 743 (25.4%) females respectively. The highest prevalence of *C. cayetanensis* infection (41.9%) found between 3 and 5 years of age in 1995 and 1998, whereas the highest rate of infection was in 6 to 8 years age in 1999 and November, 2000 (Table 1). The distribution of *Cyclospora* positive in different institution is shown in Table 2.

Distribution of *Cyclospora* in different areas, clinical presentation and other parasites

The distribution of *Cyclospora* in different areas of Nepal is shown in Fig 1. The presentation of clinical history and positive for *Cyclospora* observed in the patients shedding oocysts are shown in Table 3. There were no clinical findings distinguishing people with the infection from other patients with diarrhea. *Cyclospora* positive patients from Kanti-Children Hospital, the duration of diarrhea before admission plus days in hospital ranged from 4 to 15 days. Twelve

Table 1
Agewise distribution of *Cyclospora cayetanensis* infection.

Age (years)	April, 1995 to 1998		1999 to November, 2000	
	No. examined	Positive (%)	No. examined	Positive (%)
< 2	412	153 (37.1)	631	83 (13.1)
3-5	798	297 (41.9)	1,254	296 (23.6)
6-8	362	71 (19.6)	685	237 (34.5)
9-11	269	47 (17.5)	710	182 (25.6)
12-15	147	36 (24.5)	418	85 (20.3)
16-18	141	15 (10.6)	325	53 (16.3)
> 19	84	13 (15.5)	416	51 (12.2)
Total No.	2,123	632 (29.8)	4,439	987 (22.2)

Table 2
Institution-wise distribution of *Cyclospora cayentanensis.*

Name of Institution	(April 1995 - 1998)		(1999- November, 2000)	
	No.	*Cyclospora* positive	No.	*Cyclospora* positive
Kanti-children Hospital Tribhuvan University	1,330	403 (30.3%)	1,687	435 (25.7%)
Teaching Hospital	-	-	971	47 (4.8%)
Rural Health Clinic	550	142 (25.8%)	260	46 (17.6%)
Nepal Health Clinic (Expatriates)	243	87 (35.8%)	116	21 (18.1%)
Infectious and Tropical Diseases Research Center	-	-	1,404	438 (31.2%)
Total	2,123	632 (29.7%)	4,439	987 (22.2%)

Fig 1- The distribution of *Cyclospora* in different areas of Nepal.

children with *Cyclospora* infection alone had bloody diarrhea and three of them had *Entamoeba histolytica* trophozoites.

One or more of the following parasites were detected from 70.5% (1,143) of the 1619 infected children during excretion of *C. cayetanensis* oocysts: *Giardia lamblia* (603), *Entamoeba coli* (152), *Ascaris lumbricoides* (112), Hookworm (109), *Endolimax nana* (63), *Trichuris trichura* (60), *Hymenolepis nana* (57), *Chilomastix mesnili* (28), *Blastocystic hominis* (24), *Strongyloides stercoralis* (20), *Enterobius vermicularis* (20), *Isospora belli* (18), *E. histolytica* (16) and *Trichomonas hominis* (7). There was no difference in the distribution of coinfecting parasites between patients with diarrhea and non diarrhea ($p \geq 0.05$). *Cyclospora* were also detected from 8 malaria cases, 6 HIV positive patients, 23 tuberculosis patients, 3 in HIV and TB patients, 7 Japanese encephalitis patients (Table 3).

Infection of *Hymenolepis nana* and *Cyclospora* (Table 4)

Year and month-wise distribution of *Cyclospora cayetanensis* infection: The higher distribution of *Cyclospora* infection in Nepal is summer and rainy

Table 3
Clinical history of 1,362 *Cyclospora cayentanensis* positive patients (1997- November, 2000).

Clinical history	No. of positive cases (%)
Diarrhea	1,135 (83.3)
Abdominal discomfort	960 (70.5)
Tenesmus	825 (60.5)
Vomiting	560 (41.1)
Constipation	311 (22.8)
Flatulence	630 (46.2)
Weight loss	525 (38.5)
Fever and headache	463 (33.9)
Malaria patients	8 (0.58)
HIV positive by ELISA	6 (0.44)
Tuberculosis patients	23 (1.7)
HIV and tuberculosis	3 (0.22)
Japanese encephalitis patients	7 (0.51)
Hymenolepis nana positive cases	57 (4.2)

Table 4
Association between *Hymenolepis nana* and *Cyclospora cayetanensis* infection 1999 to November 2000.

Age (years)	No. examined	*Cyclospora* + ve (%)	*H. nana* + ve (%)
< 2	631	83 (13.1)	0 (0)
3-5	1,254	296 (23.6)	2 (1.9)
6-8	685	237 (237)	13 (12.3)
9-11	710	182 (25.6)	35 (33.1)
12-15	418	85 (20.3)	21 (19.8)
16-18	325	53 (16.3)	16 (15.1)
> 19	416	51 (12.2)	19 (17.9)
Total No.	4,439	987 (22.2)	106 (2.4)

season- June and July Fig 2. The positive rates in June, 1995, 1996, 1997, 1998 and 1999 till November 2000 were 18.6%, 16.3%, 36.9%, 34.9%, 28.1% and 50.5% respectively where as in July, 1995, 1996, 1997, 1998 and 1999 till November, 2000 were 20%, 27.2%, 51.9%, 24.4%, 31.4 and 18.6% respectively. The prevalence decreased during the winter, although there are few cases were noted during the winter in 1997, 1998, 1999 and 2000.

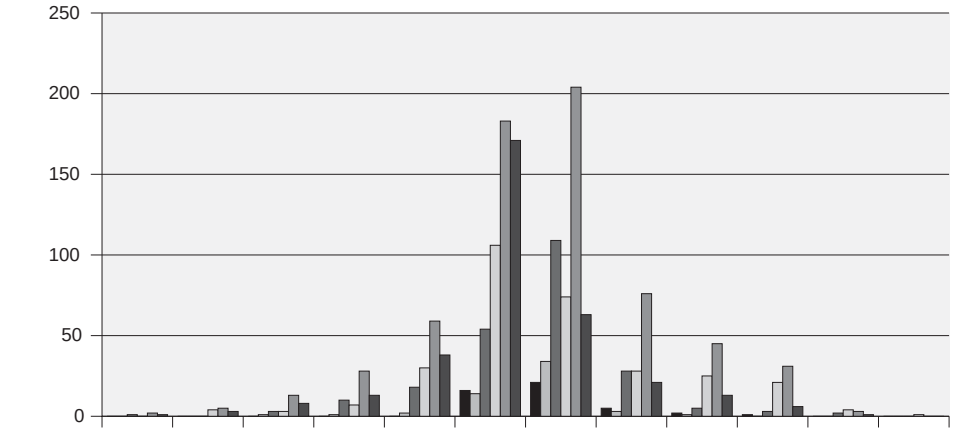
Drinking water, sewage water and vegetables

Drinking water, sewage water collected from different areas of Kathmandu valley were found to be contaminated with *Cyclospora* in June, July and August.

Green vegetables collected from 14 vegetable markets (Kathmandu, Patan and Bhaktapur) consisted of cabbage, lettuce, cauliflower, spinach, green onions, radishes, green leafy vegetables, mustard leaves and carrot in which cabbage, lettuce and mustard leaves were found to be contaminated with *Cyclospora* (Table 5). This *Cyclospora* were further confirmed by development of 2 sporocysts after 2 week incubation period in potassium dichromate solution.

***Cyclospora* like oocysts in various animals**

In this study oocyst like parasites were found in two chickens in 1998, one chicken and one dog in 1999 and one dog and two rats in 2000. In other animals the absence of *Cyclospora* oocysts in fecal samples as shown in Table 6.



	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
1995	0	0	0	0	0	16	21	5	2	1	0	0
1996	0	0	1	1	2	14	34	3	1	0	0	0
1997	1	0	3	10	18	54	109	28	5	3	2	0
1998	0	4	3	7	30	106	74	28	25	21	4	1
1999	2	5	13	28	59	183	204	76	45	31	3	0
Nov 00	1	3	8	13	38	171	63	21	13	6	1	0

Fig 2- Prevalence of *Cyclospora* infection in Nepal by month (1995 to November 2000).

Table 5
Distribution of sewage water, vegetables positive for *Cyclospora.*

	Months						
Samples	May	June	July	August	September	October	November
Sewage water	-	+	+	+	-	-	-
Cabbage	-	+	-	+	-	-	+
Lettuce	-	-	-	+	-	+	-
Cauliflower	-	-	-	-	-	-	-
Spinach	-	-	-	-	-	-	-
Green onions	-	-	-	-	-	-	-
Radishes	-	-	-	-	-	-	-
Green leafy vegetables	-	-	-	-	-	-	-
Mustard leaves	-	-	+	-	-	-	-
Carrots	-	-	-	-	-	-	

Note: + = Positive for *Cyclospora;* - = Negative for *Cyclospora*.

***In vivo* drug efficacy on mixed infection of *Cyclospora cayetanensis* and *Hymenolepis nana* patients**

The present study included 18 cases of mixed infection of *Hymenolepis nana* and *Cyclospora* with the objective to assess whether there is justifiable need for change in the management and treatment of mixed infection between *H. nana* and *Cyclospora*.

A prospective descriptive study using appropriate drug treatment methods were carried out during a period of 12 weeks between June and August, 2000. Eighteen patients confirmed by microscopic examination agreed to participate. Combined treatment of trimethoprim-sulfamethoxazole and niclosamide was given with the supervision of pediatricians and general physicians as shown in Table 7.

Table 6
Study of *Cyclospora* in various animals (1998 to November, 2000).

Animals	1998 *Cyclospora*		1999 *Cyclospora*		November 2000 *Cyclospora*	
	No.	+ve/-ve	No.	+ve/-ve	No.	+ve/-ve
Chicken	35	2	40	1	35	-
Pigs	20	-	25	-	14	-
Monkeys	15	-	13	-	12	-
Dogs	28	-	30	1	32	1+
Cats	15	-	18	-	13	-
Cows	24	-	35	-	23	-
Buffalos	25	-	34	-	26	-
Goats	26	-	48	-	31	-
Rats/House mice	8	-	15	-	27	2+
Pigeons	0	-	16	-	15	-
Total animals	196	2+ve	274	2+ve	228	3+

N = 698; *Cyclospora* positive (+) = 7; (-) Negavtive.

Table 7
Characteristics of patients with *Cyclospora cayetanensis* and *Hymenolepis nana* mixed infection according to treatment.

Case no.	Age/Sex	Niclosamide and TMP-SMZ treatment			
		10 days		15 days	
		C+/-	H+/-	C+/-	H+/-
01	8/F	-	-	-	-
02	13/M	-	-	-	-
03	16/F	-	+	-	+
04	9/M	-	-	-	-
05	22/M	+	-	-	-
06	19/F	+	-	+	-
07	5/M	-	-	-	-
08	16/F	-	-	-	-
09	27/M	-	-	-	-
10	13/F	-	-	-	-
11	6/M	-	-	-	-
12	15/F	-	+	-	-
13	12/M	-	-	-	-
14	4/M	-	-	-	-
15	31/F	-	-	+	-
16	14/M	-	-	-	-
17	10/F	-	-	-	-
18	38/M	-	-	-	-

+ = After treatment ova/ oocysts seen; - = After treatment no ova/oocyst seen.
TMP-SMZ= trimethoprim-sulfamethoxazole; H= *Hymenolepis nana*; C= *Cyclospora.*

DISCUSSION

This study upgraded our understanding of the epidemiology of *Cyclospora* in Nepal where there are repeated outbreaks of the emerging diseases associated with gastroenteritis. Although *Cyclospora* infection have been reported from all areas of the world, most of our epidemiological information comes from studies in Nepal, Haiti, Peru and United States where it is endemic (Shlim *et al,* 1991; Ortega *et al,* 1993; Cross and Sherchand, 1997). Cyclosporiasis appears to be seasonal, with peak incidence during the rainy seasons from April to June in Peru and May to September in Nepal (Ortega *et al,* 1993; Hoge *et al,* 1993). Although all age groups can acquire the disease, the highest attack rates occur among children older than 18 months (Hoge *et al,* 1995), whereas in our study the highest attack rates were found in children older than 3 years. There is no apparent immunity to infection, and reinfection can occur at all ages (Connor *et al,* 1993).

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 (Shlim *et al,* 1991; Hoge *et al,* 1995). In the United States, the outbreak of diarrhea disease associated with *Cyclospora* in 21 medical residents in 1990 was epidemiologically linked to a contaminated water supply (Huang *et al,* 1995). Subsequently, more than 1,000 confirmed cases in the US and Canada were reported (CDC, 1996). In this study oocysts of *Cyclospora* were found in sewage water in June, July and August during the high transmission period. *Cyclospora* infection occurs most commonly via contaminated water (Huang *et al,* 1995; Rabold *et al,* 1994) and the oocysts are resistant to chlorination and not readily detected by methods that are currently used to assure the safety of drinking water. Contaminated food has long been proposed as a possible route for transmission of *Cyclospora* (Connor and Shlim, 1995). 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. *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 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 contaminated hands of food handlers. Moreover, in Nepal, vegetables 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 contaminated once again. There are thousands of such instances of how food is rendered unsafe due to unhygenic 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.

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

To obtain more evidence on the source of infection, specimens from rodents, birds, insects and domestic animals need to be studied. Wider dissemination of skilled laboratory diagnosis is a prerequisite for a better understanding of the epidemiology of this infection and its association with disease. The frequency of malabsorption in clinical illness needs to be established. Detailed histopathological and electron microscopical studies on biopsy material should be done to understand the life cycle and pathogenesis of the *Cyclospora. 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* needs to be studied in the context of Nepal.

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