STUDY OF *CYCLOSPORA CAYETANENSIS* IN HEALTH CARE FACILITIES, SEWAGE WATER AND GREEN LEAFY VEGETABLES IN NEPAL

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Abstract. *Cyclospora cayetanensis,* a newly emerging parasite, is endemic in Nepal. A total of 2,123 stool specimens were collected from 3 health care facilities based on clinical symptoms during the period between 1995 to October, 1998. Out of these specimens, *cayetanensis* oocysts were found in 632 (29.8%). To identify possible sources for *Cyclospora* infection, drinking water, sewage water, green-leafy vegetables including fecal samples of various animals were collected and examined. The vegetable leaves were washed in distilled water then the washings, sewage water and drinking water were centrifuged and the sediment were examined microscopically. As a result, oocyst of *Cyclospora* were identified in sewage water and vegetable washings on four different occasions in June, August, October and November. The positive results were also confirmed as *C. cayetanensis by* development of 2 sporocysts after 2 week incubation period in potassium dichromate. A survey of 196 domestic animals from the same areas demonstrated that two chickens were positive for *Cyclospora*-like organism and others were negative. Although further studies are needed to clarify the direct link between *Cyclospora* infection and these sources, the results suggest that sewage water, green leafy vegetables are possible sources of infection and chickens could be possible reservoir host of *Cyclospora* in Nepal.

INTRODUCTTION

Human infection with the the parasitic protozoa, *Cyclospora cayetanensis,* was first described in 1979 (Ashford, 1979) and the organism recently categorized as an important gastrointestinal parasite that causes prolonged diarrhea in humans worldwide (Ortega, 1993). It was first reported from Papua New Guinea 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 (Siniah *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 *et al,* 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. Water is probably an important vehicle, either drinking parasite contaminated water directly or indirectly when water is used to grow plant foods. Water has implicated in outbreaks in the United States, (Huang *et al,* 1995) and in Nepal (Rabold *et al,* 1994; Sherchand *et al,* 1997). Food borne transmission is also suspected with reports of findings oocysts in washing of leafy vegetables in 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 (Cross *et al,* 1997; Sherchand *et al,* 1997). We therefore conducted this study to determine the prevalence of *Cyclospora* infection from patients with clinical symptoms and with diarrhea who were at Children' s Hospital, at rural health clinics and at the Nepal Health Clinic where expatriates are examined.

Additional studies on drinking water, sewage water, green-leafy vegetables and fecal samples of domestic animals were also collected within and near the houses in three study areas to determine the possible sources of infection and host or a recervior for *Cyclospora.*

METERIALS AND METHODS

The study was conducted between April 1995 and October, 1998.

Stool sampling

A total of 2,123 stools sample were collected from three different health care facilities areas: 1,330 stool samples from Kanti-Children's hospital, 550 from rural health clinic of Dhanusha district and 243 from Nepal Health Clinic who provides health service for expatriates. Stool samples collected from different areas 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 thier clinical history on the study form.

Drinking water, sewage water and green leafy vegetable sampling

From May 1997 to October, 1998, drinking water, sewage water, green-leafy vegetables were collected every month from same endemic areas as well as various places in Kathmandu valley to determine the possible source of infection. The leaves were washed in distilled water, the washing, drinking water and sewage water centrifuged and the sediment examined microscopically. Excess sediments were resuspended in 2.5% potassium dichromate solution, stored at 4°C, and the recovery of sporulation was noted.

Animals sampling

A survey was carried out of domestic animals found within and near the houses in the locations where *Cyclospora* has been studied. Their fecal samples were individually examined for the presence of *Cyclospora* by sucrose floatation and formalin-ether concentration methods.

Staining application

All the positive samples from sewage and green leafy vegetables, and fecal samples from patients and animals were confirmed by modified acid fast staining method: The samples were fixed with heat and stained with carbolfuchsin (Sigma, St Louis USA) 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 µm), 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

In the examination of stool specimens from 2,123 patients, 632 (29.8%) were found to be positive for *C. cayetanensis*. Of the 2,123 people, 1,386 were males and 737 were females. Persons aged 2 months to 49 years of age were examined (Table 1) and oocysts of the parasites were found in 391(28.2%) males and 241 (32.7%) females, respectively. The highest prevalence of *C. cayetanensis* infection (41.9%) found between 3 and 5 years of age (Table 1). The distribution of *Cyclospora* positives in different areas is shown in Table 2.

The clinical history of persons positive for *Cyclospora* shedding oocysts is given in Table 3. There were no clinical findings distinguishing people with the infection from other patients with diarrhea. In *Cyclospora* positive patients from Kanti-Children's Hospital, the duration of diarrhea before admission plus days in hospital ranged from 4 to 15 days; 26 children with *Cyclospora* infection alone had bloody diarrhea, two of them had *Entamoeba histolytica* trophozoites. In patients from rural health clinics, malnutrition was observed, however, no systematic study on malnutrition has been made.

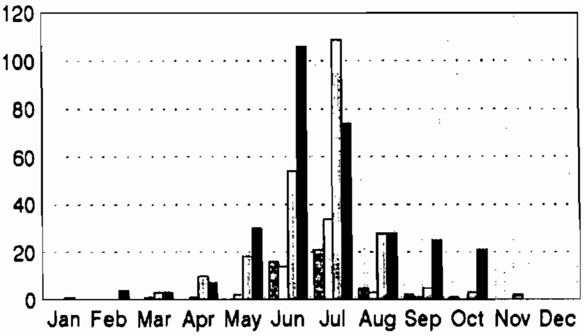
Table 1
Age-wise distribution of *Cyclospora eayetanesis* infection.

Age (years)	No. examined	Positive (%)
< 2	412	153 (37.1)
3-5	708	297 (41.9)
6-8	362	71 (19.6)
9-11	269	47 (17.5)
12-15	147	36 (24.5)
16-18	141	15 (10.6)
> 19	84	13 (15.5)
Total no.	2,123	632 (29.8)

Table 2
Area-wise distribution of *Cyclospora* positives.

Area-sampled	Total No. of samples studied	*Cyclospora* positive
Kanti-Children's Hospital	1,330	403 (30.3%)
Rural Health Clinic	550	142 (25.8%)
Nepal Health Clinic (Expatriates only)	243	87 (35.8%)

One or more of the following parasites were detected from 68.7% (434) of the 632 infected children during excretion of *C. cayetanensis* oocysts: *Giardia intestinalis* (133), *Entamoeba coli* (102), *Ascaris lumbricoides* (78), hook worm (53), *Endolimax nana* (52), *Trichuris trichiura* (43), *Hymenolepis nana* (31), *Chilomastix mesnili* (21), *Blastocystis hominis* (22), *Strongyloides stercoralis* (19), *Enterobius vermicularis* (16), *Isospora belli* (14), *Entamoeba histolytica* (9) and *Trichomonas honimis* (4). There was no difference in the distribution of coinfecting parasites between patients with diarrhea and non diarrhea ($p > 0.05$). *Cyclospora* were also detected in 5 patients with malaria (4 with *Plasmodium falciparum,* and 1 *Plasmodium vivax)* and 3 HIV ELISA positive patients (Table 3).



	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
Oct,1998	0	4	3	7	30	106	74	28	25	21	0	0

Cases in Years: 1995:45/277; 1996:56/326; 1997:233/575; 1998:298/945

Fig 1–Month-wise prevalence of *Cyclospora* infection.

Year and season-wise distribution of *Cyclospora*

The frequency distribution of *Cyclospora* in 1995, 1996, 1997 and 1998 October end were 45 (16.2%), 56 (17.2%), 233 (40.5%) and 298 (31.5%), respectively.

In Nepal in the present locations where *Cyclopora* has been studied, the prevalence tends to be higher in the summer and/or early rainy and rainy seasons: the highest rates of infection were found in the months of June and July (Fig 1). The positive rates in June, 1995, 1996, 1997 and 1998 were 35.5%, 25%, 23.2%, 35.6%, respectively where as in July, 1995, 1996, 1997 and 1998 were 46.7%, 60.7%, 46.8% and 24.8%, respectively. The prevalence decreased during the winter, although 3 cases were noted during the winter in 1997 and 4 cases in February 1998.

Table 3
Clinical history of 632 *Cyclospora* positive patients.

Clinical history	No. of positive cases (%)
Diarrhea	561 (88.7)
Abdominal discomfort	496 (78.5)
Tenesmus	464 (73.4)
Vomiting	232 (36.7)
Constipation	184 (29.1)
Flatulence	341 (53.9)
Weight loss	330 (52.2)
Fever and headache	174 (27.5)
Malaria patients	5 (0.79)
HIV positive cases	3 (0.47)

Drinking water, sewage water and vegetables

Drinking water, sewage water collected from same endemic areas of Nepal were found to be contaminated with *Cyclospora* in June, July, August and November.

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

Sampling of various animals

A survey of 196 animals in the same locations where *Cyclospora* demonstrated the presence of *Cyclospora* oocysts in fecal samples of two chickens. In other animals the absence of *Cyclopora* oocysts in the fecal samples shown in Table 5.

DISCUSSION

Although *Cyclospora* infection have been reported from all areas of the world, most of our

Table 4
Distribution of sewage water, vegetables positive for *Cyclospora.*

Samples	Months						
	May	June	July	Aug	Sept	Oct	Nov
Sewage water	-	+	+	+	-	-	-
Cabbage	-	+	-	+	-	-	+
Lettuce	-	-	-	+	-	+	-
Cauliflower	-	-	-	-	-	-	-
Spinach	-	-	-	-	-	-	-
Green onions	-	-	-	-	-	-	-
Radishes	-	-	-	-	-	-	-
Green leafy vegetables	-	-	-	-	-	-	-
Mustard leaves	-	-	+	-	-	-	-
Carrots	-	-	-	-	-	-	-

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

Table 5
Study of *Cyclospora* in various animals (September-October, 1998).

Animals	No.	*Cyclospora+/-*
Chickens	35	2 +
Pigs	20	-
Monkeys	15	-
Dogs	28	-
Cats	15	-
Cows	24	-
Buffalos	25	-
Goats	26	-
Rats	8	-
Total animals	196	2+

+ = presence of *Cyclospora*; - = Absence of *Cyclospora.*

epidemiological information comes from studies in Nepal, Haiti, Peru and United States, where it is endemic (Shlim *et al,* 1991; Ortega *et al,* 1993; Zerpa *et al,* 1995; 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) whereas in our study the peak incidence was found in the summer season: pre-rainy and rainy, in the months of June and July. 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 was found 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 United States, the outbreak of diarrheal disease associated with *Cyclospora* in 21 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. *Cyclospora* infection occurs most commonly via contaminated water (Huang *et al,* 1995; Rabold *et al,* 1994) ; they 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 of 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 of *Cyclospora* 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 sources or directly from contaminated hands of food handlers. Moreover, in Nepal, vegetables coming into the markets are dipped and rinsed in 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 unhygeinic 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 with waste disposal deposits. One of the common food contamination problems is from animals, insects and rodents and as result food becomes unfit for human consumption. Moreover, the only existing report of *C. cayetanensis* found in feces from domestic farm animals concerned a farm duck (Zerpa *et al,* 1995). In the present study, the fecal samples from two chickens were found to be positive of *Cyclospora* oocysts. So far, however, a possible infection route involving poultry-whether it be direct consumption of undercooked chicken meat, contamination of food and water sources with chicken feces, or both - remains to be determined. It should be noted that sanitary standards in poultry-breeding facilities in Nepal may not be adequate. This would account for the fact that reports implicating chickens in the transmission of *Cyclospora* (Connor and Shlim, 1995) have occurred in, or in relation to, developing countries. The *Cyclospora* found in the fecal sample of chickens in this study have the diagnostic features of *C. cayetanensis.* Nevertheless, the existence of another, not yet described, *Cyclospora* species infecting poultry, which has similar features but is different from *C. cayetanensis,* cannot be excluded at this stage. In our study, the number of oocysts recovered was not large and because low amount of feces, we could not calculate the number of oocysts passed by each chicken. The possibility that oocysts which were acquired as contamination from food or water sources area were only passing though the gut of the chickens (making the chickens a paratenic host) cannot ruled out.

Although more studies are needed to clarify the direct link between *Cyclospora* infection and these sources of animals and vegetables, the results suggests that sewage water and green leafy vegetables are possible sources of infection. The animals sources could be possible host or reservoirs of *Cyclospora,* but to support this concept more evidence fecal samples from rodents, birds, insects and large number of domestic animals require to be investigated in different areas of Nepal.

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