INTRODUCTION

Diarrhea is a major health problem in developing countries. Most acute episodes of diarrhea are due to infection of the bowel; they have an acute onset and last between two and seven days. A proportion of acute cases, about 1 in 10, become persistent, lasting more than 2 weeks, requiring medication in addition to oral rehydration therapy. Globally, an estimated 1.8 billion episodes of diarrhea occur each year leading to the death of 3 million children below age 5 years (WHO, 2002). In Nepal, the estimated number of diarrheal episodes in children below age 5 years was 11,171,000 in the year 1994 (4th after India, Bangladesh and Indonesia) and the morbidity rate was 3.3/1,000 children below 5 years of age (3rd after Bhutan and Bangladesh) (Park, 2002). The number of children below 5 years of age (total population = 3,533,962) affected with diarrheal disease in Nepal in the years 2001/2002 was 625,150 (incidence = 177/1,000) with the total number of deaths being 136 (case fatality rate = 0.2/1,000) (Department of Health Services, 2002). In western Nepal, total diarrheal episodes were 111,681/year (2001/2002) with 30 reported cases of deaths (case fatality rate = 0.1/1,000) in children below 5 years of age (Department of Health Services, 2002).

The pathogens involved in persistent diarrhea in Nepal have not previously been reported. Viruses are not commonly isolated from stools. Enterotoxigenic (ETEC) and enteropathogenic (EPEC) Escherichia coli, Aeromonas, Campylobacter and Shigella species are not usually associated with persistent diarrhea (WHO, 1998). Enteroadherent E. coli (EAEC) was isolated more frequently in the early phase of persistent diarrhea in India,
Bangladesh and Mexico (Bhan et al., 1989; Baqui et al., 1992; Henry et al., 1992). Protozoal agents, such as Giardia lamblia, Cryptosporidium spp, Entamoeba histolytica and other intestinal protozoal parasites have been associated with persistent diarrhea (Chavalittamrong and J irapinyo, 1984; De Silva et al., 1994; Bhandari et al., 1999; Park, 2002).

Socio-economic and cultural factors and lack of adequate basic sanitation have caused the children of Nepal to be vulnerable to intestinal parasitic infections. There has been no descriptive study in western Nepal to discover the protozoal profile in children with persistent diarrhea. This hospital-based study was conducted to determine the association between protozoal agents and persistent diarrhea in children below 5 years of age in this region.

**MATERIALS AND METHODS**

Diarrhea is defined as the passage of 3 or more liquid stools in a 24-hour period. Persistent diarrhea is defined as diarrhea lasting ≥14 days and acute diarrhea as diarrhea <14 days duration.

The study was carried out at the Manipal Teaching Hospital, Pokhara from April 1998 to March 2004. A total of 759 (253 x 3) stool samples were collected from 253 children under age 5 years (M=147, F=106) who were either admitted to the hospital or visited the OPD with persistent diarrhea. All stool samples were collected in waterproof screw capped plastic containers and processed immediately. Wet and iodine preparations were examined for trophozoites and cysts of protozoal parasites and eggs of helminths per standard methodology. Smears were stained by modified Ziehl-Neelsen staining procedure and observed for oocysts of Cyclospora cayetanensis and Cryptosporidium spp. The morphology of oocysts of Cyclospora cayetanensis and Cryptosporidium spp were compared with positive controls (reference slides from CDC, Atlanta). The samples were also processed for identification of suspected bacterial pathogens biochemically and serologically (in the cases of ETEC and EPEC) according to the recommended procedure (WHO, 1987).

Stool samples were also collected from 155 children under 5 years of age (M=87, F=68) who had visited or were admitted to the hospital with acute diarrhea (disease control), and from 100 healthy children below 5 years of age (M=55, F=45) from the community (normal controls) with no prior diarrheal illnesses. The controls were matched with the cases for nutritional status category (weight for age ≤90% or >90% according to Gomez’s international classification).

To study the seasonal pattern of diarrhea, each year was divided into 4 seasons: January-March (Spring), April-June (Summer), July-September (Monsoon) and October-December (Winter).

Chi-square and Student’s t-test were used to determine statistical significance.

**RESULTS**

The baseline characteristics of the cases and controls were similar (Table 1). The dominant social class within the analyzed group corresponded to the low-medium category, with an income of 32,001-62,000 Nepali rupees per annum (US$430-830/yr) and an incomplete secondary level as the maximum education achieved in at least one of the parents. The basic sanitary level of the families was poor. The children did not grow up with the habit of washing hands before eating food and used utensils that were not cleaned properly. Most children consumed unboiled or unfiltered water outside the house. The practice of using open-air toilets and open-air water sources was prevalent in society. However, no significant association between sex and nature of diarrheal episodes was found.
Of 759 stool samples, 90 (35.5%) were positive for protozoal cysts, trophozoites or oocysts, 63 (24.9%) for helminthic infections and 32 (12.6%) for bacterial infections (Table 2). In 68 samples (26.8%), no causative agents were found. We did not look for viral agents. Mixed infections were seen in 16 samples (2 samples with two types of protozoa, 6 samples with protozoa and helminthes, 3 samples with more than one type of helminth, and in 5 samples there was a mixed infection with protozoa and bacteria).

G. lamblia was the most prevalent protozoal infection in children below 5 years with persistent diarrhea (67.7%, 61/90), followed by E. histolytica (27.7%, 25/90). Four samples, 2 each, were positive for Cryptosporidium spp and C. cayetanensis. Ascaris lumbricoides was the commonest helminth (39.6%, 25/63) identified, followed by Trichuris trichiura (28.5%, 18/63), hookworm (26.9%, 17/63) and Strongyloides stercoralis (4.7%, 3/63). The distribution of 32 bacterial isolates recovered from the cases was: 24 (56.2%) EAEC, 6 (18.6%) EPEC, 3 (9.3%) ETEC, 3 (9.3%) Campylobacter spp, 1 (3.1%) Salmonella spp and 1 (3.1%) Shigella spp.

G. lamblia trophozoites were detected in a significantly higher proportion of persistent diarrheal cases (9.8%) than acute diarrheal and non-diarrheal controls (0% and 2%, respectively; p<0.001) (Table 3). Similarly, E. histolytica trophozoites were detected in a significantly higher proportion of persistent diarrheal cases (6.3%) than acute diarrheal and non-diarrheal controls (1% each). Cyclospora cayetanensis (0.7%) was isolated from only HIV-positive children, whereas in the case of Cryptosporidium spp (0.7%), one was isolated from an HIV-positive child and another from a
Table 2

Enteropathogens associated with persistent diarrhea in children (n =253) less than 5 years.

<table>
<thead>
<tr>
<th>Category</th>
<th>Pathogens</th>
<th>Total number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protozoa (n = 90)</td>
<td>Giardia lamblia</td>
<td>61</td>
<td>67.7%</td>
</tr>
<tr>
<td></td>
<td>E. histolytica</td>
<td>25</td>
<td>27.7%</td>
</tr>
<tr>
<td></td>
<td>Cryptosporidium sp</td>
<td>2</td>
<td>2.2%</td>
</tr>
<tr>
<td></td>
<td>Cyclospora cayetanensis</td>
<td>2</td>
<td>2.2%</td>
</tr>
<tr>
<td>Helminthes (n = 63)</td>
<td>Ascaris lumbricoides</td>
<td>25</td>
<td>39.6%</td>
</tr>
<tr>
<td></td>
<td>Trichuris trichiura</td>
<td>18</td>
<td>28.5%</td>
</tr>
<tr>
<td></td>
<td>Hookworm</td>
<td>17</td>
<td>26.9%</td>
</tr>
<tr>
<td></td>
<td>Strongyloides stercoralis</td>
<td>3</td>
<td>4.7%</td>
</tr>
<tr>
<td>Bacteria (n = 32)</td>
<td>EAEC</td>
<td>18</td>
<td>56.2%</td>
</tr>
<tr>
<td></td>
<td>EPEC</td>
<td>6</td>
<td>18.6%</td>
</tr>
<tr>
<td></td>
<td>ETEC</td>
<td>3</td>
<td>9.3%</td>
</tr>
<tr>
<td></td>
<td>Campylobacter sp</td>
<td>3</td>
<td>9.3%</td>
</tr>
<tr>
<td></td>
<td>Salmonella sp</td>
<td>1</td>
<td>3.1%</td>
</tr>
<tr>
<td></td>
<td>Shigella sp</td>
<td>1</td>
<td>3.1%</td>
</tr>
<tr>
<td>Unknown (n = 24)</td>
<td>(? Viruses and others)</td>
<td>68</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3

Prevalence of intestinal protozoal parasites in cases (n = 253) and controls (n = 100 each).

<table>
<thead>
<tr>
<th>Categories (Total protozoal parasites detected)</th>
<th>EH</th>
<th>GL</th>
<th>CRYP</th>
<th>CYC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persistent diarrhea (n = 90)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T C B</td>
<td>25</td>
<td>61</td>
<td>02</td>
<td>02</td>
</tr>
<tr>
<td>(Total) = 16 (6.3%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute diarrhea (n = 15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>T (Total) = 01 (1%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No diarrhea (n = 24)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T (Total) = 01 (1%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EH = Entamoeba histolytica; GL = Giardia lamblia; CRYP = Cryptosporidium sp; CYC = Cyclospora cayetanensis; T = Trophozoite; C = Cyst; B = Both trophozoite and cyst.

child suffering from protein energy malnutrition. All 4 children had persistent diarrhea.

In none of the 21 (8.3%) breast-fed children aged 0-5 months were protozoal infections detected. In most of these cases, the etiological agent remained unknown. The rates of intestinal protozoal infections in age groups 6-11, 12-23, 24-35, and 36-60 months were 13.8, 19.3, 25.2, and 33.2%, respectively. In each age group, the rate of infection was significantly higher than the previous age group. Acute infection (increased numbers of trophozoites) with both G. lamblia and E. histolytica also increased with age.
Diarrheal episodes were encountered mainly in July-September (monsoon) (44.6%), followed by April-June (34.7%), corresponding with the increased infection rate and the number of trophozoites of both G. lamblia and E. histolytica. In comparison, diarrheal cases were significantly lower in January-March (Spring) (9%) and October-December (Winter) (11.4%) compared to that of Summer and Monsoon seasons.

**DISCUSSION**

The majority (33%) of the global deaths of all types of illnesses are as a result of infectious and parasitic diseases (WHO, 1998). In Southeast Asia, diarrheal diseases constitute the largest single cause of death in children below age 5 years. Although most of the deaths are due to acute diarrhea, persistent diarrhea may account for a large proportion of all deaths. WHO and UNICEF estimated that in 1991 persistent diarrhea accounted for only 10% of diarrheal episodes, but as many as 35% of diarrheal deaths in children under 5 years of age. Evidence from studies in Bangladesh, India, Peru and Brazil indicates that approximately 45% (range 23% to 62%) of diarrhea-associated deaths were due to persistent diarrhea (Dialogue on Diarrhoea and ADDR, 1992). Amebiasis and giardiasis follow an endemic pattern and focal epidemics occur when large groups of children are exposed to common sources in water-borne and food-borne epidemics (Piya et al, 2001).

In the present study, the protozoal infection rate was higher than the bacterial and helminthic infection rates in cases of persistent diarrhea. The children probably acquired the infection from school or at home, which relates to poor quality drinking water and poor personal hygiene. Contamination of drinking water with sewage due to close running of water pipes and sewage lines and consumption of green leafy vegetables have already been reported as predisposing factors for diarrhea in Nepal (Sherchand et al, 1999; Shrestha et al, 2001).

The incidence of G. lamblia in persistent diarrhea (9.8%) in the present study was more than that reported for Bangladesh (Baqui et al, 1992; Henry et al, 1992) and Brazil (Schnack et al, 2003), but less than India (Bhandari et al, 1999; Kaur et al, 2002) and Peru (Lanata et al, 1992). G. lamblia was not responsible for acute diarrhea in the present study, although a high incidence (13%) has been documented previously for other parts of Nepal (Hoge et al, 1995). The prevalence of giardiasis was more in malnourished children in Gambia compared to healthy controls (Sullivan et al, 1990), but such an association could not be established in our study since all the cases and controls were undernourished (Grade II malnutrition).

A significantly higher proportion of those with persistent diarrhea were infected with E. histolytica compared to the acute diarrheal (6.3% vs 1%) and non-diarrheal (6.3% vs 1%) controls. A similar high association has been reported for Bangkok (6.8%) (Chavalittamrong et al, 1984), Central Africa (12.8%) (Molbak et al, 1994), India (14%) (Kaur et al, 2002), and Brazil (56.4%) (Schnack et al, 2003). E. histolytica is not always reported as a cause of persistent diarrhea in children (Bhan et al, 1989; Baqui et al, 1992; Henry et al, 1992; Bhandari et al, 1999; Kaur et al, 2002) or acute diarrhea (Hoge et al, 1995).

Cryptosporidium spp and C. cayetanensis were associated with persistent diarrhea in our study in immunocompromized children only, although infection of immunocompetent hosts has been described (Shlim et al, 1991). Cryptosporidium spp itself is known to cause persistent diarrhea in children (Baqui et al, 1992; Molbak et al, 1994) and these pathogens have been reported to have a general prevalence rate of 0.9% in Africa, especially in Morocco, South Africa and Egypt (Asfaw and Goitom, 2000). Cyclospora cayetanensis has
been reported to have a prevalence rate of 29.8% in stool samples from the general population (Sherchand et al, 1999), but 5% in children below 5 years of age with acute diarrhea in Nepal (Hoge et al, 1995).

Breast-fed children are generally known to be more resistant to protozoal infections, though isolated cases are not infrequent (Molbak et al, 1994). It is noteworthy in this context to state that there is evidence of increased risk for G. lamblia and Cryptosporidium spp infections in water storage dams and reclamation plants (Menge et al, 2001). In Pokhara, there are dams on the rivers (Seti, Kalindi and Gondoki) flowing through the region and people use the stored river water for household purposes. Heavy rainfall, low temperature and ambient humidity in Summer and Monsoon seasons create a favorable environment for the acquisition and dissemination of intestinal protozoal parasitic infections.

As giardiasis and amebiasis were observed to be associated with persistent diarrheal episodes in children below 5 years, stool microscopy should be routinely performed in these patients, and effective treatment should be instituted upon the isolation of E. histolytica or G. lamblia trophozoites. Education regarding basic sanitation and improvement of family hygiene will help in controlling the infection.

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REFERENCES


Menge JG, Haarhoff J, König E, Mertens R, van der


