SEASONAL DISTRIBUTION OF ENTEROPATHOGENS DETECTED FROM DIARRHEAL STOOL AND WATER SAMPLES COLLECTED IN KATHMANDU, NEPAL

Kazuo Ono[1], Shiba Kumar Rai[2], Masatsugu Chikahira[1], Tsuguto Fujimoto[1], Hiroshi Shibata[3], Yasunao Wada[3], Hidetaka Tsuji[1], Yoko Oda[4], Ganesh Rai[5], Chandrika Devi Shrestha[4], Kuniyoshi Masuda[1], Hari Govinda Shrestha[6], Takeo Matsumura[7], Hak Hotta[8], Takashi Kawamura[1] and Shoji Uga[9]

[1]Division of Microbiology, Hyogo Prefectural Institute of Public Health, Kobe, Japan; [2]Department of Microbiology, Nepal Medical College, Kathmandu, Nepal; [3]Clinical Laboratory, Hyogo College of Medicine, Nishinomiya, Japan; [4]Department of Pathology, Kanti Children Hospital; [5]Department of Pathology, Birendra Police Hospital; [6]Department of Pathology, Tribhuvan University Institute of Medicine, Kathmandu, Nepal; [7]Departments of Medical Zoology, [8]Microbiology, Faculty of Medicine; [9]Department of Medical Technology, Faculty of Health Science, Kobe University School of Medicine, Kobe, Japan

Abstract. A total of 334 diarrheal fecal samples (from 210 males and 124 females) collected in Kathmandu, Nepal, were studied for various kinds of enteropathogens. Overall, 33% (111/334) fecal samples were positive for one or more enteropathogens. There was no difference in detection rates between males and females. Enteropathogen detection rates in summer, winter, spring, and autumn were 61% (40/66), 52% (45/87), 31% (25/81), and 25% (25/100), respectively. Altogether eight species of bacteria, three genera of viruses, and five species of protozoan parasites were detected with considerable seasonal variations. Among the bacterial isolates, enteropathogenic *Escherichia coli* topped the list followed by *Vibrio* sp. Only one sample had *Shigella* (*S. sonnei*). Rotavirus type A was the most frequently detected among the enteric viruses, followed by human enterovirus and human adenovirus, respectively. Among the enteric protozoan parasites, *Giardia intestinalis* was the most frequently detected followed by *Cryptosporidium parvum*. Detection of bacterial and protozoan pathogens showed a slightly high tendency in the summer season compared with that in the other seasons ($p>0.05$), whereas the detection of viruses was significantly high in the winter season ($p<0.05$). Of the total 57 water samples, 43 (75%) showed one or more bacterial species out of which 51% (22/43) were *E. coli*. Among the *E. coli* isolates, 68% were EPEC. Enterohemorrhagic *E. coli* (O157) was not detected.

INTRODUCTION

It is estimated that 2.2 million people (mostly children) in the world die from diarrhea each year (WHO, 1999). The incidence is highest in children, with about 1.5 billion episodes a year, and occurs mainly in developing countries. Most of diarrheal diseases occurring in developing countries are infectious diseases. The diarrhea attack rates (episodes per child per year) in children less than five years old in developing countries varies from 6.0 to 11.9 (Guerrant *et al*, 1990). It was also observed that among urban poor the attack rate was almost double that in rural poor. This finding has been attributed to the unsanitary conditions, poverty, and malnutrition, and slum environments in urban areas.

Many reports on surveys of diarrheal diseases mainly in children have appeared from countries like Papua New Guinea (Howard *et al*, 2000), Ghana (Hori *et al*, 1994), Philip-

Correspondence: Kazuo Ono, Division of Microbiology, Hyogo Prefectural Institute of Public Health, 2-1-29 Arata-cho, Hyogo-ku, Kobe 652-0032, Japan.
Tel: +81-78-511-6640; Fax: +81-531-7080;
E-mail: ono@iph.pref.hyogo.jp

pines (Adkins *et al*, 1987), China (Kain *et al*, 1991), and Nepal (Sherchand and Shrestha, 1996b). In Nepal, of the various infectious diseases, gastroenteritis together with dysentery accounts for more than 65% of all cases (CBS, 1991). Diarrheal diseases reportedly cause over one-third of childhood deaths mainly during rainy season in Nepal (NPC/UNICEF, 1991). The causes of outbreak of diarrheal diseases were found to be associated with the poor condition or absence of a sanitary system (Rai *et al*, 1997; Matsumura *et al*, 1998), contamination of drinking water (Adhikari *et al*, 1986; Ise *et al*, 1994), and poverty. Cholera epidemic occurs annually even in the capital city of Kathmandu (Ise *et al*, 1994).

The real situation of the diarrheal diseases may not be reflected if the surveys conducted cover only one particular season. This is more evident in countries where there is a sharp seasonal change. Further, most of the previous studies have not considered environmental factors. This is true of studies in Nepal. In this study, therefore, we determined the coliform and *E. coli* bacilli from drinking water in various sites in Kathmandu, and considered these values as a bacteriological indicator of drinking water contamination, taken together with the isolation or detection of enteropathogens in diarrheal fecal samples collected in all four seasons in Kathmandu Valley, Nepal.

MATERIALS AND METHODS

Study population and specimens

A total of 334 diarrheal fecal samples collected from children attending the Out Patient Department of Kanti Children Hospital and the Birendra Police Hospital in Kathmandu were included in this study. Age of subjects included in this study ranged from less than 1 year to 15 years.The fecal samples were collected in 1996 and 1997 covering all four seasons; the summer (66 samples in July, 1996), the winter (87 samples in January and December, 1997), the spring (81 samples in May, 1997), and the autumn (100 samples in November, 1997). During this period, except in spring, a total of 57 drinking water samples, 41 from natural water sources and 16 from piped tap water, were also collected from different areas in Kathmandu Valley.

Preparation for assays

The stool samples collected were preserved in sterile plastic tubes in Carry-Blair transport medium (Carry-Blair TM) and in 2% potassium dichromate solution (PDS) for viral, bacterial and intestinal protozoan pathogens, respectively. Samples put into the sterile plastic tubes were stored at -20ºC, and those inoculated in to Carry-Blair TM and in 2% PDS were stored at 4ºC. Drinking water samples collected into sterile 50 ml tubes were stored at 4ºC. These samples were then transported to Japan under cold condition. Upon arrival in Japan, samples in sterile plastic tubes were stored at -80ºC, and those inoculated into Carry-Blair TM and in 2% PDS, together with water samples, were stored at 4ºC until tests were performed. Enrichment media like Selenite F was not employed.

Microbiological methods

Bacterial culture was performed on Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar, Salmonella-Shigella (S-S) agar, and Desoxycholate Hydrogen Sulfide Lactose (DHL) agar media (Nissui, Tokyo, Japan). Special attention was given to *Vibrio* sp, *Salmonella* sp, *Shigella* sp, and enteropathogenic *E. coli* (EPEC). Bacterial isolates were identified by standard biochemical and serological test procedures (Manual of Clinical Microbiology, 4th ed, 1985). *V. cholerae* were subjected to drug sensitivity testing against 18 drugs by the microbroth dilution method (National Committee for Clinical Laboratory Standards,1982). Viruses were detected by electron microscopy and cell culture methods. Rotavirus type A and human adenovirus were identified by enzyme linked immunoassay (Rota clone and Adeno clone, TBF, Tokyo, Japan, respectively). Enterovirus was identified by neutralization test and reverse transcription polymerase chain reaction (PCR) as described by Yoshida *et al* (1999). Intestinal protozoan parasites were detected by

direct saline and iodine wet mounts as well as by sucrose floatation technique. *G. intestinalis* and *C. parvum* were confirmed by fluorescent antibody technique using respective specific antibodies (G-C Combo, Dynal AS, Oslo, Norway).

Water samples were subjected to the isolation of coliform and *E. coli* by the defined substrate method (Stephen *et al*, 1988). *E. coli* isolates were serotyped for O antigen using specific antisera (Denkaseiken, Tokyo, Japan).

Statistical method

Chi-square test was applied to determine significant differences.

RESULTS

A total of 334 diarrheal fecal samples collected in Kathmandu, Nepal, were studied for various kinds of enteropathogens. Of the total 334 samples, 210 were from males and 124 were from females. Overall, 33% (111/334) of all fecal samples were positive for one or more enteropathogens. There was no difference in enteropathogen detection rate between male and female populations (Table 1).

Altogether eight species of bacteria, three genera of viruses, and five species of protozoan parasites were detected with considerable seasonal variations. Detection rates of bacterial and protozoan pathogens in summer were slightly high compared with those in other seasons ($p>0.05$), whereas the detection rates of viruses were dominant in winter ($p<0.05$) (Table 2). Season-wise, the enteropathgen detection rates were 61% (40/66), 52% (45/87), 31% (25/81), and 25% (25/100) in summer, winter, spring, and autumn , respectively (Table 2). *V. cholera* was detected only during the summer season, whereas other species of *Vibrio* (*V. cholerae* non O1, *V. parahemolyticus*, and *V. mimicus*) were detected only in winter. The biotypes of *V. cholerae* were O1 and El Tor Ogawa, though all isolates showed the same drug sensitivity pattern. EPEC was isolated in all seasons except spring. Both *Salmonella* and *Shigella* could not be detected in the summer season. Among the *Salmonella* organisms isolated, all were *S. typhimurium* except one strain of *S. typhi*. The only strain of *Shigella* isolated was identified as *S. sonnei*, and it was detected in the spring season, whereas the only *Aeromonas* strain was isolated in the summer season.

Table 1
Enteropathogen detection rates in diarrheal children in Kathmandu, Nepal.

Group of subjects	Total no.	No. of positive	Detection rate (%)
Children[a]	334	111	33
Male	210	70	33
Female	124	41	33

[a]Defined as less than or equal to 15.

Altogether, three genera of viruses, namely, rotavirus type A, human enterovirus, and human adenovirus, were detected at rates of 11%, 7%, and 3%, respectively (Table 2). All rotaviruses detected were Type-A. The human enteroviruses detected in this study belonged to echovirus serotypes 7, 16, 19, 20, and 30. Of these, type 16 was the most common, followed by type 30 and others (data not shown). All three types of viruses were detected in all four seasons, except adenovirus, which was not detected in autumn.

Of the five species of protozoan enteropathogens detected, *G. intestinalis* was the most common, followed by *C. parvum*, *Entoamoeba histolytica*, *Blastocystis hominis*, and *Trichomonas hominis*. *G.intestinalis* showed almost uniform distribution in all four seasons, while the others were mainly detected in the summer season. *B. hominis* was not detected in autumn whereas *T. hominis* was not detected in autumn or spring (Table 2). The frequency of all bacterial, viral, and protozoan enteropathogens was the highest among the age group of 3-5 years (42%) compared with the age groups of 6-10 years (28%) and 11-15 years (33%), and infants (33%) ($p<0.05$) (Table 3).

Table 2
Seasonal distribution of respective enteropathogens.

Enteropathogens	Total no. of positives (%)	Summer (n=66) No. of positives	Winter (n=87) No. of positives	Spring (n=81) No. of positives	Autumn (n=100) No. of positives
Bacteria					
Vibrio cholerae Ol	8 (2)	8	0	0	0
Vibrio cholerae non Ol	1 (0)	0	1	0	0
Vibrio parahaemolyticus	1 (0)	0	1	0	0
Vibrio mimicus	1 (0)	0	1	0	0
Aeromonus sp	1 (0)	1	0	0	0
Escherichia coli	12 (4)	l	3	0	8
Salmonella typhi	1 (0)	0	0	0	1
Salmonella typhimurium	4 (1)	0	2	2	0
Shigella sonnei	1 (0)	0	0	1	0
Total	30 (9)	10 (15)	8 (9)	3 (4)	9 (9)
Viruses					
Rotavirus type A	35 (11)	3	20	7	5
Human enterovirus	22 (7)	7	6	3	6
Human adenovirus	10 (3)	5	3	2	0
Total	67 (20)	15 (23)	29 (33)[a]	12 (15)	11 (11)
Protozoa					
Giardia intestinalis	15 (5)	3	4	5	3
Entamoeba histolytica	7 (2)	3	1	2	1
Cryptosporidium parvum	8 (2)	4	1	2	1
Trichomonas hominis	3 (1)	2	1	0	0
Blastocystis hominis	5 (2)	3	1	1	0
	38 (11)	15 (23)	8 (9)	10 (12)	5 (5)
Grand total	135 (40)	40 (61)	45 (52)	25 (31)	25 (25)

[a]$p < 0.05$

The pattern of coliform and *E. coli* detection in 57 water samples collected in Kathmandu Valley is shown in Table 4. In total of 57 samples examined, 43(75%) samples had one or more species of organisms, out of which 22(51%) were *E. coli*. Among the 17 *E. coli* strains isolated, over two-thirds(15) were EPEC.

DISCUSSION

In the present study, one or more enteropathogens was detected in 33% of diarrheal fecal samples studied. This rate was slightly higher than that of 29% reported from Romania (Costantineu *et al*, 1991) but much lower than that of 73.5% observed in Surabaya, Indonesia (Wasito *et al*, 1999), 58.4% in Manila, Philippines (Adkins *et al*, 1987) or 56.5% in Beijing, China (Kain *et al*, 1991). This rate was even lower than that of 43.5% found in the control group in Beijing, China (Kain *et al*, 1991). An annual variation in the detection rate of enteropathogens has been observed even in the same study population (Wasito *et al*, 1999).

In this study, we did not observe differences in the enteropathogen detection rates (overall or for particular enteropathogens) between male and female subjects. Kain *et al*. (1991) however, reported that female patients in Beijing, China, were significantly more likely to be infected with ETEC than were male patients. Among the child population, the enteropathgen detection rate and the frequency of enteropathogens were the highest in the age group of 3-5 years. This high frequency appears

Table 3
Prevalence of Enteropathogens in different age groups.

Age group (years)	Total samples	No. of positive samples (%)	No. of pathogens isolated (%)		
			Bacteria	Virus	Protozoa
Infants (< 2)	169	55 (33)	14	34	16
3 - 5	62	26 (42)[a]	8	17	12
6 - 10	76	21 (28)	6	11	7
11 - 16	27	9 (33)	2	5	3
Total	334	111 (33)	30	67	38

[a]$p < 0.05$

Table 4
Detection of coliform bacilli and *E. coli* in dinking water samples collected in Kathmandu, Nepal.

Samples	No. of samples tested	No. of positives		
		Coliform bacilli (%)	*E. coli*	
			EPEC (%)	Other (%)
Natural water[a]				
Summer	3	3	1	0
Winter	21	19	10	5
Autumn	17	11	3	1
Tap water				
Summer	7	5	1	1
Winter	4	2	0	0
Autumn	5	3	0	0
Total	57	43 (75)	15 (26)	7 (12)

[a]Natural spouts and wells.

to be associated with the crawling and oral fixation typical of children this age.

V. cholera O1, the most common *Vibrio* sp, was detected only in the summer season. This finding was in agreement with the evidence of cholera outbreak in Kathmandu Valley, though the detection rate was much higher (Ise *et al*, 1994). The spread of cholera in Kathmandu has been associated with poor sanitation and contamination of drinking water by rainwater mixed with sewage (Adhikari *et al*, 1986). Malodorous street floods up to knee level during the rainy summer season are common recent phenomena in Kathmandu Valley (Rai *et al*, 1997), and have been associated with epidemics of cholera (Ise *et al*, 1994), enteric fever and a re-emerging trend of hookworm infection (Rai *et al*, 1997). However, it was interesting that, in our study, neither *Salmonella* sp nor *Shigella* sp was detected in the summer season, during which both enteric fever and dysentery are common. Elsewhere, *Shigella* sp. has been reportedly ranked either the second or third most common enteropathogen (Adkins *et al*, 1987). One explanation for the very low frequency of *Salmonella* sp and *Shigella* sp in this study could be due to the exclusion of Selenite F enrichment media. Other species of *Vibrio* were detected only in the winter season and no explanation is available for this. Overall, the finding that EPEC was the most common bacterial isolate in this study was in agreement with findings reported previously (Wasito *et al*, 1999; Kain *et al*, 1991; Hori *et*

al, 1991). EPEC were detected in all seasons except spring. The highest frequency of EPEC found in autumn has no apparent explanation. No distribution pattern in the O antigen serotype of EPEC observed in this study contrasted with the findings in Nepal of Ise *et al* (1994) or of those in Romania of Constantiniu *et al* (1991). Of the total 216 EPEC strains isolated in Kathmandu, almost all (203) were enteropathogenic, and remaining 9, 4 and none were enterotoxigenic, enteroenvasive and enterohemorrhagic, respectively (Ise *et al*, 1994).

In this study, diarrheagenic viruses were detected in all seasons, and the frequency of detection was significantly highest in the winter, as was reported in the neighboring country of India (Nath *et al*, 1992). Of the three viral agents detected, Rotavirus type A was the most common (5-23% over the four different seasons), as was reported previously (Shetty *et al*, 1995; Guerrant *et al*, 1990). Elsewhere in Asian countries, the reported detection rates of rotavirus have ranged from 6.8 to 34.5% (Adkins *et al*, 1987; Kain *et al*, 1991; Nath *et al*, 1992; Shetty *et al*, 1995; Wasito *et al*, 1999; Howard *et al*, 2000). Previously, the prevalence of rotavirus together with adenovirus in Nepal has been reported to be 6.8% (Sherchand *et al*, 1996b). The magnitude of rotavirus infection is reportedly uniform in developed and developing countries (Guerrant *et al*, 1990). In this study, we did not detect the Norwalk-like virus, which has been reported mainly in developed countries (10-27%), and at very low rates in developing countries (1-2%) (Guerrant *et al*, 1990). However, due to lack of facilities, diarrhea due to viral infection is often undiagnosed, and therefore unreported, in Nepal.

The finding that *G. intestinalis* topped the list of protozoan parasites detected was in agreement with previously reported findings in Nepal among high school children (Rai and Gurung, 1986), in hospital-attending subjects (Rai *et al*, 1995), and in children and adults with and without abdominal discomfort (Sherchand *et al*, 1996a,b). *G. intestinalis* reportedly has remained at the top of the list for over a decade, followed by *E. histolytica* (Rai *et al*, 1995). Our present finding on *C. parvum* was almost in agreement with those previously reported (0.4-6.8%) by Sherchand *et al* (1996a, b). Silmilarly, the prevalence of *T. hominis* and *B. hominis* was also in agreement with frequencies reported previously (Sherchand *et al*, 1996a). However, the present finding on *B. hominis* was much lower than that reported in Nepal by Gianoti (1990). *Cyclospora* sp was not detected in this study though previous reports have shown its presence in Nepal (Sherchand *et al*, 1996a; Hoge *et al*, 1993).

The rate of fecal contamination of drinking water samples collected from different sites in Kathmandu Valley (75%) was in agreement with those reported earlier in Nepal; 88% in Kathmandu Valley (Adhikari *et al*, 1986) and 86% in a hilly rural village (Matsumura *et al*, 1998). This frequency has been attributed to poor or virtually non-existent sanitary conditions and/or no continuous supply of water. Fecal contamination of drinking water has long been a major public health problem in Nepal, and conditions have not improved due to the rapid population growth, spread of slums, and poor sanitation of its infrastructure.

The present study clearly showed the magnitude and pattern of enteropathogens in all four seasons, together with the status of fecal contamination of drinking water in Nepal. Hence, a comprehensive program needs to be launched to combat diarrhea-related morbidity, mortality, and its socio-economic impact in Nepal, and the results of such a program should be monitored by periodical survey.

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