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Aetiology of Diarrhoea in a Birth Cohort of Children Aged 0-2 Year(s) in Rural Mirzapur, Bangladesh

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ABSTRACT

The incidence of aetiology-specific diarrhoea and the pathogenicity of infectious agents in a birth cohort (n=252) in rural Bangladesh were determined. Stool specimens or rectal swabs were collected from diarrhoeal cases over two years and routinely on a monthly basis. Stool samples from children with diarrhoea were compared with stool samples from children without diarrhoea to calculate rates of isolation and pathogenicity of agents. In total, 1,750 stool specimens from diarrhoea patients and 5,679 stool specimens from children without diarrhoea were tested. An infectious agent was identified in 58% of the stool specimens from diarrhoea patients and 21.6% of the stool specimens from children without diarrhoea. The most commonly-isolated pathogens from all specimens were enterotoxigenic *Escherichia coli* (ETEC), enteroadherent *E. coli*, *Shigella*, *Campylobacter jejuni*, *Giardia*, and rotavirus. ETEC (ST and LT-ST toxin), enterotoxigenic *Bacteroides fragilis*, *Shigella*, and rotavirus were associated more with disease than with asymptomatic infections. Aetiology-specific infections were associated with acute episodes. The isolated enteropathogens were essentially the same as those found in other tropical rural settings. Enterotoxigenic *B. fragilis* was also identified as a pathogen. Ongoing vaccine efforts focusing on *Shigella*, rotavirus, and ETEC would be useful.

***Key words*:** Diarrhoea; Diarrhoea, Infantile; Enteropathogens; Pathogenicity; Community-based studies; Cohort studies; Bangladesh

INTRODUCTION

Acute diarrhoea is one of the leading causes of morbidity and mortality in children in most developing areas of the world. Despite the use of oral rehydration therapy, deaths due to diarrhoea in children aged less than five years are still estimated to be about two million per year (1). Besides mortality, the long-term effects of diarrhoeal illness on childhood health are extremely serious and include malnutrition and growth faltering (2). Further attention to the causes of diarrhoea is important to develop preventive measures.

Aetiology of diarrhoea is variable according to geographic and climatic conditions, host factors, and socioeconomic situations. It is important to establish the causative agents of diarrhoea across different settings and populations. Determination of enteric agents may contribute to improved surveillance systems, including early recognition of epidemics and their modes of spread, guide vaccine development and administration policies, and influence appropriate outcome measures for intervention studies.

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In the 1980s, studies of aetiologies of diarrhoeal diseases in children of developing countries, such as Bangladesh, were able to detect an enteropathogen in about half of stool specimens taken from ill subjects (3). Recent advances in microbiological techniques have greatly increased the rate of isolation of enteric pathogens, including newer ones. The enhanced laboratory capacity, coupled with its application in the context of a longitudinal study, permits a more complete picture of the epidemiology of diarrhoeal infections. The objective of the present study was to investigate the aetiological roles of a vast array of pathogens, including ones discovered relatively recently, in the context of a rural community-based birth-cohort study.

MATERIALS AND METHODS

Study site and subjects

The study site encompassed 10 villages in Mirzapur, a rural area, located approximately 60 km from Dhaka, the capital city of Bangladesh. Detailed characteristics of the villages and village inhabitants are given elsewhere (4).

During July 1993–August 1993, a door-to-door census, conducted by community health workers (CHWs), identified pregnant women or those who had the potential to become pregnant and deliver their babies. The pregnant women were invited to participate in the surveillance project. Informed consent for inclusion of their children in the project was obtained from mothers or caretakers. All study children were assured of healthcare by a paediatrician either in the home or at the Kumudini Hospital for the duration of their enrollment in the study. The Ethical Review Committee of ICDDR,B: Centre for Health and Population Research approved the study protocol, and the Johns Hopkins Committee on Human Research approved the data analysis plan.

Study participants

In total, 252 children made up the cohort, of whom 244 completed the full two-year follow-up, and 179,789 child-days of observation was made.

About 40% of the study children lived in households of more than six people, and the median monthly household income for all households was about Taka 3,000 or US$ 60. Keeping animals around the living area was common. For over 80% of the households, disposal of faeces took place in open space rather than in closed pits or sanitary latrines. Since only 30% of the households claimed ownership of a tubewell, 81% of them practised storing water for later use. Although Bangladeshi mothers generally practise partial breastfeeding for a prolonged period, exclusive breastfeeding of the infant beyond three months of age was practised by 37% of mothers in the study. More detailed data are presented elsewhere (4).

Illness and microbiology surveillance

The field team comprised a study physician and 12 local, trained female CHWs who had had previous exposure to household morbidity surveillance for diarrhoea (5). During September 1993–October 1994, newborns were enrolled into the study. From the day of birth of the study subjects, a member of the field team visited each participating home twice a week to assess the occurrence of acute respiratory and diarrhoeal symptoms since the previous visit. Follow-up activities were undertaken for each child until he/she reached two years of age. During every household-visit, the frequency and consistency of the child's stool, as noted by the caregiver, ware recorded for that day and for the previous 2-3 days. The household surveillance showed that the 252 study children had 1,916 episodes of diarrhoea over the first two years of life. The number of episodes did not vary significantly by sex (4).

Stool samples or rectal swabs were taken at the time of home-visit whenever the child was having diarrhoea or was reported to have diarrhoeal illness during the previous 2-3 days. Stool samples or rectal swabs were also collected from each child on a routine monthly basis, regardless of the presence of diarrhoea. If a child had diarrhoeal illness, the CHW provided oral rehydration solution, and the study physician administered antibiotics, if necessary. If the diarrhoea was considered to be of great severity, the caregiver was advised to visit the Kumudini Hospital, located 4-9 km from each study village, where free and immediate treatment was available for the study participants. Cultures and swabs were collected in an ice-cooler in the morning and were transported to the appropriate laboratory for processing that afternoon. An ICDDR,B parasitologist conducted parasitology examinations at a laboratory set up at the Kumudini Hospital. Bacterial and viral tests were performed at the ICDDR,B laboratories in Dhaka.

Definitions

A case of diarrhoea was defined as three or more liquid stools within 24 hours, or any number of loose stools accompanied with blood within 24 hours. Discrete diarrhoeal episodes were separated by at least three diarrhoea-free days. An acute episode was one that lasted for less than two weeks, and a persistent episode was defined as one that lasted for two weeks or longer (6). The pre-

sence of blood in stool was defined by gross appearance in stool, as noted by the mother or by the study personnel. Body temperature was measured with a thermometer, and fever was defined for analysis as a temperature of 37.8 °C or higher.

For assessing the pathogenicity of an enteropathogen, rates of isolation of pathogens from children during a diarrhoeal episode were compared with rates of isolation of pathogens from control children without diarrhoea. Controls were children without any loose stools on the day of specimen collection and three days before and three days after the collection. The presence of co-pathogens within an episode was not a reason for exclusion of a case or asymptomatic control from analysis.

Laboratory studies

Stool microbiology: Specimens were inoculated on MacConkey agar (MA), *Salmonella-Shigella* agar (SSA), tellurite-taurocholate gelatin agar (TTGA), *Campylobacter* blood-free medium with cefaperazone supplement (CBFMC) (Oxoid), tryptone blood agar with polymyxin B, irgasan, *Bacteroides* Bile Esculin agar (BBE medium), and a new medium formulated for the isolation of *B. fragilis* (7). The specimens were inoculated into selenite F broth (SFB) and bile peptone broth (BPB). BPB was sub-cultured onto TTGA after a six-hour incubation period and again onto SSA overnight. SFB was sub-cultured onto SSA after overnight incubation at 37 ^{O}C. All pathogens were initially identified by growth on selective media and colony characteristics. Aerobic pathogens were then screened using Kliger's iron agar and motility-indole urea medium. *Salmonella*, *Shigella*, and *Vibrio cholerae* were confirmed with slide agglutination tests with specific antisera. *Campylobacter* colonies from CBFMC were identified by characteristic morphology after Gram-staining, motility, oxidase and catalase tests, susceptibility to cephalothin and nalidixic acid, and hippurate hydrolysis (8).

BBE agar was used for the primary isolation of *B. fragilis* from faeces during the first half of the study. Specimens collected during the latter half of the study were tested for BF using primary isolation media developed by Dr. Jayakumar Reuben (7). A tissue culture assay using the cloned human colonic-epithelial-cell line—HT29/C1—was used for detecting enterotoxigenic *B. fragilis* in *B. fragilis*-positive specimens (9).

For recognition of diarrhoeagenic *E. coli*, three different lactose-fermenting colonies resembling *E. coli* were picked from MA and stored separately in vials containing trypticase soy broth with 15% glycerol at -70 ^{O}C. These *E. coli* colonies were later assayed for diarrhoeagenic properties with DNA probes (10).

For the detection of group A rotavirus, an ELISA based on the Dakopatts-kit was used, incorporating rabbit hyperimmune antisera (produced at ICDDR,B) and anti-human rotavirus-horseradish peroxidase conjugate (anti-human RV-HRP, Dakopatts, Copenhagen, Denmark) (11). An indirect double antibody EIA developed by Herrman *et al*. (12) was used for the detection of astrovirus.

For the detection of parasites, direct wet film of stool was prepared and checked for ova and parasites. An iodine stain was prepared for the detection of amoebic cysts and other protozoa. The formalin-ether sedimentation technique of Ritchie (13) was used for the concentration of protozoan cysts and helminth eggs.

Stool and rectal swab specimens were tested for the bacterial, viral and parasitic agents listed in Table 1. All diarrhoeal isolates and a 5% random sample of routine stools were to be tested for diarrhoeagenic *E. coli*, including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroadherent *E. coli* (EAEC), and diffusely-adherent *E. coli* (DAEC). All *Bacteroides fragilis*-positive diarrhoeal isolates and a 20% random sample of routine stools positive for *B. fragilis* were for positive enterotoxigenic *B. fragilis*.

Statistical analysis

Rates of isolation were calculated for stool specimens from children with diarrhoea and control children. To avoid detection bias from repeated sampling during a single episode, the rates were also calculated with omissions of repeat isolations within episodes.

Collecting repeat cultures during prolonged episodes was not a part of the study design. For assessment of whether specific enteropathogens were associated with persistent (lasting for 2 weeks or longer) rather than with acute episodes, episodes were classified by their final duration as either acute or persistent, and attack rates of the enteropathogens between the two groups were compared.

When examining repeat isolations of ETEC in this population, only the first-documented excretion of ETEC within an episode was used.

Data management was done at ICDDR,B using FoxPro. STATA (version 7) was used for analysis of data. Chi-square and Fisher's exact tests were used for comparisons of nominal data. Two-tailed p values and 95% confidence intervals (CIs) for odds ratios (ORs) were calculated from coefficients and standard errors of coefficients.

RESULTS

Diarrhoeal episodes

During 1,916 diarrhoeal episodes, 1,750 stool cultures were obtained; 103 (5.9%) of these were repeat specimens within the same episodes and were not included in analysis. Thus, a stool specimen was collected and tested for bacteria, viruses, and parasites for 1,647 (86%) of the 1,916 diarrhoeal episodes.

more than three days after diarrhoea had ended and were excluded. In total, 5,701 routine monthly samples were collected; 5,621 (99%) were non-diarrhoeal samples, and 80 (1%) were taken during a diarrhoeal episode. When the 80 stool specimens from routine collections from children with diarrhoea and the 1,668 stool specimens collected from children with diarrhoea during episodes were combined, there were a total of 1,748 diarrhoeal stool specimens. These 1,748 specimens were compared with 5,679 stool specimens from children without diarrhoea to calculate rates of isolation and pathogenicity of various diarrhoeal agents.

Table 1. Isolations of bacteria, parasites, and viruses in children aged 0-2 year(s) in Mirzapur, 1993-1996

Organism	Diarrhoeal stools		Non-diarrhoeal stools	
	No. of isolates	%	No. of isolates	%
	n=1,748*		n=5,679*	
Enterotoxigenic *Bacteroides fragilis*	40	2.28	15	0.26
Campylobacter jejuni	109	6.23	378	6.67
Shigella spp.	134	7.66	8	0.14
Shigella dysenteriae	14	0.80	3	0.05
Shigella flexneri	94	5.37	1	0.02
Shigella boydii	16	0.91	3	0.05
Shigella sonnei	10	0.57	1	0.02
Salmonella spp.	32	1.83	13	0.24
Salmonella GrB	16	0.91	2	0.04
Salmonella GrC	12	0.69	9	0.16
Salmonella GrD	1	0.06	1	0.02
Salmonella GrE	2	0.11	1	0.02
Vibrio cholerae O1, Ogawa	3	0.17	0	0.00
Escherichia albertii	10	0.57	3	0.05
Plesiomonas shigelloides	2	0.11	0	0.00
Giardia lamblia	231	13.20	742	13.07
Ascaris lumbricoides	185	10.57	484	8.52
Trichuris trichiuria	19	1.09	32	0.56
Cryptosporidium	11	0.63	11	0.19
Entamoeba histolytica	6	0.34	7	0.12
Hookworm	3	0.17	2	0.04
Rotavirus	89	5.09	52	0.92
	n=389*		n=558*	
Astrovirus	34	8.74	38	6.81
	n=1,351		n=355	
Diarrhoeagenic *Escherichia coli*†	632	46.78	130	36.62
	n=197*	*B. Fragilis*-positive	n=185*	*B. Fragilis*-positive
Enterotoxigenic *Bacteroides fragilis*	40	20.30	15	8.11

*Number of samples tested; †Includes enterotoxigenic *E. coli*, enterohemorrhagic *E. coli*, enteroinvasive *E. coli*, enteropathogenic *E. coli*, enteroaggregative *E. coli*, and diffusively adherent *E. coli*
E. coli=*Escherichia coli*

Specimen collection

In total, 1,726 stool samples from children with diarrhoea were collected, but only 1,668 (97%) were actually accompanied with diarrhoeal symptoms. The remaining 58 (3%) were collected on days of illness that did not meet the case definition for diarrhoea, or were taken

In total, 1,706 DNA probe assays were done to detect diarrhoeagenic *E. coli* (ETEC, EIEC, EHEC, EAEC, and DAEC). These were performed on 1,351 diarrhoeal

isolates, belonging to 1,281 separate episodes; thus, 77.2% (1,351/1,748) of the total diarrhoeal samples and 78% (1,281/1,647) of the total episodes were tested for diarrhoeagenic *E. coli*. Of normal stools, 355 DNA probe assays (6.25% of all routine stools) were tested for diarrhoeagenic *E. coli*.

Infectious agents

Overall rates of isolation for all enteric agents sought are shown in Table 1. An aetiologic agent was identified in 58.0% of the stool specimens from children with diarrhoea and 21.6% of the stool samples from children without diarrhoea. The most commonly-isolated organisms from all samples were ETEC, EAEC, *Shigella*, *C. jejuni*, *Giardia*, and rotavirus. No EHEC or EIEC were isolated.

Age-specific isolation rates of diarrhoeagenic *E. coli* are shown for ETEC and EAEC in Table 2 and for other agents in Table 3. Aetiology-specific attack rates were dependent on age. Of the 1,748 samples (1,351 diarrhoeal and 355 non-diarrhoeal) tested for diarrhoeagenic *E. coli*, 16% were positive for ETEC. Of the ETEC isolates, 88.4% (244/276) were stool samples from children with diarrhoea. Rates of isolation of diarrhoeal ETEC rose with increasing age, peaked in the 12-17-month age-group, and then declined (Table 2). For ETEC isolated from stool samples from children without diarrhoea, there was an increase in isolation after 0-5 month(s) of age; the rates then remained relatively constant (ranging from 10.6 to 11.7 of the stool samples from children without diarrhoea).

Among the ETEC isolates from the diarrhoea patients, the age-related patterns of the toxin types differed. Although LT exhibited a similar trend as other pathogens, with a peak rate among those in the 6-11-month age-group (8.8%), rates of isolation of ST peaked in the oldest age-group (8% of stool samples from children with diarrhoea tested).

The highest rates of isolation of rotavirus and diarrhoeagenic *E. coli*, occurred in the 6-11-month age-group of diarrhoea patients. Rates of isolation of *Shigella* among stool specimens increased with increasing age of diarrhoea patients. Rates of isolation of *Giardia* were seen to rise in the 6-11-month age-group and continued to increase in stool samples from both diarrhoea and non-diarrhoea patients with increasing age. Enterotoxigenic *B. fragilis* were more frequently found in *B. fragilis*-positive children aged over one year. Details

Table 2. Age-specific isolation rates of enterotoxigenic and enteroadherent *E. coli* from children, aged 0-2 year(s), with diarrhoea and without diarrhoea in Mirzapur, Bangladesh

Enteropathogen	Age-group								
	0-5 month(s)		6-11 months		12-17 months		18-23 months		0-23 month(s)		p value*
	Diarrhoea (%) (n=200)	Non-diarrhoea (%) (n=109)	Diarrhoea (%) (n=432)	Non-diarrhoea (%) (n=77)	Diarrhoea (%) (n=420)	Non-diarrhoea (%) (n=84)	Diarrhoea (%) (n=299)	Non-diarrhoea (%) (n=85)	Diarrhoea (%) (n=1,351)	Non-diarrhoea (%) (n=355)	
ETEC (all)†	14.0	4.5	16.2	11.7	21.7	10.7	18.4	10.6	18.0	9.0	<0.0001
LT only	4.0	2.8	7.6	6.5	8.8	7.1	5.7	7.1	7.0	5.6	0.35
ST only	5.5	0.0	5.1	2.6	6.7	1.2	8.0	3.5	6.3	1.7	0.0006
LT/ST	4.5	1.8	3.5	2.6	6.2	2.4	4.7	0.0	4.7	1.7	0.01
EAEC (all)‡	33.5	27.5	37.5	39.0	25.5	32.1	17.4	11.8	28.7	27.6	0.68
DA	9.0	9.1	8.6	6.5	5.7	4.8	4.3	2.4	6.8	5.9	0.55
AGGA	14.5	10.1	12.3	13.0	7.6	9.5	5.4	4.7	9.6	9.6	0.98
LA	9.0	3.7	6.3	7.8	4.8	7.1	3.0	3.5	4.6	4.8	0.87
AE	7.0	4.6	10.4	11.7	7.4	10.7	4.7	3.5	7.7	7.3	0.81

*Refers to chi-square-based p value of the difference between rates of isolation from stool samples from children, aged 0-23 month(s), with and without diarrhoea
†ETEC=Enterotoxigenic *E. coli*; LT=Heat-labile toxin; ST=Heat-stable toxin
‡EAEC: DA=Diffuse adherence; AGGA=Aggregative pattern; LA=Localized adherence; AE=Attaching and effacing adherence
E. coli=Escherichia coli

on enterotoxigenic *B. fragilis* are published elsewhere (7).

The high rate of isolation of *C. jejuni* from stool samples from diarrhoea patients of the 0-5-month age-group was significantly higher than the rate of isolation from stool samples from children of this age-group without diarrhoea (9.7% vs 3.9%; p<0.001). The rates of isolation of *C. jejuni* from stool specimens from diarrhoea patients in subsequent age-groups steadily decreased and were not significantly different. Asymptomatic carriage of the organism continued throughout the first two years of life.

To assess the pathogenicity of certain organisms, rates of their isolation in diarrhoeal episodes (excluding repeat specimens taken during a child's episode) were compared with rates of their isolation in stool specimens from children without diarrhoea. *Shigella*, rotavirus, *Salmonella*, enterotoxigenic *B. fragilis*, and ETEC were highly pathogenic. Among the ETEC specimens, however, ST only and LT/ST isolates exhibited pathogenicity; LT had not significantly different distribution among stool specimens of diarrhoea and non-diarrhoea patients. EAEC, *C. jejuni*, and *Giardia* were not found to have significantly different rates of isolation in diarrhoea cases versus non-diarrhoea patients.

The season-specific isolation rates of these organisms are presented in Figure 1. The rates of isolation of ETEC from stool specimens of diarrhoea patients peaked during the hot, dry spring months of March and April and again in the hot, wet summer months of June and July. The rates of isolation during the spring and summer were not significantly different from each other, but were significantly higher than the ETEC rate in the winter (p<0.001 for spring vs winter; p<0.001 for summer vs winter). *C. jejuni*, *Shigella*, and rotavirus were also isolated year-round. While *C. jejuni* and *Shigella* showed no distinct seasonal pattern, rotavirus-associated diarrhoea was seen more often in the winter months of October to February; the rate during this season was significantly higher than the rates of the summer and spring seasons (p<0.001 for winter vs spring; p<0.001 for winter vs summer).

From the household surveillance that enumerated the numbers of acute and persistent episodes, it was calculated that 5.8% of the 1,916 episodes lasted for 14 days or longer (3). Of the 1,647

Table 3. Age-specific isolation rates of major enteropathogens among diarrhoeal stool and non-diarrhoeal samples in children aged 0-2 year(s) in Mirzapur, Bangladesh

Potential aetiological agent	Age-group										p value*
	0-5 month(s)		6-11 months		12-17 months		18-23 months		0-23 month(s)		
	Diarrhoea (%) (n=300)	Non-diarrhoea (%) (n=1,429)	Diarrhoea (%) (n=532)	Non-diarrhoea (%) (n=1,382)	Diarrhoea (%) (n=520)	Non-diarrhoea (%) (n=1,405)	Diarrhoea (%) (n=396)	Non-diarrhoea (%) (n=1,463)	Diarrhoea (%) (n=1,748)	Non-diarrhoea (%) (n=5,679)	
Shigella spp.	1.3	0.1	7.5	0.2	7.5	0.2	12.3	0.2	7.7	0.1	<0.001
Campylobacter jejuni	9.7	3.9	7.5	11.7	5.2	6.9	3.3	4.4	6.2	6.7	0.50
Salmonella spp.	1.7	0.1	1.1	0.7	1.0	0.4	1.3	0.4	1.8	0.2	<0.001
Rotavirus	1.7	0.7	7.9	1.4	6.0	0.7	2.8	0.9	5.1	0.9	<0.001
Giardia	2.7	2.6	9.2	7.3	16.5	16.9	22.2	25.1	13.2	13.1	0.91
None	51.0	9.17	40.5	77.1	41.7	74.6	41.4	69.8	42.0	78.4	<0.001
Diarrhoeagenic *E. coli*†	(n=200)	(n=109)	(n=432)	(n=77)	(n=420)	(n=84)	(n=299)	(n=85)	(n=1,351)	(n=355)	<0.001
	47.5	32.1	53.7	50.6	47.1	42.9	35.8	22.4	46.8	36.6	
ETBF‡	(n=18)	(n=25)	(n=49)	(n=38)	(n=57)	(n=44)	(n=73)	(n=78)	(n=197)	(n=185)	<0.001
	16.7	4.0	10.2	7.9	28.1	15.9	21.9	5.1	20.3	8.1	

*Refers to chi-square-based p value of the difference between rates of isolation from stool samples from children, aged 0-23 month(s), with and without diarrhoea
†Enterotoxigenic *E. coli*, enteropathogenic *E. coli*, enteroadherent *E. coli* (diffuse, aggregative, localized, attaching and effacing)
‡Enterotoxigenic *Bacteroides fragilis* isolation rates from *B. fragilis*-positive samples
E. coli=Escherichia coli

episodes from which specimens were collected, 103 (6.3%) were persistent (Table 4).

Fig. 1. Monthly isolation rates of 4 major aetiological agents from stool specimens of children with diarrhoea, aged 0-2 year(s), in Mirzapur, Bangladesh, September 1993–October 1996

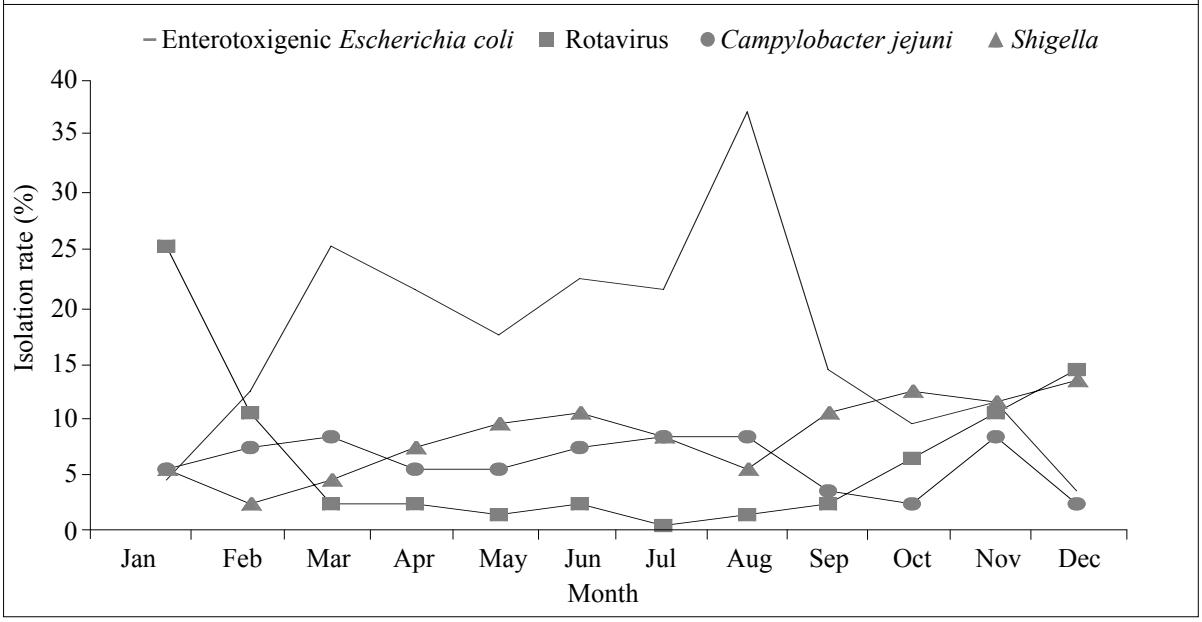

Table 4. Association of selected enteric agents with acute vs persistent episodes of diarrhoea in children aged 0-2 year(s) in Mirzapur, Bangladesh

Enteropathogen	Total no. of episodes	Acute episodes (n=1,544)		Persistent episodes (n=103)		p value*
		No.	%	No.	%	
Enterotoxigenic *E. coli*	233	223	14.4	10	9.7	0.18
Campylobacter jejuni	109	106	6.9	3	2.9	0.12
Shigella spp.	127	122	7.9	5	4.9	0.26
Rotavirus	86	84	5.4	2	1.9	0.12
E. coli with localized adherence	58	57	3.7	1	1.0	0.15
E. coli with diffuse adherence	86	78	5.1	8	7.8	0.23
E. coli with aggregative pattern	128	116	7.5	12	11.7	0.13
E. coli with attaching and effacing adherence	95	93	6.0	2	1.9	0.09

*Based on chi-square test
E. coli=Escherichia coli

In total, 141 rotavirus-associated infections were recorded. Of 111 children with rotavirus, 86 (77.7%) had only one rotavirus-associated infection. Of the remaining 25 children who had 2-3 rotavirus-associated infections each, 18 (72%) had an initial episode, followed by asymptomatic infection(s).

DISCUSSION

The main aetiological agents found in Mirzapur were essentially the same as those found in most developing countries (14) and in a birth-cohort study conducted in an Apache population in the USA (15). In addition to accomplishing more complete testing for established pathogens, this study had the unique capability of identifying the burdens of both *E. coli* that exhibit enteroadherent properties and enterotoxigenic *B. fragilis* in a rural setting.

Regarding the pathogenicity of organisms, results from Mirzapur were in agreement with those from a 1992 community study in Matlab, Bangladesh (16) which had found that, when comparing diarrhoeal cases with age-matched healthy controls, cases of diarrhoea

were associated with *Shigella* species (p=0.07) and rotavirus (p<0.05). In Mirzapur, rotavirus, *Shigella*, and ETEC-ST and ETEC-LT/ST were detected more often in stool samples from children with diarrhoea than those from asymptomatic children, results of which also correspond with findings of a longitudinal study in Brazil (17) and a hospital case-control study in Dhaka, Bangladesh (18), where ETEC, rotavirus, and *Shigella* were isolated more from diarrhoeal cases than from controls.

In a two-year prospective study of children, aged less than five years, in Argentina, the detection of rotavirus and ETEC was of greater magnitude in diarrhoeal episodes than in stool samples from asymptomatic children (19). Among the ETEC strains studied, ST was more frequently associated with diarrhoea, a pattern that was not seen for ETEC-LT. In a more comprehensive study in a periurban area of Egypt, where 242 children aged less than three years were prospectively followed (20), ST-only ETEC and not LT-only ETEC were associated with diarrhoeal symptoms. A prospective study in rural Mexico that followed children for the first two years of life (21) reported rates of ETEC by semester of life. It was found that, for infants aged 0-6 month(s), only ETEC strains producing ST-only or LT and ST were isolated more frequently from those with diarrhoea than from asymptomatic age- and sex-matched controls. For the subsequent three semesters of life, only ST/LT-producing ETEC strains were found with significantly higher frequency in children with diarrhoea than in controls. Among the ETEC found in an urban hospital case-control study in Bangladesh, Albert *et al*. reported that ST-only-producing and LT/ST-producing strains were associated with diarrhoea (22). It appears from controlled and longitudinal studies, including ours, that, although LT-only ETEC is substantially detected in very young children, it is not statistically significant with diarrhoea in infants.

As expected, ETEC were the most frequently-isolated organisms. An interesting finding in this study was that, in addition to the expected summer peak of ETEC (23, 24), there also existed a peak in the spring season. An unexpected finding in this study was the low rate of isolation of rotavirus (2%). A three-year birth-cohort study in Pakistan (25) reported 20% overall frequency of rotavirus which was less than that found in developed and some developing countries, where rates of isolation ranging from 30% to 47% had been reported. In a hospital study in Bangladesh, the rate of isolation of rotavirus among children during the first year of life was 28.7% in diarrhoea patients (26). A controlled, randomized study in ICDDR,B to determine the efficacy of bismuth subsalicylate in treating diarrhoea reported that rotavirus was found in 56% of 451 hospitalized children aged 4-36 months (27). In most studies, rates of isolation of rotavirus peaked in the first year of life and then declined, suggesting acquired immunity to re-infection. Although the overall occurrence of rotavirus was low in our study, the peak isolation rate (regardless of diarrhoeal status) was in the 6-11-month age-group and, additionally, it was found that multiple episodes per child were rarely observed. Rotavirus was seen to peak in the winter months of October through February, but was also isolated from diarrhoeal stools during the spring season.

A strength of this study was its ability to explore aspects of *E. coli* that exhibit enteroadherent properties, which have not been as well described as other *E. coli* strains. Results of two controlled studies among Bangladeshi children showed that neither diffusely-adherent nor enteroaggregative *E. coli* were associated with diarrhoea (18,22). In our study, this was also true. The pathogenic potential of these strains may be dependent on multiple and diverse serotypes, virulence factors, and plasmid profiles. For example, Giron *et al*. from Mexico suggested that many different diffusely-adherent *E. coli* clones exist that exhibit the same phenotype in tissue culture-binding assays, but could have varying pathogenic profiles (28).

For diarrhoea-associated EAEC, it has been postulated to be associated with persistent diarrhoea (i.e. lasting for 2 weeks or longer), as histopathologic lesions in the small intestine caused by this strain could account for prolonged illness. For example, Baqui *et al*., in a longitudinal study in the late 1980s, found that diffusely-adherent *E. coli* was significantly associated with persistent diarrhoea (16), and studies in rural and urban Bangladesh showed that the aggregative *E. coli* were found more often in episodes that persisted (29,30).

A study examining the relationship between this class of *E. coli* in acute versus persistent diarrhoea in rural Indian children found that, in 90% of infected cases, EAEC was the sole pathogen identified (31). Strains exhibiting localized adherence occurred more often in acute diarrhoeal cases than in controls, strains with a diffuse-adherence pattern were found with similar frequency in acute cases, persistent cases, and controls, and strains showing the aggregative pattern were found significantly more often in cases with persistent diarrhoea than in children with acute diarrhoea. Furthermore, regression analysis controlling for key confounders showed that EAEC was significantly associated with persistent, not acute, diarrhoea (odds ratio 2.2, p<0.05).

In Mirzapur, diarrhoea-associated *E. coli* strains showing localized adherent were detected more in acute epi-

sodes than in persistent episodes. Strains exhibiting diffuse adherence and aggregative patterns were detected more in persistent episodes; however, the differences in rates of isolation of these organisms among acute and persistent episodes were not statistically significant. As noted earlier, the rate of persistent diarrhoea in this cohort was relatively low (6% of all episodes). The Indian study included 240 diarrhoeal episodes, 61 (25%) of which were persistent and all of which were tested for Hep-2 cell-adherent *E. coli* (31). Although sampling for diarrhoeagenic *E. coli* testing in Mirzapur was not differential for those episodes that eventually lasted for more than 14 days and those that ended earlier in this study, almost one-quarter of all diarrhoeal isolates were not at all tested for diarrhoeagenic *E. coli*. Thus, the difference between findings from the Indian study and those from this study could potentially be due to the smaller number of total persistent cases studied. However, in a study in Peru, Lanata *et al*. found that, after controlling for age, season, anthropometric status, and antibiotic use, no specific enteropathogens were highly associated with persistent diarrhoea (32). Our results support the findings that frequent re-infections (16) and co-infections with the same organisms that produce acute diarrhoea, combined with host factors (i.e. nutritional status), may be more important than specific *E. coli* in producing prolonged diarrhoeal episodes.

There is a lack of information on antimicrobial use in this population. The predominant infections in these children are acute respiratory infections (ARI) (K.Z. Hasan. Unpublished observations). For certain ARI and diarrhoeal episodes, children would have received antibiotic treatment from the study personnel. Additionally, it is not uncommon for inhabitants of these villages to purchase antibiotics 'over-the-counter' at local shops, without a diagnosis or prescription. The indiscriminate use of antibiotics is probably widespread and may have affected rates of isolation of some bacteria.

The findings of this study reflect the problem of diarrhoeal diseases at the community level, which has implications for health planning. We present age-specific peaks of infection, which would help plan optimal strategies for vaccine administration. For example, a candidate rotavirus vaccine should be given in the first six months of life, a vaccine against ETEC may be given in the first year of life, and *Shigella* vaccine could be administered anytime in the first two years of life.

In summary, the enteropathogens isolated from children in Mirzapur, Bangladesh, were essentially the same as those found in other tropical rural settings. *Shigella*, rotavirus, *Salmonella*, and ETEC were highly pathogenic, and enterotoxigenic *B. fragilis* was identified as a significant enteropathogen contributing to diarrhoea in this community. Ongoing vaccine efforts and use of oral rehydration therapy remain important for reducing the burden of childhood acute diarrhoea in rural Bangladesh.

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