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Rotavirus-Associated Diarrhea in Rural Bangladesh: Two-Year Study of Incidence and Serotype Distribution

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Stools were evaluated from 5,811 patient visits for treatment of diarrhea in Matlab, Bangladesh, between June 1987 and May 1989. The stools were analyzed to determine the distribution of serotypes of group A rotaviruses (RV). A total of 898 stool samples (15.5%) contained RV, as determined by using an enzyme-linked immunosorbent assay. RV isolates from 855 of these samples were serotyped by using serotype-specific synthetic oligonucleotide probes. A total of 558 (65.3%) could be assigned to specific serotypes: 166 (19.4%), 228 (26.7%), 39 (4.6%), and 125 (14.6%) belonged to serotypes 1 through 4, respectively; 12 (1.4%) hybridized with more than one serotype; and 285 (33.3%) failed to hybridize. RV diarrhea was evident throughout the year, with peaks in the dry winter months and in September 1988, coinciding with a major flood. RV was isolated from 46.6% of patients between 7 and 12 months old. Among children under 24 months of age with RV diarrhea, 1.2% (10 of 828) died. The corresponding percentage for children with diarrhea from all causes is 0.9% (29 of 3,301).

Epidemiological studies on the distribution and relative frequency of the major group A rotavirus (RV) serotypes highlight regional differences and record changes with time in a given population. Understanding the dynamics of RV serotype changes in a population over time may help in the assessment of vaccine failures and successes, since heterologous protection is not easily predicted. It may also assist in the selection of appropriate serotypes for testing in the population concerned.

Four different serotypes have been reported for human group A RV, and recently a fifth and a sixth serotype were proposed; however, their epidemiologic significance is unknown (2, 17). Serotyping of RV was previously limited, because it required laborious procedures of isolation and cross-neutralization in cell culture. The use of serotype-specific monoclonal antibodies (1, 11, 19, 21) and the construction of synthetic oligonucleotide probes (18) have made it possible to serotype large numbers of RV strains with relative ease and rapidity and have helped generate important epidemiological information on RV serotypes (5, 14, 20).

Serotype-specific synthetic oligonucleotide probes can be used to serotype viruses by hybridization with RNA segments. Recent studies have suggested that this method is more sensitive than monoclonal antibody assays because double-stranded RNA segments may be more resistant than the serotype-specific protein VP7 to degradation (18). The technique can be applied to a large number of specimens and is extremely rapid, sensitive, specific, and useful for analysis of RV in epidemiologic studies (18).

In this study we examined the current contribution of RV to the total incidence of diarrhea in the Matlab area of Bangladesh, as compared with the incidence a decade earlier (4), and the relative frequencies of the serotypes encountered.

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MATERIALS AND METHODS

Population studied. All data and specimens in the study were derived from routine diarrheal surveillance approved by the human subject committee of the International Center for Diarrheal Disease Research. Stool specimens evaluated for RV were collected from 5,811 of 7,568 patients seeking treatment for acute diarrhea between June 1987 and May 1989 in all three diarrhea treatment centers in the Matlab area as part of the passive surveillance of a cholera vaccine trial carried out in 1985 (6–9, 10). The total population at that time was approximately 190,000. With a crude birth rate of approximately 40 per 1,000 per year, some 15,000 births took place over the study period.

Stools were collected on visits to the Matlab hospital or to one of two neighboring diarrhea treatment centers; the diarrhea samples were categorized as follows: (i) watery but not bloody, (ii) loose but not watery or bloody, or (iii) bloody and either loose or watery (7). Diarrhea itself was defined as the passage of three or more loose or liquid stools in any 24-h period during the illness leading to presentation for care. Classification was made on the basis of patient histories, not on the basis of direct inspection of stools. Stools were submitted for bacterial culture, and samples were stored at −20°C for detection of enteric viruses.

Bacterial culture. Stools were cultured for vibrios, salmonellae, and shigellae by standard methods described elsewhere (23).

Detection of group A RV. Stool suspensions were prepared as 10% (wt/vol) phosphate-buffered saline (pH 7.2) extracts and tested for RV by using a commercial kit (DAKOPATTS) incorporating polyclonal antibodies to group A RV.

Serotyping by RNA hybridization. The double-stranded RNA samples extracted from stools (15) were tested for hybridization with synthetic oligonucleotides constructed from the nucleotide sequences of seven separate VP7 segments representing five human group A RV serotypes (serotypes 1 through 4 and 8) and two of animal origin (serotypes 3 and 6) (18). The synthetic oligonucleotides and the meth-

TABLE 1. Occurrence of group A RV in stools from patients with diarrhea

Patient age (months)	No. of samples (%)		
	All cases of diarrhea[a]	RV positive	Cumulative RV positive
0–6	423 (7.3)	110 (12.3)	110 (12.2)
7–12	1,080 (18.6)	418 (46.5)	528 (58.8)
13 24	1,077 (18.5)	300 (33.4)	828 (92.2)
25–60	861 (14.8)	47 (5.2)	875 (97.4)
61–120	419 (7.2)	2 (0.2)	877 (97.7)
≥121	1,951 (33.6)	21 (2.3)	898 (100.0)

[a] All cases of diarrhea for which stool samples were obtained.

odology were kindly provided by P. Echeverria (AFRIMS, Bangkok).

Hybridization was performed as previously described by Sethabutra et al. (18), with modification of the hybridization temperatures to 46, 41, 41, 46, 46, 46, and 49°C. Cell culture supernatants containing RV strains RV4 (serotype 1), RV5 (serotype 2), RV3 (serotype 3), ST3 (serotype 4), and B37 (serotype 8) were included as positive controls in the respective hybridization reactions.

The probes failed to hybridize with extracts from *Shigella* spp., *Escherichia coli* isolates from five different diarrheagenic categories, *Giardia* spp., and *Entamoeba histolytica*.

PAGE. Specimens that reacted with more than one probe or did not hybridize with any of the probes were analyzed by polyacrylamide gel electrophoresis (PAGE) to determine their electropherotype. Extracted double-stranded RNAs were electrophoresed in 0.75-mm-thick 10% polyacrylamide gels for 18 h at 10 mA in the absence of sodium dodecyl sulfate (16) and then fixed and stained with silver (13).

RESULTS

The number of patient visits for treatment at the three diarrhea treatment centers during the 2-year period in which stools were tested for RV was 5,811. This was fairly evenly divided between year 1 (2,852) and year 2 (2,959). The ages of patients with acute diarrhea ranged from 9 days to 86 years; the oldest patient shedding RV was 60 years of age.

Frequency of RV diarrhea. Of a total of 5,811 stool samples evaluated for RV during the 2-year period either at the Matlab Hospital (4,599) or in one of two nearby diarrhea treatment centers (1,212), 898 (15.5%) reacted in the group A RV enzyme-linked immunosorbent assay (ELISA). Among the patient visits, 710 (15.4%) from Matlab hospital and 188 (15.5%) from the two diarrhea treatment centers were associated with shedding of RV in stools. The age distribution for individuals who were shedding RV is given in Table 1; 60.1% of the patients were male and 39.9% were female.

The monthly distribution of diarrhea (at the time of presentation) attributed to RV, in relation to the total number of diarrheal patient visits evaluated for RV over the 2-year period, is given in Fig. 1. There were slight increases in the occurrence of RV diarrhea during the dry winter months of December through February in both years. A major peak was recorded in September 1988, coinciding with a major flood in Bangladesh that affected the Matlab area. The occurrence of RV diarrhea, however, declined immediately after the flood, whereas diarrhea attributed to other causes remained high.

Temporal distribution of RV serotypes. A total of 570 (66.7%) of 855 RV strains that were evaluated for serotype hybridized with at least one of the RV serotype-specific synthetic probes; 166 (19.4%) reacted with serotype 1, 228 (26.7%) reacted with serotype 2, 39 (4.6%) reacted with serotype 3, and 125 (14.6%) reacted with serotype 4. Throughout the study period all four major serotypes were present concurrently (Fig. 2). There was a predominance of serotype 1 from September 1987 to March 1988, whereas serotype 2 was detected at a high frequency from June 1988 until February 1989. Serotype 3 was found in low numbers (4.6%) throughout the study period, and serotype 4 showed slight peaks from October to December 1987 and in May and June of 1988.

Twelve specimens (1.4%) hybridized with more than one probe, so they were subjected to PAGE. Two contained

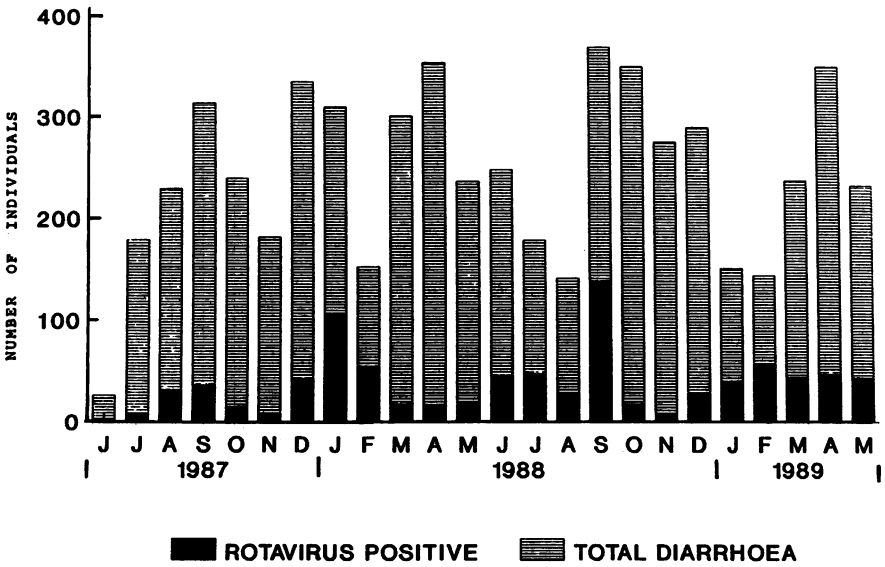

FIG. 1. Monthly distribution of detection of RV from stool specimens as a proportion of the total number of diarrheal stools in the population over the 2-year study period.

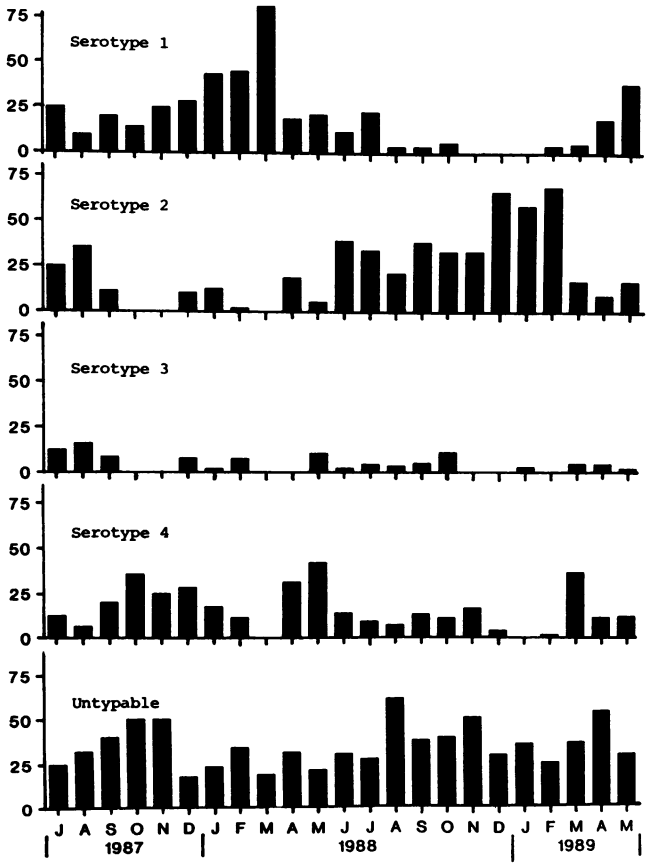

FIG. 2. Relative monthly frequency of the four RV serotypes and nontypable strains over the 2-year study period.

more than 11 bands, and eight had only 11 bands; in two specimens no pattern could be detected.

A total of 285 (33.3%) of the strains failed to hybridize with any of the seven synthetic oligonucleotide probes; their distribution throughout the study period was reasonably even (Fig. 2). Of the strains that did not hybridize, 232 (81.4%) were negative by PAGE. In 53 (18.6%) strains, an identifiable electropherotype was observed; among these, 19 had a short pattern suggesting serotype 2. The electropherotype-positive, hybridization-negative strains were evenly distributed throughout the study period.

During the study period it was noted that more than one RV serotype was often isolated from different persons with diarrhea residing in the same village at the same time (data not shown).

Types of diarrhea. Of the 898 patient visits associated with RV, 845 were for treatment of watery diarrhea and 14 were for treatment of nonwatery, nonbloody diarrhea. Among stool samples from the 39 visits by patients reporting bloody diarrhea, 20 contained other pathogens; of these, 13 contained *Shigella* spp.

Table 2 shows the occurrence of diarrhea with excretion of RV in relation to enteric bacteria in the total study population over the 2-year period, as well as the number of visits among children under the age of 2 years. Table 2 also shows the numbers of in-hospital deaths, the numbers of stools in which no pathogen was identified, and the numbers of stools with RV that also contained other pathogens.

DISCUSSION

In this report we describe the occurrence of RV diarrhea and the relative monthly frequencies of the four major RV serotypes in 5,811 stool samples from a total of 7,568 samples taken over a 2-year period from patients with diarrhea at the Matlab diarrhea treatment centers. The number of patient visits per month fluctuated markedly over the 2-year period. There was no consistent annual pattern, although there were regular waves of higher numbers of visits around September and April in both years (Fig. 1). Although the numbers of visits in which stool was evaluated for RV were similar in years 1 and 2 (2,959 and 2,852, respectively), RV was isolated from more samples in year 1 (543 isolates) than in year 2 (355 isolates). Infants aged between 7 and 12 months accounted for 46.5% of RV isolates; isolation of RV was less common in younger and older age groups. The proportion of RV-positive stools for children below 24 months of age was 32%, which was lower than the 46% reported in the same area by Black et al. (4) a decade earlier. The number of patients with diarrhea and the number shedding RV among them were the highest in September 1988, when a major flood occurred. However, although the number of diarrhea patients remained high until the end of December, the cases associated with RV sharply declined by October. The reason for this remains unclear.

Although the relative frequencies of RV serotypes fluctuated markedly, all four major serotypes were represented during the study period. Serotype 1, which predominated in the winter months of 1987 and 1988, was almost absent during the following winter, when serotype 2 was the predominant type. Strains of serotype 2, which are considered to be epidemic strains in developed and developing countries (3), were predominant from June 1988 to February 1989 but were never absent for longer than 2 months during the study. Serotype 4 was endemic throughout the study period, with increased frequencies in the autumn of 1987 and spring of 1988. Over the study period serotype 2 predominated, ac-

TABLE 2. Number of cases of diarrhea and in-hospital deaths associated with RV and enteric bacteria[a]

Patient group	n[b]	No. of patient visits (%) with the following pathogen:			No. of deaths	Case fatality rate (per 100)
		RV	Other[c]	No pathogen		
All patient visits	5,811	898 (15.5)	1,936 (33.3)	2,977 (51.2)	32	0.6
Children under 2 yrs old	2,580	828 (32.1)	462 (17.9)	1,290 (50.0)	24	0.9
Patients with RV in stool	898	839[d] (93.4)	59 (6.6)	NA[e]	10	1.1

[a] Data are from patient visits in which stool specimens were obtained over a 2-year period (June 1987 through May 1989) in the Matlab area.
[b] Number of stool specimens evaluated for RV.
[c] Other pathogens under surveillance included *Shigella* spp., *Salmonella* spp., and *Vibrio cholerae*.
[d] Number of patient visits in which no additional pathogen was detected.
[e] NA, not applicable.

counting for 40% of the typable strains, followed by serotypes 1 (29%), 4 (21.9%), and 3 (6.8%). The relative frequencies reported in Japan for 1987 through 1988 were 31.5, 34.1, 2.3, and 4.9%, respectively, for serotypes 1 through 4 (22). The 285 strains (33.3%) that failed to hybridize with the eight probes were reasonably evenly distributed (Fig. 2) throughout the study period. The proportion of strains serotyped (66.7%) by this method was marginally lower than the 70% or so achieved with the ELISA serospecific monoclonal antibody technique (21, 22). The majority of the nontypable strains in this study did not exhibit an electropherotype; those that did show patterns were similar to typed strains and therefore are not thought to be new or unidentified serotypes. Presumably the lack of sufficient double-stranded RNA was the reason for our failure to serotype the strains with hybridization or PAGE, although clearly there was sufficient viral protein for detection with the ELISA.

In more than half of the patient visits in which stool specimens were produced, no enteric pathogen was identified. Identification of diarrheagenic *E. coli*, which was not included in this study, might have decreased the percentage with no identifiable pathogen by 30 to 35%. In previous studies conducted in the same area (4, 9), enterotoxigenic *E. coli* was isolated from approximately 25% of stool samples from children with diarrhea. In another study in Bangladesh, 10.9% of diarrheal episodes in children under 12 months of age were associated with enteropathogenic *E. coli* (unpublished data).

Considering the total number of individuals in the surveillance area and all individuals presenting with diarrhea, results suggest a rate of approximately 20 visits per 1,000 villagers per year for treatment at one of the three diarrhea treatment centers. In contrast, there were approximately 124 visits per 1,000 children below 24 months old per year. Thirty-two percent of stool specimens were associated with RV, which is well within the range of 10 to 49% proposed by De Zoysa and Feachem (12) for children seeking treatment in developing countries.

The number of in-hospital deaths among persons yielding stool specimens over the study period was 32 (0.6% of the patients); 24 of the children who died were younger than 24 months old. All 10 of the deaths associated with RV occurred in children below 24 months old; for this age group, 42% (10 of 24) of all in-hospital deaths in children producing diarrheal stool specimens and 34% (10 of 29) of all in-hospital deaths were associated with RV. Our RV-associated case fatality figure of 0.17% of the total visits for treatment of diarrhea (10 of 5,811) is of the same order as that (0.2% [5 of 2,112]) reported by Black et al. (4) a decade earlier. The RV case fatality rate in the Matlab area appears to be relatively low compared with those in other developing countries, presumably because of readily available and accessible diarrhea treatment centers and the high level of health care utilization by this population. Hence it may not truly reflect the mortality rate that should be attributed to RV in developing countries.

Our study shows that the pattern of occurrence of RV diarrhea and the coexistence of the four major RV serotypes, which was observed in other countries (22), should be taken into account when formulating an effective vaccine. Since Bangladesh is unlike other geographic regions in which RV diarrhea shows strong peaks during the cold winter months, which may help in the formulation of vaccination schedules to coincide with peak incidence, it is clear that protection in children in Bangladesh will be required throughout the year.

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