Bacterial, Viral and Parasitic Aetiology of Paediatric Diarrhoea in the Highlands of Papua New Guinea

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Summary

Enteropathogens and clinical features associated with diarrhoea were investigated in 1526 children admitted over a 5-year period to the paediatric ward of a hospital in the highlands of Papua New Guinea. Overall, a recognized pathogen was isolated from 39 per cent of the children admitted with diarrhoea. The most commonly isolated agents were rotavirus (23 per cent), *Shigella* spp. (13 per cent), *Campylobacter* spp. (12 per cent), *Cryptosporidium parvum* (10 per cent) and enteropathogenic *Escherichia coli* (8 per cent). The clearest clinical associations were rotavirus with vomiting, and *Shigella* with blood and pus in the stool. A control series of children admitted with other complaints was also included, and the odds ratios for diarrhoea for the above five pathogens were 18.2, 9.6, 3.7, 2.2, and 1.6, respectively.

Introduction

Diarrhoeal diseases are a major cause of morbidity and mortality in developing countries.[1] In Papua New Guinea diarrhoeal diseases in children under 5 years of age are one of the greatest causes of hospital admissions and deaths. For the former they rank after pneumonia and, in some areas, also malaria; for the latter after pneumonia and, in some areas, also malaria and meningitis.[2,3] This study was carried out to determine the pathogens in such cases, and so help to assess treatment options.

Materials and Methods

Study population and clinical examination

Between October 1985 and March 1990, 1526 children admitted to the paediatric ward of Goroka Base Hospital with a diagnosis of diarrhoea were included in this study; of these, 1414 were under the age of 3 years. A total of 1170 children (1108 under 3 years) who were admitted with other conditions were enrolled as controls. Goroka is at an altitude of 1580 m and is the capital of the Eastern Highlands Province of Papua New Guinea. Its hospital serves the surrounding rural and peri-urban areas, as well as the urban population of approximately 25 000. Demographic and clinical variables were recorded on admission by staff of the Papua New Guinea Institute of Medical Research using standard forms. Mothers of the diarrhoea cases were asked for the duration of the illness, number of stools per 24 h, whether there had been vomiting, and whether there had been blood or pus in the stool. Dehydration was graded as absent, mild, moderate or severe using skin turgor of the abdomen, and also moistness of the mouth and sunkenness of the fontanelle if possible. Fever was defined as a temperature of 37.5°C or more. Marasmus was assessed by skin or hair discoloration, and weight for age. Ethical approval for the study was given by the Papua New Guinea Medical Research Advisory Committee.

Laboratory diagnosis

Bacteriology. A fresh stool sample or two rectal swabs in Cary–Blair transport medium were transported to the laboratory within 1 h after collection where a range of bacteriological media was inoculated. Non-lactose fermenters such as *Salmonella* and *Shigella* species were selected from MacConkey agar (Oxoid), desoxycholate citrate agar (DCA) (Oxoid) and Hektoen Enteric agar (Oxoid) after overnight incubation at 37°C. A portion of the stool sample or one of the rectal swabs was incubated in selenite selective broth (Oxoid) at 37°C overnight prior to plating out on MacConkey agar, DCA and Hektoen Enteric agar. *Campylobacter* species were cultured on Columbia agar (Oxoid) containing 5% lysed defibrinated horse blood (Gibco) and *Campylobacter* selective supplements (Oxoid). The plates were incubated in an anaerobic jar containing a Campypak gas generator (BBL) and examined after 3 days' incubation at 44°C for characteristic growth. Bacterial isolates were

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identified according to biochemical and serological procedures recommended by the World Health Organization.[4] To detect enteropathogenic *Escherichia coli* (EPEC) four lactose fermenting colonies from each stool or rectal swab was tested for slide agglutination using commercially available EPEC antisera (Wellcome).

Rotavirus. Twenty per cent suspensions of faeces in phosphate-buffered saline (PBS 0.15 M NaCl in 0.01 M phosphate, pH 7.4) were made, thoroughly mixed using a vortex mixer and clarified by low-speed centrifugation. This crude preparation was analysed for the presence of rotavirus by enzyme-linked immunosorbent assay (ELISA) and by polyacrylamide gel electrophoresis (PAGE) of extracted viral nucleic acids. The ELISA test was modified from the antibody capture method of Beards *et al*.[5] Rotavirus antigen was detected using a rotavirus group-specific monoclonal antibody (A3M4) and the conjugate used was alkaline-phosphatase conjugated with anti-mouse antibody. At the completion of the substrate reaction optical densities were read using a TiterTek MultiSkan II reader at 405 nm. The screening test was considered positive when the mean optical density of the two test wells was greater than 0.1 optical density units. The wells containing substrate only were used as plate blanks to set the plate zero level. No samples were reported positive until they had been tested in duplicate in a confirmatory blocking assay. Preparation of nucleic acids for PAGE was carried out according to a modification of a method of Herring *et al*.[6] Nucleic acid extraction was completed by the precipitation of RNA with ethanol. The RNA was pelleted, dried under vacuum, and resuspended in 30–40 ml of electrophoresis sample buffer and this was electrophoresed in a modification of the discontinuous system described by Laemmli.[7] Ten percent polyacrylamide, 0.75-mm thick slab gels with a 3% stacking gel were run overnight (16 h) at 2.25 M constant power. Gels were stained with silver nitrate then photographed.

Parasitology. Parasites were viewed under a microscope on a wet mount of a fresh stool sample in 0.9% saline solution and an iodine-stained preparation. Smears were also made, which were fixed in methanol, then stained for further and more precise identification. The stains were Gomori's trichrome stain for the identification of protozoa, and saffarinin and methylene blue for *Cryptosporidium*.

Statistical methods

Age-stratified odds ratios were calculated by the Mantel–Haenszel method with Cornfield confidence intervals.[8] To adjust for multiple explanatory variables, logistic regression was used.[9] Analysis was done using the S-PLUS and Epi Info packages.

Results

Of the 1526 diarrhoea cases, 909 were male and 617 female, while the ages ranged from 0 to 11 years, with a median of 11.6 months. Among the 1170 controls, the corresponding figures were: 712 male and 458 female; age range 0–10 years, median 7.0 months. The results of the laboratory tests are shown in Table 1. The pathogens most commonly isolated in the cases were rotavirus (from 23 per cent of those samples tested), *Shigella* spp. (13 per cent), *Campylobacter* spp. (12 per cent), *Cryptosporidium parvum* (10 per cent) and EPEC (8 per cent). Not all tests were done on all subjects, usually because of insufficient stool volume or lack of laboratory staff. Rotavirus was generally more common in the younger children (less than 24 months) and *Shigella* in the older ones (at least 12 months), while *Campylobacter* and *Cryptosporidium* showed a peak in the middle age group of 12–23 months. Of the 48 subjects with *Salmonella* spp., 11 had *S. typhi*, of whom eight were aged 24 months or more. No samples were positive for *Vibrio cholerae* or *Yersinia enterocolitica*, and only one for *Aeromonas hydrophilia*.

The importance of the pathogens in diarrhoeal aetiology was assessed by comparing their isolation rates between cases and controls, and calculating the odds ratio for each after stratifying by age (Table 1). This confirms rotavirus, *Shigella* spp., *Campylobacter* spp., and *Cryptosporidium parvum* as important causes of diarrhoea in this population. In addition, *Salmonella* spp. and *Entamoeba histolytica*, although less prevalent, are strongly associated with diarrhoeal disease. The results were not substantially changed if children with prior antibiotic history were excluded (not shown). The controls tended to be enrolled later in the study than the cases, their median admission date being almost 20 months later. Therefore, logistic regression was used to adjust for the seasonal pattern which diarrhoea follows in this area (M. Wyrsch *et al*., manuscript submitted), although this did not substantially affect the results (not shown).

Clinical features of the cases positive for any of five commonly isolated pathogens are shown in Table 2. Those with *Shigella* were by far the most likely to have blood or pus in their stool, being reported by the mothers of 70 and 52 per cent, respectively. *Shigella* was also the most strongly associated with fever, but not by such a large margin. Rotavirus was the most strongly associated with vomiting (73 per cent) and also with moderate or severe dehydration (91 per cent), although the latter was common in those positive for any of the five pathogens. The ranking of the pathogens was similar if only severe dehydration was considered. Children with *Campylobacter* were the most likely to be marasmic (11 per cent), while relatively few with rotavirus or *Cryptosporidium* were (2 per cent each). The median reported episode length was consistently 5 days. Admissions tended to be more frequent in the second and third quarters of the year, coinciding roughly with drier weather.

Discussion

In accordance with other hospital-based studies in developing countries,[10–14] EPEC, rotavirus and

TABLE 1
Isolation rates of the most common pathogens

	Number of positive samples/number tested (%), grouped by age in months						Odds ratio (95% CI)
	0–5	6–11	12–23	24–35	36+	Total	
Rotavirus							
cases	34/118 (29)	108/387 (28)	61/268 (23)	7/83 (8)	1/47 (2)	211/903 (23)	18.2 (10.8–31.8)
controls	9/413 (2)	4/304 (1)	2/152 (1)	2/78 (3)	1/57 (2)	18/1004 (2)	
Shigella spp.							
cases	5/134 (4)	30/469 (6)	68/366 (19)	29/108 (27)	29/90 (23)	153/1167 (13)	9.6 (4.7–22.4)
controls	0/254 (0)	2/195 (1)	2/104 (2)	2/60 (3)	2/47 (4)	8/660 (1)	
Campylobacter spp.							
cases	8/134 (6)	51/469 (11)	57/366 (16)	16/108 (15)	11/90 (12)	143/1167 (12)	3.7 (2.2–6.2)
controls	4/254 (2)	12/195 (6)	1/104 (1)	2/60 (3)	1/47 (2)	20/660 (3)	
Cryptosporidium parvum							
cases	6/81 (7)	24/244 (10)	22/161 (14)	4/49 (8)	1/26 (4)	57/561 (10)	2.2 (1.4–3.7)
controls	7/384 (2)	13/281 (5)	11/132 (8)	3/65 (5)	0/43 (0)	34/905 (4)	
EPEC							
cases	8/134 (6)	45/469 (10)	28/366 (8)	8/108 (7)	7/90 (8)	96/1167 (8)	1.6 (1.0–2.5)
controls	12/254 (5)	9/195 (5)	8/104 (8)	2/60 (3)	2/47 (4)	33/660 (5)	
Salmonella spp.							
cases	7/134 (5)	19/469 (4)	9/366 (2)	5/108 (5)	8/90 (9)	48/1167 (4)	8.8 (3.0–42.2)
controls	0/254 (0)	1/195 (1)	0/104 (0)	0/60 (0)	2/47 (4)	3/660 (0)	
Entamoeba histolytica							
cases	0/81 (0)	8/244 (3)	9/161 (6)	3/49 (6)	3/26 (12)	23/561 (4)	22.9 (3.8–536.2)
controls	0/384 (0)	0/281 (0)	1/132 (1)	0/65 (0)	0/43 (0)	1/905 (0)	
Any of the above[a]							
cases	63/179 (35)	253/608 (42)	219/477 (46)	62/144 (43)	48/112 (43)	645/1520 (42)	
controls	32/490 (7)	38/358 (11)	24/171 (14)	9/87 (10)	8/62 (13)	111/1168 (10)	
Total in study							
cases	180	610	480	144	112	1526	
controls	492	358	171	87	62	1170	

[a] Not all tests were done for all subjects.

TABLE 2
Number with clinical feature/number examined (%), for children with the most commonly isolated pathogens.[a] *Clinical features related to pathogens isolated*

	Rotavirus	*Shigella*	*Campylobacter*	*Cryptosporidium*	EPEC	All subjects
Blood in stool	22/211 (10)	107/153 (70)	30/142 (21)	11/57 (19)	21/95 (22)	342/1512 (23)
Pus in stool	32/210 (15)	79/152 (52)	25/142 (18)	12/57 (21)	18/95 (19)	311/1502 (21)
Vomiting	154/210 (73)	42/149 (28)	60/142 (42)	26/57 (46)	51/95 (54)	698/1505 (46)
Fever	89/209 (43)	103/150 (69)	81/140 (58)	33/57 (58)	50/93 (54)	944/1496 (63)
Marasmus	4/201 (2)	13/137 (9)	13/121 (11)	1/57 (2)	6/87 (7)	105/1374 (8)
Dehydration (moderate or severe)	192/211 (91)	127/153 (83)	114/143 (80)	50/57 (88)	84/96 (88)	1194/1525 (78)
Median days with diarrhoea	5	5	5	5	5	5
Median stools in previous 24 h	4	5	4	3	4	4

[a] Some denominators are slightly less than the number positive in Table 1 because of missing data.

Campylobacter were frequently isolated enteropathogens. *Shigella* was also common, as in some (but not all) of such other studies. *Cryptosporidium parvum* has been studied less, but we have found that it is also common. We tested for heat labile toxin production by enterotoxigenic *Escherichia coli* (ETEC-LT), by both co-agglutination and gene probe. Both methods demonstrated the presence of ETEC-LT in this population, although poor agreement between them prevents us from confidently quantifying the prevalence. Miwatani *et al*.[15] have already reported both heat labile and heat stable toxin of ETEC elsewhere in Papua New Guinea.

Rotavirus was the pathogen most frequently isolated in cases until 2 years of age, having more than four times the rate of any other until 6 months of age, and more than double until 1 year. Moreover, this is likely to be a clinically important cause in view of its high odds ratio (i.e., the much lower isolation rate in controls) and the high occurrence of vomiting and dehydration in those testing positive. This emphasizes the importance of vaccines, which have been undergoing field trials for several years and are now close to being licensed.[16] This study was designed to move subsequently into appropriate vaccine trials, although these have now been greatly delayed. The dominance of rotavirus at lower ages has been reported less often than the subsequent rapid decline.[12,17] However, some caution needs to be taken comparing the isolation rates of different tests, because the sets of children on whom they were done do not always coincide closely. *Shigella*, the next most commonly isolated pathogen, also appears to be of clinical importance given the high odds ratio, and the associated levels of blood and pus in stools, as described elsewhere.[12,18] These two pathogens showed the clearest clinical associations, and are two of the three that a recent World Health Organization multicentre study[12] concluded should be targeted for intervention (the third being ETEC).

The proportion of cases from whom any pathogen was isolated was relatively low at 42 per cent. However, one quarter (375/1515) had previous antibiotics recorded in their personal 'health book' (clinic attendance record), and this should also be borne in mind when comparing the isolation rates of bacterial and other pathogens. The role of multiple infections is made more difficult to assess by the fact that different subjects had different combinations of tests done. However, considering the five most common pathogens, no unexpectedly numerous combinations were seen, the prevalences being either roughly as expected under independence, or somewhat less. Accordingly, the most common double infections were combinations with rotavirus, the prevalence of which in EPEC-positive cases was 25 per cent (compared to 23 per cent overall). The prevalence of rotavirus in those with *Campylobacter*, *Cryptosporidium* and *Shigella* was 15, 13 and 5 per cent respectively.

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