CHARACTERISTICS OF CHILDHOOD DIARRHEA ASSOCIATED WITH ENTEROTOXIGENIC *ESCHERICHIA COLI* IN MALAYSIA

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Abstract. Amongst 107 diarrheal cases studied a bacterial agent was isolated from 71 (66%) cases of which 60 (85%) were due to a single agent and the remaining 11 (15%) were of mixed infections. Enterotoxigenic *Escherichia* coli (ETEC) was isolated from 65 cases. Other pathogens isolated included *Salmonella* spp, *Shigella* spp and rotavirus. There was a higher isolation rate of ETEC from females and rotavirus from males. The infection rate was found to higher for the 0-2 year age group as compared to the 3-5 year age group. Amongst the ETEC isolated the STa 2 toxotype was the predominant type.

INTRODUCTION

Acute diarrheal disease is a major cause of malnutrition, morbidity and mortality amongst children under 5 years of age in many developing countries (Guerrant *et al*, 1983; Bern *et al*, 1992). The incidence of diarrheal disease in this age group varies geographically with children having more episodes of diarrhea per year in Latin America (3.9) and Africa (2.7) as compared to those in Asia (2.0). With a global median of 2.7 episodes of diarrhea per child per year, this is equivalent to a global estimate of 1 billion episodes each year, in children under 5 years of age (Bern *et al*, 1992).

In etiological studies performed worldwide, the pathogens most frequently isolated as causes of acute diarrhea amongst children under 5 years of age in developing countries include rotavirus, enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *Escherichia coli* (EPEC), *Shigella* spp and *Campylobacter jejuni* (WHO, 1986). The most frequently isolated agent in many geographic regions is enterotoxigenic *E. coli*, followed by rotavirus (Echeverria *et al*, 1985; WHO, 1986; Geyer *et al* 1993). Other enteropathogens although implicated, occur to a lesser extent.

The diarrhea due to ETEC has been classified as watery diarrhea similar to that of cholera, but of less severity (Bacqui *et al*, 1991; Huilan *et al*, 1991). The incidence of diarrhea due to ETEC was found to be highest amongst the 0-2 years old (Black *et al*, 1981). Beyond this age, there seems to be an increase in the proportion of asymptomatic infections. The aim of this study was to determine the prevalence of ETEC and other enteropathogens amongst acute diarrheal cases in children 0-5 years of age, in Malaysia.

MATERIALS AND METHODS

Bacterial strains

107 cases of acute gastroenteritis amongst children under 5 years of age, inpatients or attending the outpatient clinic at the University Hospital, Kuala Lumpur, were studied from February to May 1991. The stools of these children were screened for the presence of enteropathogens which included *Salmonella* spp, *Shigella* spp, *Vibrio* spp, enteropathogenic *E. coli* (in children less than two years), *Campylobacter* spp (only in stools that were present with blood) and rotavirus.

The bacterial enteropathogens were isolated using MacConkey agar (Oxoid, UK), thiosulfate-citrate-bile salts sucrose agar (TCBS,Oxoid, UK), deoxycholate citrate agar (DCA, Oxoid, UK) and blood agar. However, *Campylobacter* spp were isolated using Skirrow's medium (Oxoid, UK) at 42°C, under microaerophilic conditions. Identification of all pathogens were done using standard biochemical (Cowan, 1981) and immunological methods at the Department of Medical Microbiol-

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ogy, University of Malaya. For the detection of enterotoxigenic *E. coli*, 5 lactose-fermenting colonies on MacConkey agar plates, presumptive of *E. coli* were selected at random, pooled and reconfirmed using standard biochemical tests.

Bacterial strains used as controls for ETEC included *E. coli* strain E 40/90 (LT-positive) *E. coli* strain E 41/89, (ST-positive) (Instiute for Medical Research, Kuala Lumpur, Malaysia). *E. coli* strain 25922 (ATCC, USA), was included as the non-ETEC control.

All strains were subcultured onto fresh nutrient agar slants (NA, Oxoid, UK) every three months and stored at 4°C or room temperature during the course of this study. Control strains were stored in nutrient broth containing 20% glycerol at -70°C.

Stool types

The consistency, presence of blood or mucus and color of the stools was recorded.

ETEC detection

DNA-DNA hybridization assays were performed using LT, STa1 and STa2 DNA probes. The LT probe comprised of a 800 base-pair *Hin III* fragment derived from recombinant plasmid pAT153H6; the STa1 probe comprised a 157 base-pair *Hin I* fragment derived from pST285; and the STa2 probe comprised a 600 basepair *Hin III* fragment derived from pSR287. The DNA probe fragments were excised from the purified plasmid DNA by digestion with the respective restriction endonucleases and separated from the vector by agarose gel electrophoresis. The fragments were then electro-eluted from the gel, purified using phenol-chloroform extraction, ethanol precipitated and radio-labeled *in vitro* using ^{32}P by the random oligo-priming method (Feinberg and Vogelstein, 1983) to a specific activity of 10^7 cpm/μg. Colony hybridizations were performed as described by Moseley *et al* (1980). Briefly, the strains were spot-inoculated on to nutrient agar, cultured at 37°C and then transferred on to Hybond-N nylon membranes (Amersham International). The bacterial colonies were then lysed, the released DNA denatured (denaturing solution; 1.5M NaCl, 0.5 N NaOH) and neutralized (0.5M Tris, 1.5 M NaC1, 10m M EDTA). Following neutralization, single stranded DNA were immobilized on the filters using UV irradiation (colony-side down) for 3-5 minutes. Prehybridizations were performed using BLOTTO (0.25% skimmed milk, 6 x SSC, 0.1% SDS), for 4 hours at 60°C, followed by hybridizations with the labeled probes, overnight, using fresh BLOTTO (10 ml/100 cm^2 membrane) at 60°C. Following hybridizations, the filters were washed and the bound DNA probe detected by autoradiography.

Rotavirus detection

Detection rotavirus was performed using the Rotalex kit (Orion Diagnostica) on direct fecal samples. Briefly, according to manufacturer's instructions, each fecal specimen was diluted using rotalex buffer (1:9) in a test tube and vortexed. The suspension was then centrifuged for 10 minutes at 3,000 rpm after which 2 drops of the supernatant was mixed with 1 drop of Rotalex latex reagent on a test card and placed on a rotary shaker. A positive result was indicated by agglutination occurring within 4 minutes of mixing.

RESULTS

Pathogens and stool type

Amongst the 107 diarrheal cases studied, a bacterial agent was isolated from 71 (66%) cases, of which 60 (85%) were due to a single causative agent and the remaining 11 (15%) cases were of mixed infections (Table 1). Of these cases ETEC was isolated as a single cause from 54 (90%) cases and *Salmonella* spp from 6 (10%) cases. The remaining ETEC were isolated from all of the 11 cases of mixed infections; with *Salmonella* spp, 5 cases; *Shigella* spp, one case; and rotavirus, 5 cases.

Salmonella spp was detected in 11 cases, *S. typhi* was detected in one case while the remaining strains consisted of the food poisoning serotypes of *Salmonella*, which included *S. bareilly* (2), *S. kentucky* (1) and *S. weltevreden* (1). The remaining 6 strains were untypable. The *Shigella* spp detected in one case was identified as *S. sonnei*. There were no bacterial pathogens isolated from 30 of the remaining cases.

Detection of rotavirus was performed on only 70 specimens. Of the 11 cases positive for rotavirus, 5 were present together with ETEC and in the remaining 6 no other pathogen was isolated.

Table 1

Bacterial enteropathogens and stool type in 107 children with acute diarrhea.

Pathogen	No. detected	Stool type		
		Watery	Bloody	Normal
Single				
ETEC	54	13	3	38
Salmonella spp	6	1	0	5
Multiple				
ETEC and *Salmonella* spp	5	1	0	4
ETEC and *Shigella* spp	1	1	0	0

Stools obtained from the children were observed for their characteristics and reported as being watery, bloody or normal (soft/semi-solid without blood) (Table 1). In cases with single cause, of 54 cases with ETEC infections 38 (70%) had normal stools; 13 (24%) had watery stools while bloody stools were present in only 3(6%) of them. In cases with rotavirus as the single pathogen, 4 of 6 (67%) had normal stools while the other two cases presented with watery stools. In mixed infections of the five cases with ETEC and rotavirus, three children had watery stools whereas two had normal stools. Amongst the five cases with ETEC and *Salmonella* spp, a watery stool was found in only one case whilst all the rest were "normal". Similarly in the single case of ETEC and *Shigella* spp the patient had watery stools.

Age and sex distribution

There was a higher preponderance of isolating a bacterial agent amongst females than males although the male to female ratio of the study population was 1.6:1 (Table 2). Detection of ETEC was significantly higher in females at 75.6% (31/41) as compared to detection in males at 51.5% (34/66; X^2 test with Yates' correction, $p < 0.05$). *Salmonella* spp was detected in 12.2% (5/41) of females in comparison to 9.1% (6/66) in males but this difference was not statistically significant (X^2 test with Yates' correction, $p < 0.05$).

Conversely the detection of rotavirus performed amongst 41 males and 29 females indicated a higher infection rate amongst males at 17.1% (7/41) as compared to 13.8% (4/29) in females. This difference was again not statistically significant (X^2 test with Yates' correction, $p < 0.05$).

Table 2

Distribution of bacterial enteropathogens by sex in 107 children with acute diarrheal disease.

Enteropathogens	No.[a](%) detected	
	Males (n = 66)	Females (n = 41)
ETEC	34 (51.5)	31 (75.6)
Salmonella spp	6 (9.1)	5 (12.2)
Shigella spp	0 (0.0)	1 (2.4)

[a]: There is an overlap of figures because some children had multiple pathogens.

The distribution of enteropathogens by age indicated that 74.7% of cases in the 0 - 2 years age group were infected with an enteropathogen compared to 61.5% in the 3 - 5 years age group. The rate of infection with ETEC in the 0 - 2 years age group was 61.3% (46/75) compared to 50.0% (13/26) in the 3 - 5 years age group (Table 3), but this difference was not statistically significant (X^2 test with Yates' correction, $p < 0.05$). The same was true for *Salmonella* spp, with an infection rate of 12.0% (9/75) in the 0 - 2 years group versus 7.7% (2/26) in the 3 - 5 years group. Conversely, for rotavirus, the infection rate was higher in the 3 - 5 years age group at 23.5% (4/17) compared to 14.0% (7/50) in the 0 - 2 years age group, but both differences were

Table 3

Distribution of bacterial enteropathogens by age in 107 children with acute diarrheal disease.

Enteropathogens	No.[a](%) detected		
	Age (years)		
	0-2 (n = 75)	3-5 (n = 26)	Unknown (n ≤ 5)
ETEC	46 (61.3)	13 (50.0)	6 (100.0)
Salmonella spp	9 (12.0)	2 (7.7)	0 (0.0)
Shigella spp	0 (0.0)	0 (0.0)	1 (16.7)

[a]: There is an overlap of figures because some children had multiple pathogens.

statistically not significant (X^2 test with Yates' correction, $p < 0.05$).

Distribution of LT genes, ST genes and toxotypes of ETEC

Amongst the ETEC isolated from the 65 children with ETEC-associated diarrhea, the STa2 toxotype either as a single toxotype or in combination with the LT and/or STa1 was significantly high amounting to 69.2% (Table 4). STa2-ETEC toxotype was isolated from 43.1% of the cases. While LT, STa1, LT/STa2 and STa1/STa2 toxotypes occurred in an almost equal distribution of 13.8%, 12.3%, 13.8% and 10.8% of ETEC cases respectively. LT/STa1 toxotype was infrequently detected (4.6%) with LT/STa1/STa2 toxotype being present in only one case.

Table 4

Toxotypes of ETEC detected in 65 children with ETEC associated diarrhea.

Toxotype	No. (%) detected
LT only	9 (13.8)
STa1 only	8 (12.3)
STa2 only	28 (43.1)
LT/STa1	3 (4.6)
LT/STa2	9 (13.8)
STa1/STa2	7 (10.8)
LT/STa1/STa2	1 (1.5)
Total	65 (99.9)

DISCUSSION

In etiological studies carried out worldwide, the pathogens implicated most frequently as causes of acute diarrhea in children under 5 years, in developing countries, are rotavirus, ETEC, EPEC, *Shigella* and *Campylobacter jejuni* (WHO, 1986). In all these studies, there was no marked differences in the geographical distribution of the most frequently isolated enteropathogen. In most cases, at hospitals with laboratories capable of comprehensive diagnostic services, a pathogen could be identified in 60-65% of cases of acute diarrhea (WHO, 1986).

In this study, an enteropathogen was identified in 77/107 (72.0%) cases of acute diarrhea. Most of these cases were associated with at least one enteropathogen. Enterotoxigenic *E coli* (ETEC) accounted for 65 of 107 (60.7%) of the cases that were studied and was the sole pathogen identified in 54 of 107 (50.5%) of the stools examined. *Salmonella* spp (10.3%) and *Shigella* spp (1 case) infections accounted for the remaining cases with positive cultures. Detection of rotavirus was only performed on stools of 70 cases and a positivity rate of 15.7% was obtained.

In comparison, in a surveillance study performed in Bangladesh, by Black and co-workers (1980), 70% of diarrheic children under 2 years had an enteropathogen identified in their stool. ETEC was detected in about 25% of these cases while rotavirus was the most frequently isolated enteropathogen with an isolation rate of 46%. *Shigella* spp and *Salmonella* spp were only detected in 5% and < 1% of cases respectively. In another study in northeastern Thailand, ETEC was identified in 53% of the cases and *Shigella* spp in 15% of the cases. However the presence of rotavirus and *Salmonella* spp was not studied (Echeverria *et al*, 1985). In another study, in South Africa, 77% of the cases had a causative agent identified and ETEC accounted for 39% of the cases (Geyer *et al*, 1993). Rotavirus was identified in 12.8% of cases and *Campylobacter* in 15.4% of cases.

In all these studies, it was encouraging to note that the isolation rate of an enteropathogen was possible in more than 70% of diarrheal cases. However the isolation rate of each enteropathogen varied suggesting the predominance of different enteropathogens in different geographical locations. At the same time the ETEC infection rates observed in the present study were much higher than those described in Bangladesh, Thailand and South Africa.

The detection of ETEC has frequently been associated with watery stools similar to cholera (Baqui *et al*, 1991; Huilan *et al*, 1991). In this study, only 13 of 54 (24.1%) cases with ETEC as a single pathogen, and five cases involving mixed infection had watery stools (Table 1). Similar figures were obtained in a study by Black *et al* (1980) in Bangladesh. However, in a later study, in Bangladesh, involving patients of all groups (Baqui, 1991), 88% of ETEC cases had watery diarrhea. In this study, there were three cases which presented with bloody

diarrhea from which ETEC was isolated. This was an unusual finding, since ETEC has not normally been associated with "blood-in-stools", This may have occurred as a result of the presence of other invasive pathogens which were not included in this study, such as enteroinvasive or enterohemorrhagic *E. coli* which invade mucosal cells or produce toxins that damage cells resulting in hemorrhage, hence the bloody stools.

An unusual finding in this study was, that in the majority of children where ETEC was the sole pathogen isolated, 70.1% (38/54) had "normal", *ie* soft/semi-solid stools. The time at which the stool sample was collected in relation to the disease, could account for this finding as ETEC infection can often present as a self-limiting disease. It may also be possible that asymptomatic carriage of ETEC within a population could have occurred and therefore the detection of ETEC may not necessarily be associated with diarrheal disease (Vadivelu *et al,* 1989).

The preponderance of females, found in this study 75.6% females against 51.5% males, amongst ETEC-infected children was statistically significant. These findings were similar to that of a study in China (Kain *et al*, 1991). The difference in infection rates between male and female children could be due to variation in host susceptibility to ETEC infection. It is postulated here that the microbial flora and the local immunity in the gut environment of male children could be different from that in females, giving rise to better protection against intestinal colonization by ETEC in males.

The proportion of diarrheic children involved in this study was three times higher in the 0 to 2 years group as compared to the 3 to 5 years group (Table 3). The higher incidence of diarrhea in the younger age group could possibly be related to the status of development of active immunity in the prevention intestinal colonization, in this young age group (Black *et al*, 1981); thus resulting in the increase in the proportion of asymptomatic infections beyond two years of age. The present study also showed that ETEC was detected at a higher frequency from patients in the age group of 0 - 2 years (61.3%) than from the 3 to 5 years (50.0%) group (Table III), but the difference in the percentage of detection was not statistically significant. These findings were similar to that of Baqui and co-workers (1991) and Black and co-workers (1980).

In the present study, the distribution of LT, STa1 and STa2 genes independently was not found to be significantly different among the age groups. However, the most common toxotype detected was the STa-only toxotype, followed by LT/STa and LT-only toxotypes (Table 4). It was found that the STa2 subtype was the more predominant of the STa, representing 43.1% of all toxotypes detected. This finding is in keeping with another study done in Malaysia on diarrheal cases involving children and adults by Cheong *et al* (1990), where it was reported that STa-only ETEC was the most common toxotype identified, followed by LT-only and LT/STa ETEC and that of Vadivelu and co-workers (1989) in which the STa2 toxotype was most predominant.

The proportion of ETEC cases that produce STa-only (either/both STa1 and STa2), LT-only or both shows geographical variation. In Iran STa-only ETEC were most prevalent, followed by LT/STa producing ETEC and LT-only ETEC (Katouli *et al*, 1988) but a study in India documented the predominance of LT-only strains (Sen *et al*, 1984). Similarly, in China, Kain and co-workers (1991) found that most of the ETEC strains detected were LT-only strains. In Bangladesh LT/ST strains were dominant in one study (Sack *et al*, 1977), but in another study it was found that in children under 2 years, the highest percentage of infection with ETEC involved STa-only ETEC (15%) while LT-only ETEC involved about 5% of infections and LT/STa-producing ETEC were seen in 7% of infections (Black *et al*, 1980). It may thus be concluded that the toxotypes associated with ETEC diarrhea vary with geographical regions.

Although ETEC was associated with 60.7% of acute diarrheal cases, this may not trully reflect the disease due to ETEC as this study did not include matched controls to indicate the prevalence of asymptomatic ETEC infection. Although other enteropathogens such as rotavirus, *Salmonella* spp and *Shigella sonnei* were identified, the frequency of detection was much lower than that of ETEC. In comparison with other studies performed in this geographical region the detection of ETEC in this study is the highest. In addition, the detection of ETEC (70.1%) involving normal stool suggests that the presence of ETEC may be due to asymptomatic carriage rather than a true infection.

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