Prevalence of enteroaggregative and other HEp-2 cell adherent *Escherichia coli* in asymptomatic rural south Indians by longitudinal sampling

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Abstract

Enteroaggregative and other HEp-2 cell adherent *Escherichia coli* can produce acute and persistent diarrhoea in children and adults, but their prevalence in asymptomatic individuals in the community is not known. In this study, faecal specimens were obtained at 3–4 monthly intervals from 349 subjects constituting a 20% age-stratified sample of a rural community for a period of two years. HEp-2 cell adherent *E. coli* were found in 210 subjects, and repeat isolations of enteroaggregative *E. coli* belonging to the same serogroup were found in 12.6% of children less than 12 years of age, indicating that this organism can asymptomatically colonise the intestinal tract. These children may act as a reservoir of infection for the community.

Introduction

Although *Escherichia coli* are an important part of the normal faecal flora, several strains of *E. coli* carry virulence factors which may cause diarrhoea. These include *E. coli* that are designated enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohaemorrhagic (EHEC), diffusely adherent (DAEC) and aggregatively adherent (EAggEC) (Levine, 1987; Nataro, 1995). These categories exhibit distinct clinical syndromes associated with their virulence factors, and laboratory diagnosis of infections by these organisms depend on identification of certain characteristics such as serotypes, toxin production and interactions with epithelial cells.

The development of a tissue culture adherence assay using HEp-2 or HeLa cells showed that *E. coli* adhered to these cells in more than one pattern (Cravioto *et al.*, 1979; Scaletsky *et al.*, 1984; Nataro *et al.*, 1987). The adherence pattern of enteropathogenic *E. coli* (EPEC) which have been known for 50 years to cause an acute watery diarrhoea (Bray and Beavan, 1948) has been described as 'localized' adherence (LA) based on the presence of clusters of bacteria or microcolonies on the surface of the HEp-2 cells (Scaletsky *et al.*, 1984).

In contradistinction to EPEC and non-adherent strains, some non-EPEC did not adhere in the characteristic microcolonies. Instead these strains displayed a phenotype described as diffuse adherence (DA) with dispersed bacteria adhering all over the cell in no particular pattern. These have been shown to be associated with acute diarrhoea

in travellers, Mayan and Bangladeshi children (Giron *et al.*, 1991; Baqui *et al.*, 1992), but have not been implicated in diarrhoeal disease in other studies (Cravioto *et al.*, 1991; Kang *et al.*, 1995; Albert *et al.*, 1996). The most recently identified adherence pattern is aggregative adherence, where the bacteria are arranged in a 'stacked brick' formation with adherence to glass and tissue culture cells in parallel rows. EAggEC have been extensively studied in persistent diarrhoea in children in Asia, Central and South America (Bhan *et al.*, 1989; Nataro, 1995). In spite of extensive research in the decade since these organisms were first described, many questions regarding the mode of transmission and prevalence remain unanswered. During the present work faecal excretion of HEp-2 cell adherent *E. coli* was examined by repeated sampling of a cohort of asymptomatic rural South Indians.

Materials and methods

Subjects

A 20% stratified random sample of Ka village, 25 km from Vellore, with a population of approximately 1,400 was kept under surveillance for diarrhoeal disease as part of ongoing research by our department. An earlier study in the same village (Rajan and Mathan, 1982), showed that 14.8% of the population sampled were asymptomatic carriers of *Campylobacter* species and 18.1% had other enteric pathogens.

Sample collection

For this study, stool samples were collected every 3 to 4 months from each member of the families and investigated for bacteria and parasites. At least 5 ml of faeces was provided by each individual in a screw-capped container which was transported on ice to the laboratory within 2 h and processed immediately. Isolates obtained during a 2 year period, from 1 January 1994 to 31 December 1995 were included in the adherence assays. *Escherichia coli* isolates were tested in the adherence assay immediately after culture and identification. A total of 349 subjects from 67 families gave at least three samples and 1,270 *E. coli* isolates were obtained from 1,475 samples from these subjects.

Identification of enteric pathogens

The laboratory procedures for the identification of enteric pathogens are described below.

Bacterial culture

The following primary media were used for bacterial pathogen isolation: one blood agar plate, two MacConkey agar plates (one incubated at room temperature), one desoxycholate-citrate agar plate

and one xylose-lysine desoxycholate agar plate. One tube of selenite enrichment broth was subsequently subcultured after 16 h incubation onto *Salmonella-Shigella* agar. *Campylobacter* on Butzler's medium was incubated at 42°C in an atmosphere of 5–7% oxygen and 10% carbon dioxide. Species of the following genera of enteric bacteria were recorded: *Salmonella*, *Shigella*, *Vibrio*, *Aeromonas*, *Yersinia* and *Campylobacter*. Colonies of suspected enteropathogens were further identified and characterized by appropriate standard biochemical tests according to the Manual for Laboratory Investigation of Acute Infectious Diarrhoea (WHO, 1983). *Salmonella, Shigella* and *Vibrio* species were serotyped using antisera from Difco Laboratories, Detroit, Michigan, U.S.A.

At least five colonies of non-mucoid lactose fermenters on MacConkey agar plates were serogrouped to identify enteropathogenic serotypes by slide agglutination using polygrouping antisera (Biotec Laboratories, England), and individual EPEC antisera (Wellcome, England). All isolates which showed the localized adherence pattern in the HEp-2 cell adherence assay were serogrouped as above.

Isolates from patients who had the aggregative adherence pattern identified more than once from their samples were initially grouped with commercial antisera and then sent to the Central Public Health Laboratory, Colindale, England, for confirmation of serogrouping.

Identification of parasites

Direct saline and iodine preparations of the stool samples were examined by light microscopy at x10 and x40 magnifications. About 2 g of the stool sample was also processed for concentration by the formol-ether technique, and examined with saline and iodine preparations. Smears were prepared from the deposit after concentration and stained with safranin-methylene blue, a modified acid-fast stain, for *Cryptosporidium* oocysts (Mathan *et al.*, 1985).

Adherence assay

The adherence assay was carried out as previously described (Kang *et al.*, 1995). Briefly, HEp-2 cell monolayers were grown overnight on ten spot multi-test slides (Flow Laboratories) and washed three times with minimum essential medium (MEM; Gibco). Then 40 µl of the overnight bacterial culture grown in Luria broth was added to 0.5 ml of MEM containing 2% foetal calf serum and 1% methyl-alpha-mannoside, and 25 µl was overlaid onto each test spot. The slides were then incubated at 37°C with 5% carbon dioxide for 3 h, washed three times with MEM, fixed with 70% ethanol and stained with 10% Giemsa solution. The slides were examined by light microscopy, using an oil immersion objective.

Results

The 349 individuals studied had a high prevalence of potentially pathogenic bacteria and parasites. *Campylobacter* spp were isolated more than once from 28 subjects (8.0%) in four patients. Various species of shigellae were isolated from thirteen subjects (3.7%). Parasites were found in 168 patients (48.1%), and the most frequently identified were *Giardia* (61), *Cryptosporidium* (36) and hookworm ova (36). Other parasites were *Entamoeba histolytica* (21), *Hymenolepis nana* (14), *Strongyloides* (10), pinworm (7), *Trichuris* (3) and *Ascaris* (1). Of 1,270 isolates of *E. coli* obtained from these people, 802 were nonadherent, 347 were diffusely adherent, 66 were aggregatively adherent and 55 were locally adherent (Table 1). A total of 139 subjects did not have adherent *E. coli* in any sample.

Isolates of EAggEC (66 *in toto*) were obtained from 34 individuals. A total of seventeen subjects from eight families had EAggEC isolated at least twice during the study period (Table 1). Of these, eleven children were under 12 years of age (Table 2). EAggEC were seen in all samples from five subjects, of whom four were under 12 years of age. On serogrouping of the isolates from the seventeen subjects with repeat EAggEC isolations, the isolates from four individuals were all rough mutants and O antigens could not be determined. Isolates from the other thirteen individuals were O3 (3 subjects), O6 (3 subjects), O55 (3 subjects), O86 (2 subjects), O125 (1 subject) and O126 (1 subject). All repeat isolates belonged to the same serogroup as the original isolates. EAggEC were isolated at least once from one other family member in all twelve children under 12 years with repeated EAggEC isolation. The incidence of two or more isolations of EAggEC was significantly higher ($p < 0.01$) in the two age groups under 12 years (Table 2).

EAggEC were isolated only once in samples from seventeen subjects, and in twelve of these no other adherent *E. coli* were identified in any samples. A total of 55 isolates of LAEC were obtained from 31 individuals, and 38 (69%) belonged to the classical EPEC serotypes. Thirteen subjects from eleven families had LAEC isolated at least twice during the study (Table 1). In nine people, the LAEC isolates agglutinated with EPEC antisera, and of these in eight subjects, the repeat LAEC isolates belonged to the same serotype as the first LAEC isolate. In one individual, the first isolate was O111:H2, but the second isolate did not agglutinate with any of the antisera used, although it had a localized adherence pattern.

LAEC were seen in all four samples from one 17-year-old subject, and all of these were O127:H6. LAEC were isolated only once in samples from eighteen individuals, and in eight patients no other

Table 1 Adherence patterns of *Escherichia coli* isolated from a stratified random sample of an asymptomatic rural population by longitudinal sampling

Adherence pattern	Total number of isolates	Total number of subjects showing each adherence pattern: Single isolate	Two or more isolates
Diffuse	347	93	62
Localized	55	18	13
Aggregative	66	17	17
Non-adherent	802		

The adherent *E. coli* were isolated from 210 of the 349 individuals who gave 1,475 stool samples from which 1,270 *E. coli* were isolated.

adherent *E. coli* were identified in any samples. The incidence of two or more isolations of LAEC was higher in the age group under 5 years, but lower in the older age groups (Table 2).

A total of 347 of DAEC were obtained from 155 subjects. Single isolations of DAEC were more common than repeat isolations in all age groups (Table 2). Among the individuals who had two or more isolates with the same adherence pattern, familial clustering was seen only in EAggEC, with seventeen subjects belonging to eight families. EAggEC colonization was higher in children below 12 years, than in older children and adults (Table 2).

Table 2 Age distribution of isolates fo EAggEC and LAEC in a stratified random sample of an asymptomatic rural population

Age (years)	n	Isolate from single sample only: EAggEC	LAEC	DAEC	Isolates from two or more samples: EAggEC	LAEC	DAEC
0–4.9	52	2 (3.8%)	2 (3.8%)	13 (25.0%)	7 (13.4%)	3 (5.7%)	9 (17.5%)
5–11.9	43	3 (6.9%)	4 (9.3%)	12 (27.9%)	5 (11.6%)	2 (4.6%)	6 (13.9%)
12–18	69	2 (2.9%)	6 (8.7%)	20 (29.0%)	2 (2.9%)	3 (4.3%)	13 (18.8%)
>18	185	10 (5.4%)	6 (3.2%)	20 (28.9%)	3 (1.1%)	5 (2.7%)	25 (18.9%)

Table 3 Community-based studies on the epidemiology of EAggEC in children with diarrhoea and control children

Reference	Country/duration of diarrhoea	Number of patients/isolates	Number with EAggEC	Number of control/isolates	Number with EAggEC
Nataro *et al.* (1991)	Chile	253	84 (33%)	134	20 (15%)
Bhan *et al.* (1989)	India				
	<14 days	179	23 (13%)	201	20 (10%)
	>14 days	61	18 (30%)		
Cravioto *et al.* (1991)	Mexico				
	Acute	579	49 (8%)	100	5 (5%)
	Persistent	57	29 (51%)		
Henry *et al.* (1992)	Bangladesh				
	<14 days	28	5 (18%)	46	9 (20%)
	>14 days	62	16 (27%)		
Present study multiple samples	India			52	9 (17%)

Table 4 Hospital-based case-control studies on the epidemiology of EAggEC in children with diarrhoea and control children

Reference	Country/ duration	Number of patients	Number with EAggEC	Number of controls	Number with EAggEC
Bhan *et al.* (1989b)	India	92	18 (20%)	92	6 (6%)
Gomes *et al.*	Brazil	100	10 (10%)	100	8 (8%)
Wanke *et al.* (1991)	Brazil				
	Acute	50	4 (8%)	28	2 (5%)
	Persistent	40	8 (20%)		
Bhatnagar *et al.* (1993)	India	254	46 (12%)	107	4 (3.7%)
Gunzburg *et al.* (1993)	Australia	68	12 (18%)	70	15 (21%)
Brook *et al.* (1994)	UK	135	12 (9%)	46	4 (9%)
Morelli *et al.* (1994)	Italy	112	7 (6%)	56	1 (2%)
Paul *et al.* (1994)	India				
	Secretory	159	17 (11%)		
	Nonsecretory	174	3 (2%)	25	1 (4%)
Albert *et al.* (1995)	Bangladesh	451	43 (10%)	602	67 (11%)
Fang *et al.* (1995)	Brazil				
	Acute	52	24 (46%)	42	13 (31%)
	Persistent	56	38 (68%)		
Kang *et al.* (1995)	India	794	60 (7.6%)	566	22 (3.9%)

Discussion

These studies on the epidemiology of HEp-2 cell adherent *E. coli* are the first community wide assessment of the prevalence of these organisms in asymptomatic individuals. The results show that they are not only widely prevalent in the apparently asymptomatic rural population in southern India, but are also capable of colonizing the gut of these subjects for up to 2 years. Since the first report of EAggEC from Chile (Nataro *et al.*, 1987), a number of papers have been published on the prevalence of EAggEC and other HEp-2 cell adherent *E. coli* in childhood diarrhoea and appropriate controls.

In a previous paper on a stratified random sample of Ka village (Rajan and Mathan, 1982), it was found that the overall percentage prevalence in asymptomatic individuals belonging to all age groups of *Campylobacter* spp was 14.8%, while that of other bacterial pathogens was 18.1%, with a higher incidence of pathogens in the preschool age group. Rajan and Mathan (1986) examined multiple samples and isolated bacterial intestinal pathogens from 20.5% of stool specimens, but HEp-cell adherent *E. coli* were not examined. In the present work, the prevalence of conventional bacterial pathogens, including *Campylobacter*, was 12%.

In geographical areas where there is a high background prevalence of potentially pathogenic organisms, a single isolation of a particular organism from a subject may simply be a reflection of the contaminated environment with the presumptive pathogen just transiting the gut lumen, rather than evidence of colonization of that particular individual. However, when two or more isolates of the same adherence pattern and belonging to the same serogroup in three or more stool samples taken 4–6 months apart are regarded as evidence of colonization, then almost 25% of individuals in our rural population appeared to be colonized by HEp-2 cell adherent *E. coli*.

There was a striking difference in the pattern of isolation of the HEp-2 cell adherent *E. coli*. Repeat isolations of EAggEC were significantly more common in children aged <12 years (Table 2), with EAggEC also showing clustering in families. Single isolates of EAggEC were more in the older age group (>18 years). In children <5 years of age, two or more isolates of LAEC were more frequently seen than single isolates, but in the older age groups, single isolates were found in a higher percentage of the sample than repeat isolates. In all age groups, the percentage of subjects with single isolates of DAEC was higher than that of subjects with repeat isolations. It is possible that the single isolations were due to acquisition of the organism from the environment. The repeat isolation of EAggEC belonging to the same serogroups seemed to indicate that children may

be truly colonized by EAggEC. Young children may be an immunologically naive population, unable to prevent colonization by these organisms, while repeated exposure may lead to the development of an immune response which prevents colonization in adults.

In previous studies, the prevalence of EAggEC in control children in the community (Table 3) ranged from 5% in Mexico (Cravioto *et al.*, 1991) to 20% in Bangladesh (Henry *et al.*, 1992), and the results reported here fall in this range. When the prevalence of EAggEC in the controls was examined in hospital based studies (Table 4), it ranged from 2% (Morelli *et al.*, 1994) to 31% (Fang *et al.*, 1995). Our previous work on hospital based controls showed a 3.9% prevalence of EAggEC (Kang *et al.*, 1995), much lower than that recorded here in a community based study. Two possible explanations for this are (1) urban rural environmental differences, since the majority of the hospital based children were from the town of Vellore; (2) the fact that these isolates had been stored for nearly 10 years before this phenotype was tested and it may have lost the specific characteristic, whereas in the present results, the adherence assays were done immediately on isolation of the *E. coli*.

Among bacteria which produce diarrhoea, adherence to the host is of paramount importance in establishing colonization, and in symptomatic infections, facilitating dissemination, toxin delivery and host cell lysis. A study of faecal excretion patterns facilitates the determination of true asymptomatic colonization in an endemic area with high prevalence rates of enteroadherent *E. coli*.

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