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Characterization of Enteroaggregative *Escherichia coli* Isolated from South Indian Subjects in Health and Disease

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Microbial Ecology in Health and Disease 2002; 14: 42–49

Objective: To characterize enteroaggregative *Escherichia coli* (EAEC) isolated from children and adults with different forms of diarrhoea, and also from controls. *Design:* A panel of 40 EAEC isolates from children with acute diarrhoea, adults affected in an epidemic of acute diarrhoea associated with EAEC and isolates from control children and adults were analyzed by adherence pattern, serotyping, DNA probing, haemagglutination, fluorescence actin staining (FAS), internalization/invasion and secretory activity in Ussing chambers in an attempt to distinguish isolates from symptomatic individuals from those obtained from asymptomatic excretors. *Setting:* A predominantly rural community of 95000 in South India kept under surveillance for diarrhoeal outbreaks. *Results:* Predominantly cellular adherence was seen in 27/40 (67.5%), adherence to glass in ten (25%) and a honeycomb pattern in three (7.5%). Of 40 isolates, 13 were rough and O untypable, and the remaining belonged to nine different O serogroups. Thirty-one isolates (77.5%) hybridized with the pCVD 432 probe. Erythrocytes from one or more of four species were agglutinated by 33 (82.5%) isolates. All isolates were negative in the FAS assay and a total of 17 (42.5%) isolates were internalized by HEp-2 cells at levels above 5%. In Ussing chamber experiments, an increase in short circuit current was seen with all ten isolates from patients affected by the outbreak and 14 of 20 other isolates from children with acute diarrhoea and controls. *Conclusions:* Characterization of EAEC from sporadic cases of diarrhoea and controls showed that they were a heterogeneous group of organisms, only some of which possess virulence factors such as toxin production and invasiveness. This differed from the isolates obtained from the diarrhoeal outbreak, which belonged to only two serotypes, had similar haemagglutination patterns and invasive ability. *Key words:* enteroaggregative *Escherichia coli*, virulence, India.

INTRODUCTION

Escherichia coli are an important part of the normal fecal flora, but several classes of *E. coli* have been identified as potentially diarrhoeagenic as they carry identifiable virulence markers. These include *E. coli* that are designated enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohaemorrhagic (EHEC) and enteroadherent (1). The enteroadherent *E. coli* can be further subdivided according to the characteristic adherence pattern to tissue culture cells into locally adherent (LAEC), diffusely adherent (DAEC) and aggregatively adherent (EAEC) (2). EAEC have been implicated in acute and persistent diarrhoea in developed and developing countries, causing an illness that has predominantly secretory effects. EAEC isolates appear to be a heterologous group of strains and so far no genotypic or phenotypic markers have been identified that distinguish pathogenic strains from those that are non-pathogenic.

In this study, we analyzed phenotypes of virulence and other markers in an attempt to distinguish diarrhoeagenic EAEC from non-pathogenic isolates, using a panel of 40 EAEC isolates from children with acute diarrhoea, adults affected in an epidemic of acute diarrhoea associated with EAEC and isolates from control children and adults.

MATERIALS AND METHODS

Bacterial isolates

The panel of EAEC consisted of isolates from ten children with acute diarrhoea and ten matched case controls, ten adults affected in an acute epidemic of diarrhoea and ten adult controls from asymptomatic individuals in a rural area. The isolates from children with acute diarrhea and controls were obtained as part of a WHO sponsored study between 1983 and 1985 (3). These were obtained from children between 1 and 35 months of age with acute watery diarrhoea of less than 72 h duration and age and sex matched children without gastrointestinal symptoms

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presenting to the hospital. Laboratory procedures were carried out as described earlier (3), although adherence assays were not carried out at this time. From each diarrhoeal and control sample, at least five colonies of non-mucoid lactose fermenters grown on MacConkey agar were stored in nutrient agar stab cultures at 4°C. Samples from adults with diarrhoea were obtained from individuals affected during an outbreak of diarrhoea caused by EAEC linked to a water source (4). A total of 11 isolates of EAEC were obtained, and ten were chosen for this study. Isolates from controls were obtained from a study on asymptomatic rural south Indian that were sampled longitudinally for 2 years for HEp-2 cell adherent *E. coli* (5). Type strain 17-2 (serotype O3:H2), isolated from a South American child with diarrhoea was used as a control (1).

Adherence assay

All *E. coli* from children which had been stored in nutrient agar at 4°C were recovered by culture in Luria Bertani broth at 37°C for 16 h. Isolates from diarrhoeal and control adults were tested after primary isolation and identification, without further subculture or storage. The isolates were tested in a modified adherence assay using HEp-2 cells grown on multitest slides, which was carried out as previously described (5). All experiments were repeated at least three times. The criterion for identification of *E. coli* as aggregatively adherent was the characteristic stacked brick arrangement (2). The three distinct forms of aggregative adherence which have been described were used for further classification of adherence, namely, predominantly cellular adherence, adherence predominantly to glass (6) and the recently described honeycomb pattern (7), which has large open spaces bordered by bacteria arranged in parallel rows.

Serotyping

Of the 40 isolates, 28 (all isolates from children and eight of ten isolates from control adults) had their O serogroups determined at the Central Public Health Laboratory, Colindale, London, UK and 12 (all isolates from diarrhoeal adults and two of ten isolates from control adults) were grouped and typed at the Labortorio de Salud Publica, Mexico City, Mexico.

DNA probing

Colony blots of the 40 EAEC isolates and 32 additional isolates from the study on acute diarrhoea in children, which showed the localized or microcolony type (n-10), diffuse (n-10) and 'mixed' pattern (n-12) of adherence were hybridized with the EAgg, DA, EAF and *eae* probes (a gift from D. Acheson, Tufts New England Medical Center, Boston, MA, USA) as previously described (5). The probes were prepared from recombinant plasmids that contained the probe DNA fragments as inserts. The EAgg probe is a 700 bp *Eco*RI–*Pst*I insert in pCVD432 (8). The DA probe is a 390 bp *Pst*I fragment in pSLM 862 (9). The EAF probe was a 1 kb *Sal*I–*Bam*HI fragment cloned into pCVD 315 (10). The *eae* probe was a 1 kb *Sal*I–*Stu*I fragment on pCVD434 (11).

Fluorescence actin staining (FAS) assay

The FAS assay was done for the isolates as described earlier (12), with accumulation of actin at the site of localized adherence being identified by fluorescein-isothiocyanate staining. EPEC strain 2348/69 was used as a positive control in all experiments. In addition to the 40 EAEC isolates, ten isolates each showing localized and diffuse adherence as well 12 isolates showing 'mixed' adherence isolated from children with acute diarrhoea (3) were tested in the FAS assay. All experiments were repeated at least three times.

Toxin testing in Ussing chambers

Ussing chambers were used to study secretory activity of culture supernatants of EAEC using rabbit ileal mucosa as described earlier (13). A positive and negative control were included in each set of experiments with plain LB broth serving as negative control and the culture supernatant of EAEC strain 17-2 as positive control, in these studies the controls from the asymptomatic adults were not studied. The potential difference (PD) and short circuit current (I_{sc}) were measured after 5 min to allow equilibration and subsequently, every 15 min for 2 h. The change in short circuit current was calculated and graphs plotted for the change against time. Each experiment was repeated at least three times, and significance of differences in short-circuit current were assessed by application of Student's t-test.

Haemagglutination

Slide haemagglutination using rat, human (group O), rabbit and sheep erythrocytes was done on 40 isolates of EAEC (14). Both mannose resistant (MRHA) and mannose sensitive haemagglutination (MSHA) patterns were determined. The effect of physical agents such as heat and shaking was examined by growing bacteria at 42°C and on a rotatory shaker. To determine whether the haemagglutinins were cell-associated or free in the supernatant, cultures were centrifuged and the supernatants checked for haemagglutination activity by the microtitre assay. The supernatants of bacteria grown as for slide agglutination were prepared by centrifuging the cultures at 3000 rpm for 20 min at room temperature. Serial doubling dilutions were made from neat supernatant in PBS with and without 2% D-mannose in 25 μl volumes after which an equal volume of 2% rat or human erythrocytes were added to each well. The plate was incubated at 4°C for 4 h and the results read.

Internalization assay

The HEp-2 cell invasion assay using gentamicin to kill extracellular bacteria was used to determine the internalization potential of 40 isolates of EAEC as previously described (15). The EPEC strain 2348/69 which has previously been shown to be internalized at levels above 5% (16) was used as a positive control and *E. coli* K12 was used as a negative control.

RESULTS

Serotyping

Nine of ten EAEC from the diarrhoeal outbreak belonged to a total of two serotypes, with five isolates of O125:H12, four isolates of OR:H4, and the remaining isolate being untyped. Among the 31 other isolates, two were O93:H16 (one each from a child with acute diarrhoea and control child), two were O6:H16 (one each from a control child and control adult). Including the four OR:H4 isolates from the diarrhoeal outbreak, 13 isolates were rough and O non-typable. The O serogroups of the eight remaining isolates were O26 (two isolates), O55, O88, O97, O126 (two isolates) and O127.

Hybridization with DNA probes

Of the 40 EAEC isolates tested, 31 (77.5%) hybridized with the pCVD 432 probe, as did type strain 17-2 (Table I). Hybridization with the probe was seen in 8/10 isolates from children with acute diarrhoea, 9/10 isolates from patients during the outbreak of prolonged diarrhoea and 14/20 isolates from controls. None of the EAEC hybridized with the other three probes. As expected, the LAEC hybridized with the EAF (9/10) and eae (10/10) probes. DAEC hybridized with the DA probe (8/10). The mixed pattern of adherent *E. coli* showed variable hybridization with all the probes (Table I). The results of the DNA hybridization were compared with those of the adherence assay and the 31 probe-positive isolates showed cellular adherence in 26, adherence to glass in two and a honeycomb pattern in three isolates. The nine probe-negative isolates showed adherence to cells in one and to glass in eight.

Fluorescence actin staining assay

The results of the FAS assay showed no actin accumulation in HEp-2 cells at the sites of adherence of the type strain 17-2, the 40 isolates showing the aggregative pattern and the ten isolates with the diffuse pattern. All ten LA strains were FAS positive, with accumulation of filamentous actin, detected by intense fluorescence at the site of microcolony formation and adherence to the HEp-2 cell. Six isolates with a mixed, predominantly LA, pattern were also positive by the FAS assay and hybridized with the *eae* probe, while six isolates with a mixed pattern were negative.

In vitro toxin testing

An increase in short circuit current was consistently seen with the type strain and with all ten isolates from patients affected by the outbreak (Fig. 1a) and 14 of 20 other isolates from children with acute diarrhoea and controls (Fig. 1b and c). The increase in short circuit current in these 24 isolates was significantly different ($p < 0.001$–0.05) from the negative controls included in each experiment.

Haemagglutination

None of the 40 isolates of EAEC agglutinated with rabbit RBCs, although agglutination was seen with type strain 17-2 (Table II). A total of 30 (75%) isolates showed rapid HA with rat RBCs, of these 26 were MRHA, and four MSHA. In the microtitre assay, all of the eight isolates

Table I

DNA probing by colony blot hybridization of tissue-culture adherent E. coli

Adherence pattern	Number of isolates	Hybridization with DNA probes			
		EAgg	EAF	eae	DA
Aggregative	40	31	–	–	–
Localized	10	–	9	10	–
Diffuse	10	–	–	–	8
Mixed	12	2	6	6	1
Mixed	12	2	6	6	1

The aggregative adherence pattern refers to the isolates tested in this study. Hybridization patterns were compared by representative isolates from children with acute diarrhoea that had localized, diffuse and 'mixed' patterns of adherence (5).
EAgg probe, 700 bp *Eco*RI–*Pst*I insert in pCVD432 (8).
EAF probe, 1 kb *Sal*I–*Bam*HI insert in pCVD 315 (10).
eae probe, 1 kb *Sal*I–*Stu*I insert in pCVD434 (11).
DA probe, 390 bp *Pst*I insert in pSLM 862 (9).

tested were able to cause haemagglutination of rat RBCs at dilutions of more than one in 320. Of a total of 25 (62.5%) isolates that agglutinated human RBCs, 17 caused MRHA and eight of the remaining 23 showed MSHA. Sheep RBCs were agglutinated by 16 (40%) of the isolates, with 11 showing MRHA and five MSHA. All haemagglutinins appeared cell-associated, since no supernatants showed haemagglutination with erythrocytes of any species. Expression of haemagglutinins was dependent on temperature, with lack of haemagglutination with cultures grown at 42°C. There was no difference in the haemagglutinating abilities of cultures grown with and without shaking.

The isolates from the outbreak showed two patterns of haemagglutination, with five isolates of serotype OR:H4 all showing the same pattern of MRHA of rat RBCs and MSHA of human RBCs and five isolates of serotype O125:H12 showing MRHA of human, rat and sheep RBCs (Table III).

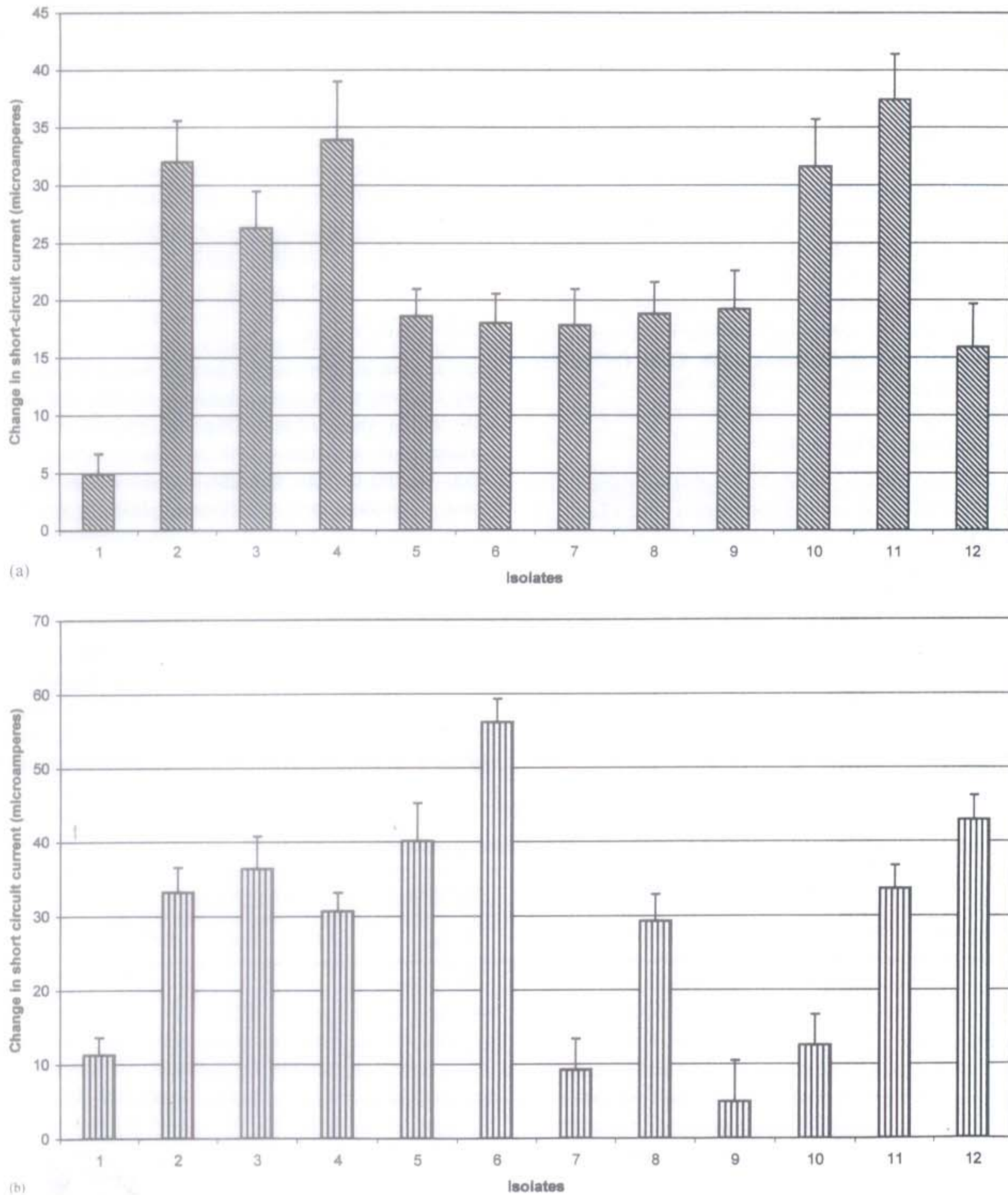

Fig. 1.

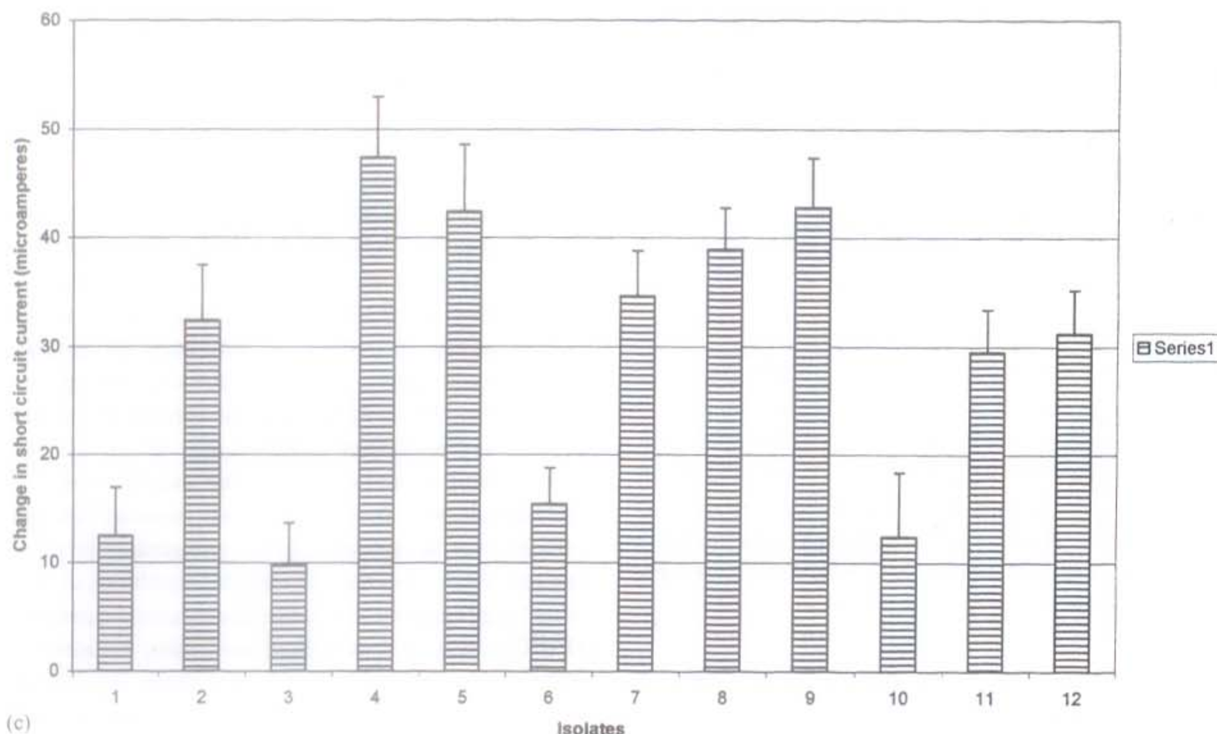

Fig. 1. Change in short circuit current across rabbit ileal mucosa mounted in Ussing chambers 60 min after addition of cell-free culture supernatants of isolates of enteroaggregative *E. coli*. Positive and negative controls included with each set of experiments were EaggEC strain 17-2 and Luria broth are numbered 1 and 2 in each set, respectively. Each bar represents the mean of three separate experiments with an isolate. All ten isolates from the diarrheal outbreak (a) and seven isolates each from children with acute diarrhoea (b) and control children (c) showed an increase in short-circuit current that was higher than the mean and two standard errors of the corresponding negative control. Isolates from adult controls were not tested.

Patterns of adherence

The type strain 17-2 showed adherence predominantly to the HEp-2 cells with little adherence to the surface of the slide between cells. The 40 isolates tested also showed predominantly cellular adherence, with honeycomb adherence being seen only in the samples from children, two from children with acute diarrhoea and one from a control (Table IV). The isolates from the outbreak of diarrhoea showed adherence predominantly to cells.

Internalization

In most previous studies on invasion/internalization, and in our study, EPEC strain 2348/69 has been shown to be internalized at levels above 5% (16). The type strain 17-2

Table II

Haemagglutination patterns of 40 clinical isolates of enteroaggregative E. coli

Subject group	Haemagglutination								
	Human			Rat			Sheep		
	HA	MR	MS	HA	MR	MS	HA	MR	MS
Acute diarrhoea	8	7	1	6	6	0	5	4	1
Diarrhoeal outbreak	10	5	5	10	10	0	5	5	0
Controls, children	5	5	0	7	3	4	3	1	2
Controls, adults	2	0	2	7	7	0	3	1	2
Total (n-40)	25	17	8	30	26	4	16	11	5

HA, total number showing haemagglutination.
MR, mannose-resistant, showing haemagglutination in the presence of mannose.
MS, mannose-sensitive, haemagglutination inhibited in the presence of mannose.

Table III

Patterns of mannose-resistant haemagglutination seen among 40 isolates of enteroaggregative Escherichia coli

MRHA			Pattern	Number of isolates (%)
Human	Rat	Sheep		
+	+	+	I	8 (20)
+	+	−	II	4 (10)
+	−	−	III	3 (7.5)
−	+	−	IV	14 (35)
+	−	+	V	2 (5)
−	−	−	VI	9 (22.5)

and 23 of the isolates were internalized at less than 5%, and all ten isolates from the diarrhoeal outbreak fell in this category (Table IV). Fourteen isolates were internalized at a level of 5–10%. Three isolates, all from children with acute diarrhoea, showed internalization levels of over 10%.

DISCUSSION

A variety of tests were been employed on a panel of EAEC isolated from patients and controls, in an attempt to identify a marker or markers that would enable us to distinguish diarrhoea associated EAEC from other isolates.

The first generation probe utilized in this study had been shown to be 89% sensitive in detecting EAEC isolates from Chile and India (8), but has been shown to have a sensitivity of less than 50% when tested against isolates from Thailand (17), and a sensitivity of 68.1% against isolates from Bangladesh (18). The 77.5% sensitivity seen here is slightly higher than that seen in most other studies, but the failure of the probe to identify all EAEC isolates suggests that EAEC in this area possibly produce more than one genetically heterologous adhesins that confer an aggregative adherence pattern. Among the other probes used, the *eae* probe (10/10) was more sensitive for LAEC than the EAF probe (9/10). The use of DNA probes helped in assigning isolates showing mixed patterns of adherence to specific classes of *E. coli*, but this has limited clinical significance.

The FAS assay was negative in all isolates that gave a clear AA adherence, but was positive in six isolates that had appeared aggregatively adherent in some areas of the slide, indicating that this test might be useful in distinguishing *E. coli* that show a mixed pattern of adherence. Early studies using this assay have consistently shown that isolates with AA pattern of adherence are negative (12, 19). While it appears that FAS positive isolates are never EAEC, this test does not help in distinguishing diarrhoea associated EAEC from others.

In Ussing chambers, secretory activity was seen on testing culture supernatants of all isolates from patients affected during the diarrhoeal outbreak, but not of all isolates from patients with acute diarrhoea or controls. Enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) was first described in Ussing chamber studies using rabbit ileal mucosa and culture supernatants of EAEC strain 17-2 (20). After the original description of EAST1 (20), few studies have actually tested EAEC isolates in Ussing chambers for secretory activity. Most subsequent reports have detected EAST1 gene sequences in *E. coli* isolates other than EAEC (21), and have found that these gene sequences are widely distributed among enterobacteriaceae. Recently, it has been reported that a 108 kDa heat-labile protein, designated Pet, induces cytotoxic effects and also functions as an enterotoxin, inducing short circuit current changes in rat jejunal mucosa in Ussing chambers (22), but this has been described so far only in strain O42, while EAST1 appears to be more wide-spread. Although one study has shown that EAST1 probe positivity is in concordance with secretory activity in Ussing chambers (23), this toxin does not appear to be a useful

Table IV

Characterization of 40 enteroaggregative Escherichia coli isolates from patients and controls by six different techniques

Isolates obtained (n) from	Adherence pattern			DNA	FAS	UC	Results of MRHA patterns						Invasion (%)		
	Ce	Gl	Ho				I	II	III	IV	V	VI	<5	5–10	>10
AD (n-10)	6	2	2	8	0	7	3	0	0	5	0	2	2	5	3
DO (10)	9	1	0	9	0	10	5	0	0	5	0	0	10	0	0
CC (10)	6	3	1	7	0	6	0	2	1	2	0	5	6	4	0
CA (10)	6	4	0	7	0	ND	0	2	2	2	2	2	5	5	0

Adh. patt, adherence pattern; Ce, cellular; Gl, glass; Ho, honeycomb.
DNA, colony hybridization with EAgg DNA probe from pCVD 432.
FAS, fluorescence actin staining assay; UC, secretory activity in Ussing chambers.
MRHA, mannose-resistant haemagglutination; ND, not done.
AD, isolates from children with acute diarrhoea; DO, isolates from diarrhoeal outbreak.
CC, control children; CA, control adults.

test to distinguish EAEC from other diarrhoea associated *E. coli*, or to distinguish diarrhoea associated EAEC from other isolates.

Determination of haemagglutination patterns did not demonstrate any clearly distinguishable differences between EAEC from patients and controls. None of the 40 isolates studied agglutinated rabbit erythrocytes which is in agreement with previous findings of EAEC haemagglutination (14, 24). The other three species derived red cells were agglutinated, with a total of six patterns of MRHA. In Quadri's detailed study (14) on haemagglutination patterns of 41 EAEC isolates, they used six types of RBCs and divided the isolates into 18 patterns based on MRHA. If the isolates from that study are classified by our patterns, pattern I would detect in 24/41 isolates, whereas in our study it is seen in 8/40 isolates. When the isolates obtained from the diarrhoeal outbreak are examined separately, they show two patterns of haemagglutination, which correspond with their serotypes.

In the only previous study on internalization of EAEC, 45% of 60 isolates were recoverable at 5% of the original inoculum or more (15). In this study, 42.5% of 40 isolates were internalized at levels above 5%, but again, internalization did not help in distinguishing diarrhoea associated EAEC isolates from those from controls.

Characterization of EAEC from sporadic cases of diarrhoea and controls showed that they were a heterogeneous group of organisms, only some of which possesses virulence factors such as toxin production and invasiveness (Table IV). This differed from the isolates obtained from the diarrhoeal outbreak, which belonged to only two serotypes, had similar haemagglutination patterns and invasive ability. A phylogenetic analysis of EAEC showed that they are heterogeneous with respect to chromosomal and plasmid-borne genes, but the majority houses a member of a conserved family of virulence plasmids (25). Our study and others seem to indicate that identification of genotypic and phenotypic markers of EAEC may be of use only in an outbreak setting or for characterization of a panel obtained from a particular geographic area (26). Despite extensive investigations, no single character or virulence factor has so far been identified as pathognomic of diarrhoeagenic EAEC.

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