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The significance of enteroaggregative *Escherichia coli* in the etiology of hospitalized diarrhoea in Calcutta, India and the demonstration of a new honey-combed pattern of aggregative adherence

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Abstract: Previous studies have identified enteroadherent *Escherichia coli* that exhibit localized adherence, diffuse adherence and atypical diffuse adherence as diarrhoeagenic agents associated with infantile diarrhoea in Calcutta, India. In this study, a DNA probe specific for enteroaggregative adherence was used to determine the etiological significance of enteroaggregative *E. coli* in the causation of diarrhoea. From a total of 330 strains of *E. coli* recovered from 159 cases of acute secretory diarrhoea and 174 cases of invasive diarrhoea, 20 strains hybridized with the probe, whereas of the 25 *E. coli* strains recovered from 25 healthy controls only 1 strain hybridized with the probe. Of the 21 probe positive strains, 19 adhered to HeLa cells in the typical stacked-brick pattern while 2 strains recovered from 2 cases of secretory diarrhoea adhered to the glass surface in a hitherto undescribed formation which we have termed, based on the appearance, as the honey-comb pattern. The enteroaggregative *E. coli* strains identified in this study did not produce any conventional enterotoxins and were significantly associated with patients with secretory diarrhoea (10.7%) than with invasive diarrhoea (1.7%). The results of this study indicate that enteroaggregative *E. coli* play a causal role in acute secretory diarrhoea in this part of the world which lends credence to the involvement of a potent toxin in the pathogenesis of EAggEC mediated infections.

Key words: Enteroaggregative *Escherichia coli*; Honey-comb pattern; DNA probe; Secretory diarrhoea

Introduction

Beginning as an additional means to support serogrouping for authenticating the identification of enteropathogenic *Escherichia coli* (EPEC) strains, tissue-culture adherence of isolates which do not belong to the classical EPEC O serogroups has now spawned yet another group of diarrhoeagenic *E. coli*. Based on the pattern of adherence to eukaryotic cells, *E. coli* strains iso-

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lated from patients with diarrhoea have been classified into three groups viz., localized adherence, enteroaggregative adherence and diffuse adherence [1].

Among strains exhibiting different patterns of adherence, enteroaggregative *E. coli* (EAggEC) have been increasingly recognized as one of the etiologic agents of persistent diarrhoea especially in children in developing countries [2]. Though the exact mechanism of pathogenesis of EAggEC is unknown, preliminary insights have been gained by electron microscopic and pathophysiological studies which suggests that EAggEC may be a large-bowel pathogen which colonize by a fimbrially mediated adhesion mechanism. EAggEC have been documented to elaborate a heat-stable enterotoxin [3] and a heat-labile toxin antigenically related to *E. coli* hemolysin [4]. EAggEC are characterized by their typical mannose resistant 'stacked-brick' like lattice pattern of adherence to HeLa and HEp-2 cells in tissue culture [1]. A ~ 60 MD plasmid reportedly confers the expression of the aggregative phenotype [1,3].

Epidemiological studies from India [2] and Chile [5] have incriminated EAggEC in paediatric diarrhoea. In an earlier study on enteroadherent *E. coli* conducted in Calcutta, we were unable to detect the aggregative pattern of adherence among *E. coli* strains recovered from hospitalized infants aged below 6 months or from aged-matched controls [6]. Recently a 1 kb fragment from the plasmid of 17-2, a prototype EAggEC strain from Chile, was found to be a highly specific DNA probe for identifying EAggEC [7]. The availability of the probe allowed us to study isolates recovered from hospitalized cases of secretory and inflammatory diarrhoeic patients with the view to evaluating the role of EAggEC as an etiologic agent of hospitalized diarrhoea in Calcutta.

Materials and Methods

Source and characterization of strains

A total of 330 strains of *E. coli* recovered from 159 cases of acute secretory diarrhoea and from 174 cases of invasive diarrhoea admitted to the Infectious Diseases Hospital, Calcutta between July 1989 and November, 1991 were examined in this study. Another group of 25 strains from 25 healthy subjects from our field surveillance area were included as controls. The fecal samples examined from all the above categories were collected in sterile MacCartney bottles and immediately transported to the laboratory for bacteriological analysis of enteropathogens using standard techniques (WHO Manual, 1983). Lactose fermenting colonies from MacConkey agar (Difco Laboratories, MI, USA) were picked, biochemically characterized as *E. coli* and maintained in nutrient agar slants at room temperature to subsequently ascertain the virulence status of the isolates.

Toxin production

All the 330 strains of *E. coli* isolated in this study were initially examined for the heat-stable enterotoxin (ST) by the suckling mouse assay (WHO Manual, 1983), for heat-labile enterotoxin (LT) by the Chinese hamster ovary cell-line assay (WHO Manual, 1983) and for verotoxin (VT) production by the Vero cell-assay [8]. The broth media used for assessing production of ST, LT and VT were casamino acid yeast extract broth (casamino acid 2 g, yeast extract 0.6 g, NaCl 0.25 g, K_2HPO_4 0.872 g, $MgSO_4 \cdot 7H_2O$ 103 μg, $MnCl_2 \cdot 4H_2O$ 8.3 μg, distilled water 100 ml), tryptic soy broth (Difco) and Penassay broth (Difco), respectively.

EAggEC DNA probe

The failure of our previous efforts to detect the aggregative phenotype in the adherence assay prompted us to search for EAggEC using the recently developed highly specific EAggEC DNA probe. The EAggEC DNA probe comprised of an approximately 1 kb *Eco*RI-*Pst*I digestion fragment of the plasmid of the prototype EAggEC strain 17-2 contained in pCVD432 subcloned into the *Bam*HI site of the vector pUC19. All the 660 *E. coli* strains isolated from the various categories of samples were first examined by this probe.

Preparation of plasmid DNA and labelling the probe

Plasmid DNA was isolated by the alkaline lysis method [9]. Restriction endonucleases were used

according to the recommendations of the suppliers (Genei, Bangalore, India). Gel purified restriction fragment was prepared by standard techniques [9] and the purified DNA fragment was labeled in vitro with α^{32}P deoxynucleotide triphosphate (BRIT, Bombay) by nick translation. Labeled DNA was purified by Sephadex G 50 chromatography. The double stranded labeled DNA was heat denatured and used as the probe.

DNA colony blot hybridization test

Test organisms were inoculated onto the nitrocellulose filters placed on LB agar and incubated at 37°C overnight. The membranes containing the freshly grown strains were placed successively on the top of a denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 7 min, twice on a neutralization solution (0.5 M Tris-HCl [pH 7.2], 1.5 M NACl) for 4 min each, and finally on 2 × Sodium Saline Citrate solution (SSC, where 1 × SSC contains 0.15 M NaCl and 0.015 M sodium citrate). The membranes were air dried and baked at 80°C for 2 h. Colony blots of the test strains immobilized on nitrocellulose filters were incubated at 65°C for 2 h in the following hybridization solutions: 4 × SSC, 5 × Denhardt solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% nuclease free bovine serum albumin Fraction V) and fragmented heat denatured 100 μg/ml Salmon sperm DNA. The filters were then transferred to fresh hybridization solution containing 10^6 cpm of heat denatured DNA probe and incubated at 65°C for 18 h. The filters were washed twice at 65°C in 2 × SSC containing 0.1% sodium dodecyl sulfate (SDS) for 20 min each, twice in 0.2 SSC with 0.1% SDS for 20 min each, rinsed in 0.2 × SSC at room temperature and air dried. The filters were exposed to X-Omat-R X-Ray film to obtain autoradiographic image.

Adherence assay

Strains of *E. coli* which tested positive with EAggEC probe were tested for their ability to adhere to HeLa cells in the presence of D-mannose by the method of Craviato et al. [10]. Briefly, 20 μl (approx. 10^7 cfu) of bacterial culture, grown overnight in tryptic soy broth (Difco) with agitation at 37°C, was added to HeLa cells grown to approx. 60% confluence on plastic Petri dishes containing sterile glass cover slips. The inoculated tissue culture cells were incubated for 3 h at 37°C in 5% CO_2 in air. Adherence to HeLa cells was also examined in the presence of 1% mannose. After incubation, the cover slips were washed with PBS (pH 7.2), dried in air, fixed in methanol for 2 min, stained with 1% Giemsa in methanol and examined under bright-field illumination. The pattern of bacterial adherence to HeLa cells was evaluated according to the published description [1].

Serotyping

O and H antigens of EAggEC strains were determined using standard agglutination methods.

Antimicrobial susceptibility

The EAggEC strains isolated in this study were further investigated for their in vitro susceptibility to antimicrobial agents which included older and newer classes of quinolones, broad spectrum penicillin, chloramphenicol, tetracycline, aminoglycoside and trimethoprimsulphamethoxazole using the disc diffusion method [11].

Results

Of the 159 strains recovered from cases of acute secretory diarrhoea, 17 strains hybridized with the EAggEC DNA probe while of the 174 strains from cases of non-secretory diarrhoea, 3 strains hybridized with the same probe. One strain from the 25 healthy control subjects hybridized with the EAggEC probe. Among the 17 cases of secretory diarrhoea from which EAggEC was isolated, 11 were solely infected by EAggEC while in 6 cases EAggEC was isolated in association with other enteropathogens like *V. cholerae* O1 (4 cases), *V. cholerae* O1 and *V. mimicus* (1 case) and *V. cholerae* non-O1 (1 case). All the 3 cases of non-secretory diarrhoea from which EAggEC was isolated were solely infected by EAggEC.

EAggEC were, therefore, detected in faecal samples from 6% (20/333) of patients and were the only potential pathogen detected in 4.2%

(14/333) of patients. The EAggEC strains identified in this study did not produce any conventional enterotoxins and were significantly associated with patients with secretory diarrhoea (10.7%) than with invasive diarrhoea (1.7%). The other enteropathogens isolated from the 159 secretory diarrhoea cases were *V. cholerae* O1 (59.7%), *V. cholerae* non-O1 (8.2%), enterotoxigenic *E. coli* (3.7%), EPEC (8.8%), *S. typhimurium* (1.9%), *V. parahaemolyticus* (3.1%) and in 12 cases, we were unable to detect a known etiologic agent. Among the 174 cases of invasive diarrhoea examined in this series the other enteropathogens isolated were *Shigella* spp. (54.6%), *V. parahaemolyticus*, *Plesiomonas shigelloides* and *Acinetobacter calcoaceticus* (1.1% each) and in 70 cases we were unable to detect a defined etiology.

Of the 20 EAggEC strains isolated, ONT(not typable):H21 was the dominant serotype (11 cases) followed by O86:H18 (3 cases), ONT:H7 (2 cases) and one each of O131:H12, ONT:H27, ONT:H40 and O92:HNT. The strain from the healthy control was found to belong to the serotype ONT:H1. Both the strains belonging to the serotype ONT:H7 and the single strain belonging to the serotype O92:HNT were isolated from cases of invasive diarrhoea. Further, serotyping of multiple strains taken from 16 patients from whom EAggEC was detected, revealed that all strains from a single case invariably belonged to the same serotype indicating that infection was caused by a single serotype in each case.

Retrospective analyses by the HeLa cell adherence assay of the 21 strains of EAggEC which hybridized with the probe revealed complete concordance between the probe and adherence assay. All probe positive strains exhibited aggregative adherence in the HeLa cell assay. There was, however, two distinct patterns of enteroaggregation; of the 21 strains examined, 19 showed the typical stacked bricked pattern of aggregation both on the surface of the HeLa cells as well as on the glass coverslip free from the cell (Fig. 1A) as originally described [1]. The other pattern of aggregation shown by 2 strains of *E. coli* from 2 cases of acute secretory diarrhoea was different, but again with bacterial cells adhering to the HeLa cell surface as well as to the glass surface. Based on the appearance of this pattern of aggregation, we have termed this as a honey-comb pattern of aggregation (Fig. 1B). The honey-comb pattern of aggregative adherence was consistently reproducible. Interestingly, the serotypes of the two strains showing the honey-comb pattern of

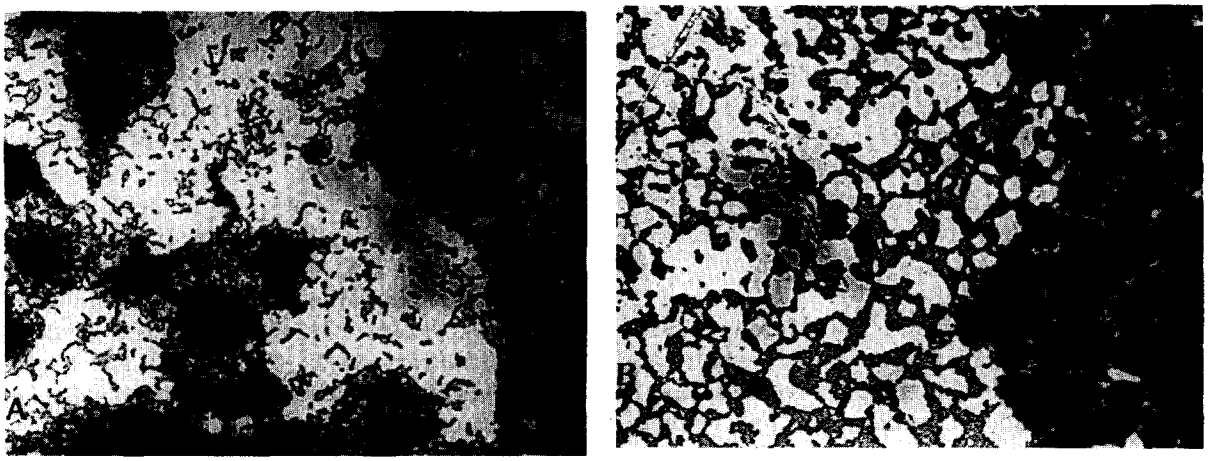

Fig. 1. Photomicrograph showing the typical 'stacked-brick' pattern (**A**) of adherence to HeLa cells of an EAggEC strain and the newly described honey-comb pattern (**B**) of aggregation of an EAggEC strain.

Table 1

Clinical profile of patients solely infected with enteroaggregative *Escherichia coli*

Characteristics	No.	Percent
Stool character:		
Watery	11	78.57
Dysenteric	3	21.42
Abdominal pain:	13	92.85
Dehydration:		
None	3	21.42
Some	1	7.14
Severe	10	71.42
Duration of diarrhoea before hospitalization (h):		
Mean ± SD	14.90 ± 2.70	
Temperature (> 38.8°C)	10	71.42

aggregative adherence were O131 : H12 and O? : H27 and were different from the serotypes of strains showing the stacked brick pattern.

Age distribution and the clinical presentation of the 14 patients solely infected with EAggEC ranged between 7 months and 50 years (mean ± SD = 16.25 ± 14.36). Of the 14 patients, 10 had fever and 13 complained of abdominal pain (Table 1). All the EAggEC strains examined in this study gave negative responses in the suckling mouse assay, CHO and Vero cell assays indicating that none of them produced heat-stable, heat-labile or Vero toxins. All the 21 strains of EAggEC examined were resistant to tetracycline, one-third of the strains were resistant to chloramphenicol, ampicillin and cotrimoxazole while all the strains were sensitive to furazolidone, gentamycin, nalidixic acid, norfloxacin and ciprofloxacin.

Discussion

Previously referred to as enteroadherent *E. coli* or enteroadherent-aggregative *E. coli*, EAggEC are now discerned as the fifth category of diarrhoeagenic *E. coli*. Epidemiological studies have implicated EAggEC as a cause of diarrhoea in infants and young children in India [2]. However, in another setting, a yearlong case-control study of infantile diarrhoea in Bangkok seemed to indicate that *E. coli* with diffuse or aggregative adherence to HeLa cells or that which hybridized with the F1845 (probe for fimbrial adhesin responsible for the diffuse HEp-2 adherence [12]) and EAggEC probes were not associated with infantile diarrhoea [13]. The purpose of this study was to establish the etiologic role of EAggEC in the causation of diarrhoea in Calcutta. A previous investigation on the role of enteroadherent *E. coli* in the less than 6 months age-group had yielded inconclusive results since we were unable to detect the aggregative type of adherence by the HeLa cell assay [6]. Therefore, in this study, we resorted to the use of the EAggEC probe constructed to identify aggregative pattern of adherence in *E. coli* strains recovered from all age-groups and from both acute and inflammatory hospitalized diarrhoea cases.

While there was no apparent predilection for any particular age-group, our studies clearly suggest that EAggEC play a causal role in acute secretory diarrhoea but not in inflammatory diarrhoea. These results are in tandem with recent reports which are beginning to indicate that the pathogenicity of EAggEC is mediated by a toxin because they do not induce the attaching and effacing lesion characteristic of EPEC [14]. Further, experimental EAggEC infection in isolated rabbit and porcine intestinal loops appear to indicate the EAggEC may elaborate a toxin that damages enterocytes [15]. It has recently been demonstrated that EAggEC elaborate a low molecular mass partially heat-stable enterotoxin which induces ion transport alterations consistent with a secretory response [3]. A heat-labile toxin antigenically related to *E. coli* hemolysin which could promote cellular changes in vitro and these changes may be important factors in the development of diarrhoea in vivo has also been documented [4]. The association of one or both of these factors in the pathogenesis of EAggEC mediated secretory diarrhoea seems likely because none of the 21 strains recovered in this study produced any of the conventional heat-labile, heat-stable or vero toxins.

The different categories of diarrhoeagenic *E. coli* are known to belong to distinct O : H

serotypes. In this study, 5 of the EAggEC strains were distributed among three serogroups O131, O86 and O92 while in the remaining 16 the O antigen was untypable. *E. coli* strains of serogroups O86 and O92 have also been isolated from different geographical locations [14] from infants with acute and persistent diarrhoea suggesting that the aggregation phenotype is conserved among certain specific serotypes of *E. coli*. However, in contrast to the Chilean EAggEC strains [14] in which the most common H antigen was type 33, the predominant H antigen encountered was type 21 among the 11 EAggEC strains in this study; however, all these strains were O antigen untypable.

The fact that all the probe-positive EAggEC strains examined in this study correlated with the retrospectively performed tissue culture cell adhesion assay indicates that the EAggEC DNA probe is a sensitive method for identifying *E. coli* strains with the aggregative adherence property. At this time we are unable to comment on the specificity of the probe because all the probe-negative strains were not examined by the HeLa cell assay. The transfer of 55–65 MDa plasmid into a non-aggregative *E. coli* HB101 strain by genetic manipulations and subsequent expression of the aggregative phenotype indicates the importance of the plasmid in encoding some putative virulence properties of EAggEC. However, what seems interesting is the ability of the EAggEC probe to pick up strains which showed the honey-comb pattern of adherence as well as the typical stacked-brick pattern. It is apparent that there might be some other factor having homology at the DNA level with the probe. It remains to be seen whether the atypical phenotype is linked to the same plasmid as found in the typical EAggEC. Detailed genetic analysis and cloning are in progress to identify the putative factor(s).

Clinically, the disease caused by EAggEC was not distinctive and cannot be differentiated from that caused by other toxigenic etiologies. The fact that all the patients solely infected with EAggEC were moderately to severely dehydrated again suggests the involvement of a potent toxin. The illnesses associated with EAggEC differed from other toxigenic bacterial etiologies only in having a tendency to a higher frequency of abdominal pain and to the presence of fever > 38.8°C. Clinical isolates of EAggEC isolated from patients with diarrhoea from Mexico, Chile and Peru showed marked drug resistance to sulphamethaxozole, ampicillin and chloramphenicol [16] which is very similar to that observed in this study. Broad spectrum gentamycin and quinolones showed good activity against EAggEC.

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