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Examination of archetypal strains of enteropathogenic *Escherichia coli* for properties associated with bacterial virulence

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Summary. Nine strains of *Escherichia coli* isolated from infants with diarrhoea between 1947 and 1960 and designated "enteropathogenic" were examined for phenotypic and genetic characters associated with virulence. Each strain belonged to a different serotype. All the isolates were historically significant in that they were amongst the first strains of *E. coli* reported to be causally associated with infantile diarrhoea. Five strains possessed the virulence properties of class I enteropathogenic *E. coli* (EPEC). All these strains were isolated originally from symptomatic children during outbreaks of diarrhoea. Two isolates from sporadic cases of diarrhoea fulfilled the criteria for classification as class II EPEC. One strain was identified as enteroaggregative *E. coli* and the other carried no known virulence-associated properties. These findings indicate that most early isolates of *E. coli* which were designated "enteropathogenic" were indeed EPEC, as currently defined.

Introduction

Escherichia coli was suspected as a cause of diarrhoea because of its association with nosocomial outbreaks of severe neonatal diarrhoea during the 1940s and 1950s.[1] These strains were designated enteropathogenic *E. coli* (EPEC) to distinguish them from strains of *E. coli* that caused urinary and other extra-intestinal infections.[1,2] At the time, no virulence factors were known for these bacteria and they were identified by O-serogrouping alone. This practice was predicated on the observation that some serogroups were associated more frequently with outbreaks of diarrhoea than others.[1]

The subsequent discovery of enterotoxigenic *E. coli* (ETEC) and enteroinvasive *E. coli* (EIEC), which were identified by their ability to secrete enterotoxins and to invade epithelial cells, respectively, cast doubt on the pathogenicity of EPEC, because the latter possess neither of these traits.[1,3] Furthermore, the observation that ETEC and EIEC do not belong to the same serogroups as those that include EPEC brought into question the value of serogrouping as a diagnostic tool.[3–6] Some investigators postulated that the original EPEC strains isolated during the 1940s and 1950s were not pathogens, or were strains of ETEC or EIEC that had lost their virulence determinants.[1]

More recently, however, detailed examination of serogrouped EPEC strains from various sources has shown that they are a distinct category of enteric pathogens that are characterised in part by their ability to produce distinctive microscopic lesions in the intestinal mucosa.[7,8] The capacity to produce these lesions, known as attachment and effacement or microvillus dissolution, is associated with a chromosomal locus, termed *eae*.[9] A closely related gene is found in another pathogenic category of *E. coli*, designated enterohaemorrhagic *E. coli* (EHEC), which also produce attaching-effacing lesions but can be distinguished from EPEC by their ability to produce large amounts of one or more cytotoxins, known as verotoxins or Shiga-like toxins (SLT).[10–13] The ability of *E. coli* to produce attachment-effacement lesions can be demonstrated by microscopical examination of the intestinal mucosa from susceptible animals,[7,8,11] or, *in vitro*, by a fluorescent actin staining (FAS) technique, which relies on the ability of the bacteria to induce polymerisation of actin in cultured epithelial cells.[14,15]

A second genetic locus, found in most EPEC strains, is the EPEC attachment factor (EAF). This is located on a plasmid that regulates expression of *eae* and encodes the ability of bacteria to adhere to cultured epithelial cells in a distinctive pattern known as localised adherence.[9,16] EPEC that carry EAF are designated class I EPEC.[16] These bacteria tend to be associated with outbreaks of diarrhoea, although they are also common in sporadic cases. Class II EPEC are EAF-negative and are generally found in sporadic cases of diarrhoea only.[16]

The recognition of virulence properties in EPEC has permitted these bacteria to be identified in terms of

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their ability to produce attachment-effacement lesions in animals (or a positive FAS test) and their failure to elaborate SLT. Class I EPEC strains are further defined by their ability to adhere to cell culture in a localised pattern. The identification of the genes required for these various virulence-associated properties has led to the development of DNA probes to identify EPEC.

We have used these tools to characterise nine strains of *E. coli* of different serotypes that had been designated "enteropathogenic" when isolated originally from infants with diarrhoea between 1947 and 1960. All of these strains were of historical significance in that they were amongst the original isolates that led to the recognition of *E. coli* as a cause of diarrhoea. The aim of this study was to determine whether these bacteria fulfilled the criteria for classification as EPEC as currently defined.

Materials and methods

Bacteria

The bacterial strains investigated are listed in the table. Each belonged to a different serotype. All the strains had been reported previously as amongst the first isolates of that particular serotype in infants with diarrhoea.[1,17–25] Six strains were obtained from symptomatic infants during outbreaks of diarrhoea between 1947 and 1960. Three were isolated from sporadic cases. Before testing, all strains had been stored at ambient temperature on Dorset egg medium and subcultured fewer than three times during 30–40 years. Bacteria were encoded so that the investigators who performed the virulence assays were unaware of their origin and their behaviour in other assays.

Bacterial adhesion to cell culture

The pattern of bacterial adherence to HEp-2 cells was determined by the 3-h "CVD" method reported previously.[26,27] Bacteria were designated non-adherent if $< 5\%$ of 200 cells examined had five or more attached bacteria. The fluorescent actin staining (FAS) assay was performed with 3-h and 6-h incubation periods, as described by Knutton *et al.*[15]

Assay for attaching and effacing capacity

The ability of *E. coli* to produce attachment and effacement lesions *in vivo* was determined in ligated loops of rabbit ileum. These were constructed in 2-kg female New Zealand White rabbits, which had been starved of food for 24 h. Rabbits were anaesthetised with sodium pentobarbitone 50 mg/kg given intravenously and lignocaine hydrochloride locally. A paramedian incision was made and the terminal ileum was identified and doubly ligated close to the appendix, with care not to interfere with the blood supply. A 1-ml suspension of *c.* 10^7 bacteria, grown overnight at 37°C in L-broth and washed in phosphate buffer, was inoculated into a 10-cm segment of intestine. Each segment was doubly ligated at both ends, and separated from adjacent experimental segments by *c.* 2 cm of uninoculated intestine. Up to 10 loops were constructed in each rabbit and each strain was examined in at least two separate animals. At the conclusion of the operation, the abdomen was closed in layers and the animals were left for 18 h. Rabbits were killed by a lethal injection of sodium pentobarbitone; the intestine was removed, incised along the antimesenteric border and fixed in glutaraldehyde solution. Tissues were processed for light and electron-microscopy as described previously.[8]

Assay for cytotoxin

The ability of bacteria to produce cytotoxin (SLT) was examined in Vero cells by the method of Marques *et al.*[28]

Hybridisation with DNA probes

Selected DNA probes were investigated for their ability to hybridise with the test bacteria at high stringency (incubation at 68°C and inclusion of a final wash in low salt buffer at 68°C) by the colony blotting technique described previously.[29,30] The DNA probes were derived from the following plasmids (the corresponding virulence-associated property is shown in parentheses): pCVD403 (heat-labile enterotoxin type I of ETEC); pCVD402 (heat-stable enterotoxin of ETEC, human subtype); pCVD404 (heat-stable enterotoxin of ETEC, porcine subtype); pCVD419 (adhesion-association plasmid of EHEC); pCVD432 (adhesion-associated plasmid of enteroaggregative *E. coli*); pCVD434 (*eae* gene, associated with the attaching-effacing ability of EPEC and EHEC); pJPN16 (EAF, associated with the localised-adherence phenotype of class I EPEC); pJN37-19 (SLT-I of EHEC); pNN110-18 (SLT-II of EHEC); and pRM17 (enteroinvasive capacity of EIEC).[9,29,31,32] Appropriate positive and negative control strains were included in all assays.

Results

The results of the assays for virulence-associated determinants are shown in the table. Generally, there was excellent agreement between the results of the phenotypic and genotypic assays. All seven strains that hybridised with the *eae* probe produced attachment and effacement lesions in rabbit ileum and gave positive results in the FAS assay. One of the strains, E611 (O126:H2), gave a positive result in the FAS assay only after incubation with the cell culture for 6 h. This was necessary because the bacteria had not adhered to the cells in sufficient numbers after 3 h to allow the assay to be interpreted with confidence.

Table. Characters of *E. coli* strains investigated

Strain no.	Serotype	Country of origin	Source	Year isolated	Adherence to HEp-2 cells		Attaching and effacing in rabbit ileum	Hybridisation with DNA probe for
					Pattern	FAS		
F41[17]	O26:K60(B6):H−	Denmark	Sporadic diarrhoea	Before 1950	LA	+	+	*eae*
B. coli β[18,19]	O55:K59(B5):H6	Scotland	Diarrhoea epidemic	1947	LA	+	+	*eae*, EAF
E990[20,21]	O86:K61(B7):H−	England	Diarrhoea epidemic	1950	LA	+	+	*eae*, EAF
F1961[21]	O86:K62(L):H2	Denmark	Sporadic diarrhoea	Before 1954	AA	−	−	AA
Stoke W[18,19]	O111ab:K58(B4):H−	Scotland	Diarrhoea epidemic	1947	LA	+	+	*eae*, EAF
E464[22]	O125ab:K70(B15):H19	England	Diarrhoea epidemic	1949	NA	−	−	—
E611[22]	O126:K71(B16):H2	England	Sporadic diarrhoea	1949	NA	+	+	*eae*
E56/54[23]	O128ab:K67(B12):H2	England	Diarrhoea epidemic	1953	LA	+	+	*eae*, EAF
C771[24]	O142:K86(B):H6	Indonesia	Diarrhoea epidemic	Before 1960	LA	+	+	*eae*, EAF

FAS, fluorescent actin staining; LA, localised adherence; NA, not adherent (3-h assay); AA, aggregative adherence; *eae*, *E. coli* attaching-effacing gene; EAF, EPEC attachment factor; SLT, Shiga-like toxin.

Accordingly, this strain was classified as non-adherent in the 3-h HEp-2 cell assay.

All the bacteria that hybridised with the EAF probe showed localised adherence to HEp-2 cells. One strain, F41 (O26:H−), which was not recognised by this probe, also demonstrated localised adherence.

Strain F1961 (O86:H2) hybridised with the probe derived from the plasmid of a strain of entero-aggregative *E. coli* (EAggEC) and was the only strain to demonstrate an aggregative pattern of adherence to HEp-2 cells. None of the strains produced detectable amounts of SLT or hybridised with the probes for SLT-I, SLT-II or the EHEC adherence factor (CVD419). No strain was recognised by the probes for *E. coli* enterotoxins or invasion-associated genes.

Discussion

The results of the assays for virulence determinants permitted the classification of the *E. coli* strains into four groups: class I EPEC, class II EPEC, EAggEC and non-pathogenic. Of the nine strains investigated, five were identified as class I EPEC because they adhered to HEp-2 cells in a localised pattern, gave positive results in the FAS assay, produced attachment-effacement lesions in rabbit ileum, and hybridised with DNA probes derived from *eae* and EAF. These strains were "*B. coli β*" (O55:H6), E990 (O86:H−), Stoke W (O111:H−), E56/54 (O128:H2), and C771 (O142:H6). All these isolates were originally obtained from affected infants during outbreaks of diarrhoea (table). Of the three strains from sporadic cases, two were class II (EAF-negative) EPEC and one was EAggEC.

One class II EPEC strain, F41 (O26:H−), adhered strongly to HEp-2 cells in a localised pattern, but was not recognised by the EAF probe. This result was unexpected, because all of the class II EPEC strains we have examined previously adhere poorly to HEp-2 cells (unpublished data). *E. coli* F41 may carry a novel adhesin, genetically unrelated to EAF, but analogous to that reported recently in some localised-adherent, EAF-negative EPEC strains in serogroups O55, O111 and O128.[33,34]

One of the strains, F1961 (O86:H2), isolated from a sporadic case of diarrhoea, was categorised as EAggEC by virtue of its characteristic pattern of adherence to cell culture and its ability to hybridise with the corresponding DNA probe. This strain represents the first documented isolate of EAggEC, a pathogenic category of *E. coli* which was described only recently.[35] The finding that this strain belongs to an EPEC serotype is noteworthy in light of the recent report by Scotland *et al.*[34] that a number of putative EPEC strains in serotypes O44:H18, O111ab:H21 and O126:H27, obtained from patients with diarrhoea in the UK, were in fact EAggEC.

One of the strains examined in this study, E464 (O125:H19), possessed none of the known virulence-associated factors of diarrhoeagenic *E. coli*. This isolate, originally designated the "Canioni strain", was obtained during a nursery outbreak of infantile diarrhoea in London during 1949.[22] An unusual feature of this outbreak was that the putative aetiological agent colonised a number of babies without causing diarrhoea until 8 weeks after it had been introduced into the nursery. This suggests that it probably was not the cause of the patients' symptoms. Other evidence against this strain being a pathogen is the fact that bacteria of this serotype have not been identified in association with outbreaks of diarrhoea since the original report in 1952.[25,36]

In conclusion, we have shown that of nine arche-

typal strains of "enteropathogenic" *E. coli* isolated before 1960, five were class I EPEC, as defined currently. Two strains, from sporadic cases of diarrhoea, failed to hybridise with the EAF probe, and accordingly were designated class II EPEC. Of the two non-EPEC strains, one was identified as EAggEC; the other carried no known virulence determinants. None of the strains in this study possessed the virulence-associated properties of ETEC or EIEC, and none produced SLT characteristic of EHEC. Our findings are in broad agreement with those of Moyenuddin *et al.*,[36] who investigated the adhesive properties of strains of *E. coli* obtained during outbreaks of diarrhoea in the USA from 1934 to 1987, and found that most putative EPEC strains displayed localised adherence to HeLa cells and hybridised with the EAF probe. Taken together, the results of these two investigations indicate that most strains of EPEC designated "enteropathogenic" when first identified prior to 1960, were in fact EPEC as defined currently. Our findings also confirm the suggestion that class I EPEC are more likely to be associated with epidemics of diarrhoea than class II strains.[16]

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