Identification of enteropathogenic *Escherichia coli* isolated in Britain as enteroaggregative or as members of a subclass of attaching-and-effacing *E. coli* not hybridising with the EPEC adherence-factor probe

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Summary. Strains of *Escherichia coli* from sporadic cases of diarrhoea and belonging to serotypes O44:H18, O55:H7, O111ab:H21, O111ab:H25 or O126:H27 were examined for virulence properties. With the exception of O111ab:H25 these are considered to be classical enteropathogenic *E. coli* (EPEC) serotypes. The strains had been isolated in Britain from the faeces of children < 3 years old. Of the serotypes examined, 7 of 13 O44:H18 strains, all of 10 O111ab:H21 strains and 13 of 21 O126:H27 strains belonged to the enteroaggregative class of *E. coli* (EAggEC) that attached to HEP-2 cells in the characteristic aggregative pattern and hybridised with the EAggEC probe. They also caused mannose-resistant haemagglutination of rat erythrocytes, a property which may be a useful marker for their identification. Strains of O44:H18 with similar properties were also isolated from three small outbreaks in Britain, one of which involved elderly patients. EAggEC have not been considered previously as aetiological agents of diarrhoea in developed countries and have rarely been reported as belonging to EPEC serotypes. All 15 O55:H7 strains and seven of eight O111ab:H25 strains were also considered to be potentially diarrhoeagenic as they gave localised attachment (LA) to HEP-2 cells that resulted in a positive fluorescence actin-staining test. This test is considered to correlate with the attaching-and-effacing virulence mechanisms of EPEC *in vivo*. None of the strains in this study hybridised with the EPEC adherence-factor (EAF) probe. Neither the aggregative EPEC nor the LA-positive EAF-negative EPEC described here would be identified in epidemiological surveys when the EAF probe is used in the absence of cell tests.

Introduction

Some classes of *Escherichia coli* causing diarrhoea in man possess well-characterised virulence properties such as production of heat-labile enterotoxin (LT), heat-stable enterotoxin (ST_A) or Vero cytotoxin (VT). For the enteropathogenic *E. coli* (EPEC), the virulence mechanisms are less clear. In most clinical laboratories, EPEC are identified because they belong to certain O serogroups that have been associated with outbreaks of infantile diarrhoea and it is probable that only a proportion of strains belonging to these O serogroups are virulent.¹ Strains of *E. coli* have been characterised by the pattern of their attachment to tissue-culture cells that can be localised (LA), diffuse (DA) or aggregative (AggA).^{2–4} LA has been shown to be an important property of EPEC.^{2,5,6} An LA-positive strain E2348/69 (*E. coli* O127:H6) was confirmed as diarrhoeagenic in human-volunteer

studies and in this strain a particular plasmid was necessary for full virulence and for good LA.^{5,7} A 1-kb probe, the EPEC adherence-factor (EAF) probe developed from this plasmid,⁸ has been used in a few laboratories for epidemiological studies as a means of recognising pathogenic strains.

We have used the EAF probe to examine strains belonging to 13 different EPEC serogroups and isolated from cases of diarrhoea in Britain;⁹ only 23 of 353 strains hybridised with the EAF probe. It was noted that the EAF-positive strains did not include strains of serotypes O44:H18, O55:H7, O111ab:H21, O111ab:H25 and O126:H27 which accounted for 20% of the EPEC strains examined. With the exception of O111ab:H25, these serotypes are included in the list of classical EPEC serotypes.¹ Strains belonging to these five serotypes have now been characterised further with respect to other putative virulence factors that have been described for diarrhoeagenic *E. coli*.

In EPEC infections large numbers of bacteria attach to the small intestine. There can be localised

destruction (or effacement) of the microvilli and bacteria causing these lesions have been called attaching and effacing *E. coli* (AEEC).¹⁰ AEEC affect the status of actin in infected tissue-culture cells and this can be visualised in the fluorescence actin-staining (FAS) test.¹¹ To date only strains giving LA have been shown to give positive results in the FAS test^{11,12} but these include strains that hybridise with the EAF probe and others that do not. For example, strains of EPEC serogroup O128 giving LA may be EAF-positive^{6,13,14} or EAF-negative¹⁴ and, as both classes are positive in the FAS test, both are probably AEEC. Most strains of O26:H11, which is considered an EPEC serotype, are EAF-negative but LA-positive and FAS-positive, and so are probably also AEEC.¹² Thus, there exists a subclass (or subclasses) of AEEC comprising strains that are EAF-negative. We have examined strains of O44:H18, O55:H7, O111ab:H21, O111ab:H25 and O126:H27 for their pattern of attachment to tissue-culture cells and for their reaction in the FAS test.

The role of *E. coli* giving an aggregative pattern of attachment to tissue-culture cells (EAggEC) in diarrhoea has been a matter of some controversy. They have been associated with diarrhoea in children in India where the number of isolations was significantly greater from cases than from controls.^{15,16} In contrast, cases and controls yielded similar numbers in studies in Brazil and Chile.^{17,18} These differences may in part be due to the difficulties reported in the assessment of attachment as LA, AggA or DA in tissue-culture tests. Different test procedures have been used and Vial *et al.*¹⁹ concluded that recognition of the aggregative pattern is best with an initial incubation period of 3 h rather than a 30-min period. We have always used the 3-h initial incubation period and have also found it advantageous to include a second 3-h incubation period for the easy identification of some groups of LA-positive strains.^{12,14} A probe has been developed to detect EAggEC²⁰ and, in addition to determining the pattern of attachment of the strains in the present study, they were also tested for hybridisation with this probe.

Materials and methods

Bacterial strains

For the main study, the strains of *E. coli* were isolates from sporadic cases of diarrhoea in Britain since 1985 and belonged to serotypes O44:H18, O55:H7, O111ab:H21, O111ab:H25 and O126:H27. All were isolated from the faeces of children <3 years old. The numbers of each serotype are shown in table I. Additional strains belonging to these serotypes from our culture collection that had been isolated from outbreaks of diarrhoea were also tested.

For comparison, the adhesive properties of strains from the faeces of 32 healthy children were deter-

mined. A representative of each serotype excreted by the child was tested and there were 50 strains belonging to 40 different serotypes. All strains that adhered to HEp-2 cells were tested for hybridisation with the EAggEC probe.

Adhesion and fluorescence actin-staining tests

The strains were tested for attachment in the presence of D-mannose 1% w/v to HEp-2 cells in a 6-h test at 37°C as described before;²¹ the cells were washed after the first 3-h incubation period. At least 100 cells were examined and the pattern of attachment assessed as LA, AggA or DA. For the FAS test the procedure of Knutton *et al.* was followed in which the cell monolayer was fixed, permeabilised and treated with fluorescein isothiocyanate (FITC)-labelled phalloidin at the end of the 6-h test.¹¹ The monolayer was then examined for areas of intense fluorescence; their position was compared by phase-contrast microscopy with that of attached bacteria.

Haemagglutination tests

The strains were tested for agglutination of bovine, guinea-pig, human and rat erythrocytes.²¹ For these tests, strains were grown on Mueller-Hinton agar or subcultured twice in Mueller-Hinton broth. Agglutination that occurred in the presence of mannose 0.5% was recorded as mannose-resistant haemagglutination (MRHA) and agglutination that was inhibited by the presence of mannose as mannose-sensitive haemagglutination (MSHA). Strains were also tested after growth on colonisation factor antigen agar containing bile salts 1.5% w/v,²² which has been used for optimal expression of haemagglutinins of enterotoxigenic *E. coli*, but the haemagglutination of strains in this study was poorer than that of strains grown on Mueller-Hinton agar and the results are not given.

DNA-hybridisation tests

DNA-hybridisation tests were performed under stringent conditions as described before.²³ The EAF probe was a 1-kb *SalI*-*Bam*HI fragment.⁸ The VT1 and VT2 probes were 0.75-kb *Hinc*II and 0.85-kb *Ava*I-*Pst*I fragments, respectively.²⁴ These probes were labelled with deoxyadenosine 5'- α -[³⁵S] thiotriphosphate. Some EAF- and VT-probe hybridisation results have been reported in an earlier paper.⁹ The EAggEC probe was a 1-kb *Eco*RI-*Pst*I fragment²⁰ labelled with digoxigenin-dUTP.²⁵ Non-radioactive alkaline phosphatase-labelled synthetic oligonucleotide probes were used to detect ST_A and LT genes according to the manufacturer's protocols (SNAP hybridisation system, Dupont); the ST probe comprised ST_{A1} and ST_{A2} sequences (at our request).²⁶

Table I. Properties of *E. coli* from sporadic cases of infant diarrhoea

Serotype	Number of strains	Number of strains adhering to HEp-2 cells	Properties of adherent strains			
			Pattern of adherence	Hybridisation with		FAS test
				EAF probe	EAggEC probe	
O44:H18	13	7	AggA	—	+	—
O111ab:H21	10	10	AggA	—	+	—
O126:H27	21	13	AggA	—	+	—
O55:H7	15	15	LA	—	—	+
O111ab:H25	8	7	LA	—	—	+

AggA, aggregative pattern; LA, localised pattern.

FAS test, fluorescence actin-staining test.

Results

Strains giving localised attachment

Strains from sporadic cases of diarrhoea giving localised attachment were found in serotypes O55:H7 and O111ab:H25 (table I). The strains did not hybridise with the EAF probe (or with EAggEC, ST, LT and VT probes). For two strains of *E. coli* O55:H7, the percentage of cells with attaching bacteria was 16% and 29%; for the remaining 13 strains the percentage ranged from 40 to 100, with a mean of 60. For all strains the clumps of bacteria usually contained > 50 bacteria. Outbreak-associated *E. coli* O55:H7 strains were isolated from one of three infants with diarrhoea in one hospital in 1979 and from two of three babies with diarrhoea in a second hospital in the same year. A faecal specimen from the asymptomatic mother of one baby who was excreting also yielded the organism. All these outbreak-related strains were LA-positive, attaching to > 52% cells, but EAF-negative. In contrast, only one of the O111ab:H25 strains from sporadic cases of diarrhoea adhered in large numbers to many HEp-2 cells (72%). The other six strains adhered to between 1% and 11% of cells, with a mean of 6%; bacteria were present in localised clumps of usually < 10 bacteria. A similar pattern of attachment was given by *E. coli* O111ab:H25 strains

isolated from two neonates in a special-care baby unit in 1984. Although the numbers of attaching bacteria differed, the localised attachment of bacteria of both serotypes O55:H7 and O111ab:H25 resulted in intense fluorescence in the FAS test.

Strains giving aggregative attachment

Strains from sporadic cases of diarrhoea giving an aggregative pattern of attachment were found in serotypes O44:H18, O111ab:H21 and O126:H27 (table I). All aggregative strains hybridised with the EAggEC probe (but not with the EAF, ST, LT and VT probes). The strains were negative in the FAS test. Aggregative *E. coli* O44:H18 strains with similar properties were also isolated from: (i) four infants (one with bloody diarrhoea and one with non-bloody diarrhoea) in a children's hospital in 1984; (ii) from nine children (three with diarrhoea) and four staff members in a day nursery in 1980; and (iii) from five elderly patients (all > 68 years) with diarrhoea in a hospital in 1980.

Haemagglutination tests

Table II shows the haemagglutination results for all aggregative strains from sporadic cases of diarrhoea

Table II. Haemagglutination (HA) of aggregative *E. coli* strains

Serotype	Number of strains*	HA of erythrocytes indicated after growth							
		in MH broth				on MH agar			
		bovine	guinea-pig	human	rat	bovine	guinea-pig	human	rat
O44:H18	6	—	—	—	MR	—	—	—	MR
	4	—	—	—	MR	—	—	—	—
O111ab:H21	10	MR	MS	MR	MR	—	MS	—	MS
O126:H27	13	MR	—	MR	MR	—	—	MR	MR

MS, mannose-sensitive haemagglutination; MR, mannose-resistant haemagglutination; —, no haemagglutination; MH, Mueller Hinton broth or agar.

*Three of the six O44:H18 strains are representative strains from the three small outbreaks; all other strains were from sporadic cases of diarrhoea.

and for one representative O44:H18 strain from each of the above three outbreaks. All strains gave MRHA of rat erythrocytes after growth in broth, but they differed with respect to MRHA of the erythrocytes of other species and in the reactions of solid- or liquid-grown cultures. All O111ab:H21 and O126:H27 strains gave MRHA of bovine and human erythrocytes after growth in broth, although for the latter strains the MRHA of bovine cells was weaker. Only O126:H27 strains consistently gave MRHA when grown on agar and MRHA of rat erythrocytes was stronger than that of human cells.

Non-adhering strains, or those giving LA, gave no haemagglutination or MSHA only. Broth cultures were more likely to give MSHA than agar-grown bacteria, and MSHA of guinea-pig or rat erythrocytes was more common than that of bovine or human erythrocytes. These are characteristics of MSHA due to type-1 fimbriae. The aggregative strains of O44:H18 and O126:H27 did not show evidence of production of type-1 fimbriae after two passages in broth, whereas all the aggregative strains of O111ab:H21 did.

Tests with strains from healthy children

The strains isolated from healthy children did not give localised adhesion to HEP-2 cells. Two gave an aggregative pattern of attachment with > 50 bacteria attaching to a cell in either chains or aggregates. Both were of serogroup O81; one was of flagellar-type H27 and the other was non-motile. Neither hybridised with the EAggEC probe or gave haemagglutination of any of the erythrocyte species tested. Fourteen strains gave diffuse attachment but for only two strains were there > 20 bacteria attached to each cell.

Discussion

We have examined strains isolated from infants with diarrhoea in Britain belonging to serotypes O44:H18, O55:H7, O111ab:H21, O111ab:H25 and O126:H27, and have shown that the majority have *in vitro* properties that can be related to diarrhoeagenic ability. Strains of O44:H18, O111ab:H21 and O126:H27 were EAggEC as shown by both hybridisation and aggregative attachment to cells in tissue culture. Strains of O55:H7 and O111ab:H25 had properties characteristic of AEEC—they caused an accumulation of polymerised actin beneath bacteria attached in localised clumps in a tissue-culture test.

Flagellar (H) types are only rarely determined in studies of EPEC and so previous reports on the properties of other bacteria belonging to the serotypes we have examined are few and the numbers of strains studied small. In most of these papers the properties examined were not comprehensive. One O111ab:H21 strain and one O126:H27 strain have previously been reported to give AggA.²⁷ With these two exceptions, other strains belonging to the serotypes shown to be

EAggEC in our study have previously been reported to give DA, DA and LA or to be non-adherent,^{28–32} but several of these studies were performed before AggA was recognised as a distinct pattern. In addition, there is one report of an EAF-positive strain of O126:H27 but the pattern of attachment was not stated.³³ *E. coli* O55:H7 strains have been reported to give LA,^{26,29,30} DA³⁰ or no attachment.^{29–31} Strain 660–79 of this serotype was LA-positive, FAS-positive¹¹ and it colonised cultured duodenal mucosa giving AE lesions;³⁴ it was not stated whether this strain was EAF-positive. Three O55:H7 strains and one O111ab:H25 strain have been described that were LA-positive, FAS-positive and EAF-negative;²⁷ the attachment of all four strains was stated to be poor whereas the O55:H7 strains studied in the present paper attached well. The present work is the first to describe potentially diarrhoeagenic properties for a significant number of strains belonging to these various serotypes.

LA-positive strains of O111ab:H25, with one exception, attached to a smaller number of HEP-2 cells than O55:H7 strains. Nevertheless, adhesion of bacteria of both serotypes resulted in good fluorescence in the FAS test. Knutton *et al.*²⁷ reported that some strains giving poor LA to tissue-culture cells were able to attach to enterocytes in numbers similar to those achieved by strains giving good LA. Thus, it seems possible that strains of both classes are diarrhoeagenic and the reason for the difference in attachment *in vitro* is not known. Genes controlling the ability to give good LA are plasmid-encoded in the EAF-positive strain E2348/69 and the EAF-negative O128:H2 strain E25253,^{7,14,35} but the ability to cause actin accumulation after attachment appears to be chromosomally encoded.^{11,14} It will be of interest to see whether the properties of the O55:H7 and O111ab:H25 strains are under similar control and whether a gene-probe recently described for the FAS property³⁶ will detect both EAF-positive and EAF-negative EPEC. Certainly, we can conclude that with the use of the EAF probe alone some subclasses of AEEC will not be recognised.

Only two strains isolated in our small study of 32 healthy children in Britain gave AggA and none gave LA. The two aggregative strains, both of serogroup O81, did not hybridise with the EAggEC probe. Previous studies have shown that not all aggregative strains hybridise with the EAggEC probe indicating that these organisms are heterogeneous.^{18,20} We do not know if the heterogeneity is related to the ability to cause diarrhoea or not. If only some strains giving AggA are pathogenic this may account for the significant numbers isolated from control groups in some epidemiological surveys. Fourteen strains from healthy children gave diffuse attachment; it is still a matter of controversy as to whether strains of *E. coli* that give diffuse attachment can cause diarrhoea.³⁷

Our results confirm earlier reports¹¹ that EAggEC are negative in the FAS test. An aggregative strain

(221) isolated from an adult with diarrhoea in Mexico has been shown to cause diarrhoea in adult volunteers.³⁸ This strain, like most EAggEC reported to date, did not belong to an EPEC serogroup and was judged to be most closely related to serotype O78:H33/35; we have recently shown (unpublished results) that it belongs to serotype O92:H33. In the present study, we have shown that strains belonging to the classic EPEC serotypes O44:H18, O111ab:H21 and O126:H27 may be EAggEC. Most EAggEC have been isolated during studies of infant diarrhoea. Strain 221 was isolated from an adult and some O44:H18 strains in our study were from elderly patients. Methods to identify EAggEC should probably be included in surveys of diarrhoea of all age groups to see if they are a significant cause of such disease.

Nineteen of 40 EAggEC isolated in Chile gave MRHA of human or bovine erythrocytes or both.¹⁸ Agglutination of rat erythrocytes was not tested. We chose to include rat erythrocytes in the range of bloods studied as Old *et al.*³⁹ had reported that two aggregative strains of serogroup O78 gave MRHA of rat erythrocytes; these strains also agglutinated erythrocytes of several other species. In our study, all of the aggregative strains isolated from the faeces of sick children, but not two strains from healthy children, gave MRHA of rat erythrocytes and, although the

relationship of this property to AggA needs to be confirmed, it may be a useful means of identifying these strains. EAggEC did not give identical patterns of agglutination and, interestingly, the patterns were closely related to the serotype of the strains. As yet, we do not know the reason for these differences.

EPEC remain an important cause of infant diarrhoea in developing countries. In developed countries there has been much discussion as to the continued need to serogroup strains of *E. coli* from children with diarrhoea because the number of serious outbreaks due to these organisms has decreased dramatically in the last 20 years. Nevertheless, some laboratories continue to search for EPEC. It is now possible, using in-vitro tests, as described in this paper, to assign some of these strains to definite classes of diarrhoeagenic *E. coli*. It is probable that alternative tests to those described will be developed soon that will enable clinical laboratories to isolate and identify such strains. We conclude that it is important to identify EPEC strains belonging to the EAggEC and LA-positive FAS-positive EAF-negative classes, as, in Britain, based on the results of this and our earlier communication,⁹ these are probably of greater importance than the LA-positive FAS-positive EAF-positive class.

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