BACTERIAL PATHOGENICITY

Use of gene probes and adhesion tests to characterise *Escherichia coli* belonging to enteropathogenic serogroups isolated in the United Kingdom

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Nine hundred and twenty-five *Escherichia coli* isolates from cases of diarrhoea in the United Kingdom and belonging to enteropathogenic *E. coli* (EPEC) O serogroups were examined for virulence properties. The tests included adhesion to HEp-2 cells, the fluorescence actin staining (FAS) test (which correlates with the ability to cause attaching and effacing lesions) and DNA hybridisations with probes to detect sequences for *eaeA* (*E. coli* attaching and effacing factor), EAF (EPEC adherence factor), verocytotoxins VT1 and VT2, enteroaggregative *E. coli* and diffusely adherent *E. coli*. The O serogroups examined were 18, 26, 44, 55, 86, 111, 114, 119, 125, 126, 127, 128 and 142. Six hundred and sixty strains (71.4%) hybridised with at least one of the DNA probes. Over 80% of strains in O serogroups 26, 55, 119, 125, 127 and 142 and 41% of strains of serogroups 86, 111, 114, 126 and 128 hybridised with the *eae* probe and most showed localised attachment and were FAS-positive. However, <10% of these *eae* probe-positive strains hybridised with the EAF probe. Eighty-four of 232 strains in O serogroups 44, 86, 111, and 126 were enteroaggregative. VT genes were detected in 57 of 402 strains in O serogroups 26, 55, 111 and 128. Identification of EPEC by serogrouping was shown to be an effective method of identifying strains with pathogenic potential, although the organisms were diverse in their properties.

Introduction

Enteropathogenic strains of *Escherichia coli* (EPEC) were first recognised to cause infantile enteritis in the 1940s. They were shown to belong to a restricted range of O serogroups and commercial antisera became available for their identification [1, 2]. Later it was shown that some strains of *E. coli* caused diarrhoea because they produced enterotoxins, or produced a verocytotoxin (VT), or were able to invade epithelial cells [3]. These groups of pathogenic *E. coli* were termed enterotoxigenic *E. coli* (ETEC), verocytotoxin producing *E. coli* (VTEC) or enteroinvasive *E. coli* (EIEC), respectively. However, characteristic EPEC strains isolated from outbreaks of diarrhoea did not belong to any of these groups, although occasionally strains belonging to EPEC serogroups and isolated from sporadic cases of diarrhoea did produce enterotoxins or VT [4].

A property possessed by typical EPEC strains was the

ability to adhere in characteristic localised clusters to HEp-2 cells grown in tissue culture [5]. This adherence was shown subsequently to be accompanied by structural changes in the cells which resulted in the accumulation of polymerised actin beneath the attached bacteria, visualised by the use of fluorescein isothiocyanate-labelled phalloidin in the fluorescence actin staining (FAS) test [6]. These in-vitro tests with HEp-2 cells were shown to correlate with the ability of typical EPEC to cause attaching and effacing (AE) lesions of intestinal epithelial biopsy material. The ability of such EPEC strains to adhere well *in vitro* was shown to depend on the possession of a plasmid. An *E. coli* adherence factor (EAF) probe was developed from gene sequences carried on a plasmid in strain E2348/69 (serotype O127:H6) [7]. Subsequently, chromosomal gene sequences were shown to be necessary for the formation of AE lesions as judged by the FAS test and the *eae* (*E. coli* attaching and effacing) probe was developed from the same *E. coli* strain [8]. In addition to the localised attachment (LA) shown by typical EPEC, other patterns of adhesion to HEp-2 cells have been recognised. These are the aggregative and diffuse patterns; strains

exhibiting these types of attachment have been termed enteroaggregative *E. coli* (EAggEC) and diffusely adherent *E. coli* (DAEC) [4].

Strains in EPEC O serogroups 18, 26, 44, 55, 86, 111, 114, 119, 125, 126, 127, 128 and 142 that were isolated from cases of diarrhoea and received by the Laboratory of Enteric Pathogens in the years 1985–1993 were examined. They were tested for adhesion to HEP-2 cells and for hybridisation with several probes used to identify the different classes of diarrhoeagenic *E. coli*.

Materials and methods

Bacterial strains

The strains were received from laboratories in many different areas of the United Kingdom. They were isolated from the faeces of patients of all ages with diarrhoea during the period 1985–1993; 867 (94%) of the 925 strains were from children <4 years old. The strains were identified and serotyped with antisera to O antigens 1–173 and H antigens 1–55 [9]. Some of the strains have been included in previous studies [10–12].

DNA hybridisation

Strains were tested by colony hybridisation [13]. The VT1 probe was a 0.75-kb *Hinc*II fragment derived from strain H19 (*E. coli* O26:H11) and the VT2 probe was a 0.85-kb *Ava*I–*Pst*I fragment derived from strain E32511 (*E. coli* O157:H–) [14]. The VT probes were labelled with fluorescein-dUTP or digoxigenin and used as described previously [15, 16]. The *eae* and EAF probes were derived from strain E2348/69 [7, 8]. The EAggEC probe was that described by Baudry *et al.* [17] and the DAEC probe was a 370-bp *Pst*I fragment within the *daaC* gene of strain C1845 [18]. The *eae*, EAF, EAggEC and DAEC probes were labelled with fluorescein-dUTP. Hybridisation and washing were under stringent conditions with the following exceptions. For the EAggEC probe, membranes were washed twice at room temperature for 15 min in $2 \times$ SSC, SDS 0.1% and then in $5 \times$ SSC, SDS 0.1% for 15 min at 54°C followed by 30 s at room temperature in $2 \times$ SSC, SDS 0.1%. Membranes from DAEC probe tests were washed twice at room temperature for 15 min in $2 \times$ SSC, SDS 0.1% and then in $0.5 \times$ SSC, SDS 0.1% at 68°C for 15 min.

Adhesion tests

Adhesion to HEP-2 cells was tested in the presence of D-mannose 1% w/v as described previously [19]. The pattern of adhesion was assessed as localised, diffuse or aggregative at the end of a 6-h test. All the strains that showed no adhesion were tested in at least two experiments. The FAS test was that of Knutton *et al.* [6].

Results

Testing of strains with DNA probes

Of the 925 *E. coli* strains isolated in the UK and belonging to 13 O serogroups tested for hybridisation with the *eae*, EAF, EAggEC, DAEC and VT probes, 660 (71.4%) strains hybridised with at least one probe (Table 1).

The largest number of strains (520) hybridised with the *eae* probe. Within each serogroup the positive strains were usually of particular O:H combinations and there were also some non-motile strains. The *eae*-positive serotypes most commonly found were O26:H11, O26:H–, O55:H7, O55:H34, O55:H–, O111:H25, O119:H2, O125:H6, O127:H40, O127:H45, O127:H–, O128:H2 and O128:H8. These 13 serotypes accounted for 80% of the *eae*-positive strains although there was a total of 51 *eae*-positive serotypes identified. The *eae* genes were found in all serogroups except O18 and O44. Results of the EAF probe tests showed that 49 strains were positive and all except one O18:H4 strain also hybridised with the *eae* probe.

One hundred and twenty-one strains were EAggEC-probe positive. All these strains gave negative results with the *eae* probe and the majority belonged to distinct O:H serotypes such as O44:H18, O111:H21, O126:H27 and O128:H35. Some of the EAggEC probe-positive strains also hybridised with the DAEC probe as observed in a recent study of O44:H18 strains [20]. In addition to these strains that hybridised with both the EAggEC and DAEC probes, there were 10 strains that hybridised with the DAEC probe and not the EAggEC probe (Table 1). Four of these 10 DAEC probe-positive strains, all of which were of serotype O55:H–, were unusual in that they hybridised with the *eae* probe.

VT genes were detected in 57 strains of serogroups O26, O55, O111 and O128; 41 of these VTEC strains were in serogroup O26 and most were O26:H11, as found in previous studies [12, 21]. Forty-five of the 57 VTEC strains also hybridised with the *eae* probe and the remaining 12 *eae*-negative strains were in serogroup O128.

Two hundred and sixty-five strains did not hybridise with any of the probes and 90 of these belonged to serogroup O18 (Table 2). The remaining probe-negative strains belonged to all 12 serogroups but there were no more than eleven strains of any individual O:H serotype.

Adhesion to cell cultures

Selected strains were examined for attachment to HEP-2 cells and the results were correlated with those of the hybridisation tests (Tables 3 and 4). All 47 strains

Table 1. Properties of strains that hybridise with DNA probes

Serogroup	Number positive/ total tested	Number of strains	Hybridisation with					H type (number)
			<i>eae</i>	EAF	EAggEC	DAEC	VT	
O18	1/91	1		+				4(1)
O26	123/128	81	+					8(2), 11(18), -(61)
		41	+				+	11(35), 21(1), 32(1), -(4)
		1	+	+				2(1)
O44	14/21	2			+			18(1), 41(1)
		12			+	+		7(1), 18(11)
O55	96/116	80	+					7(35), 34(22), 35(2), 42(1), -(20)
		4	+	+				-(4)
		4	+			+		-(4)
		2	+				+	7(2)
		5			+			4(1), 10(2), 12(1), -(1)
		1			+	+		27(1)
O86	25/40	11	+					8(9), 45(1), -(1)
		3	+	+				34(1), -(2)
		10			+			2(2), 6(2), 11(1), 18(3), 21(1), -(1)
		1				+		18(1)
O111	79/92	31	+					25(31)
		5	+	+				-(5)
		1	+					-(1)
		42			+			7(2), 12(2), 21(21), -(16), ?(1)
O114	17/33	9	+					19(1), 49(8)
		6	+	+				2(5), -(1)
		1			+		+	-(1)
		1			+	+		9(1)
O119	29/31	24	+					2(22), 6(1), -(1)
		5	+	+				6(4), -(1)
O125	48/61	41	+					6(38), -(3)
		5	+	+				21(1), -(4)
		2			+			9(2)
O126	49/79	4	+					6(4)
		9	+	+				2(2), -(7)
		32			+	+		27(31), -(1)
		4				+		27(2), 33(1), 43(1)
O127	55/69	50	+					4(1), 40(13), 45(12), -(24)
		4	+	+				6(1), -(3)
		1			+			21(1)
O128	104/140	76	+					2(54), 8(12), 11(1), 12(1), 47(1),
		2	+	+				-(7)
		1	+				+	2(1), -(1)
		12					+	25(1)
		12			+			2(11), -(1)
		1				+		7(1), 35(11)
O142	20/24	16	+					10(1)
		4	+	+				6(2), 10(1), 23(1), 34(6), 35(2),
								36(2), 52(1), -(1)
								4(1), 6(3)

examined that were *eae*- and EAF-probe-positive showed localised adhesion, although there was considerable variation in the percentage of HEp-2 cells with adherent bacteria. Of the 321 strains that

hybridised with the *eae* probe but were EAF-negative, 201 showed some LA. The percentage of cells that showed LA ranged from 1 to 92 and was usually lower than adhesion observed with the *eae*-positive, EAF-

Table 2. Flagellar (H) types of strains that did not hybridise with probes

Serogroup	Total	H types (number)	Number of non-motile strains
O18	90	1(6), 4(2), 5(3), 7(41), 8(1), 14(2), 49(1),	34
O26	5	32(4)	1
O44	7	5(1), 18(5), 41(1)	0
O55	20	1(1), 9(2), 12(5), 22(1), 24(1), 27(5), 32(4), 45(1)	0
O86	15	4(1), 8(1), 10(1), 18(1), 20(1), 27(2), 30(2), 43(1)	5
O111	13	12(3), 19(1), 45(4)	5
O114	16	4(6), 9(4), 10(1), 25(2)	3
O119	2	4(1)	1
O125	13	19(2), 21(1), 30(9)	1
O126	30	2(4), 6(1), 7(1), 8(1), 20(1), 21(5), 27(11), 45(1), 49(1), 51(1)	3
O127	14	4(2), 5(1), 21(2), 40(6)	3
O128	36	1(9), 2(1), 9(1), 12(5), 19(1), 27(1), 35(6), 45(1), 46(1), 47(1), 49(1)	8
O142	4	4(1), 11(1), 38(2)	0

Table 3. Examination of *eae*-positive strains and correlation with EAF probe and cell tests

Serogroup	H type (n)	Total	EAF probe	Percentage of cells with LA*		Number of strains with no attachment
				Range	Mean	
O26	—	24	—	1–32	9.5	3
	11	18(13VT+)	—	1–52	13.6	0
	32	1	—			1
O55	—	4	+	96–100	99	0
	—	4†	—			Diffuse adhesion
	7	31	—	1–56	20.2	7
	—	14	—	1–68	12.8	6
	34	18	—	1–52	10.0	7
	42(1) 35(1)	2	—			2
	34(1) —(2)	3	+	32–56	45	0
O86	8	10	—	1–50	25	8
	45	1	—	21		0
	—	5	+	46–100	79	0
O111	25	30	—	1–24	4.9	15
	—	1VT+	—	4		0
	2(5), —(1)	6	+	20–100	54	0
O114	49	8	—	1–18	5.3	4
	19	1	—	17		0
	6(4) —(1)	5	+	13–70	34	0
O119	2	21	—	1–21	6.5	10
	—	1	—			0
	6	1	—	72		0
O125	21(1) —(4)	5	+	6–52	29	0
	6	19	—	2–24	6.4	6
	—	3	—	1–29	15	1
O126	2(2) —(7)	9	+	4–58	25	0
	6	4	—	3–11	6	1
	6(1) —(3)	4	+	40–100	77	0
O127	—	22	—	2–7	4.5	20
	45	12	—	1–5	2.5	4
	40	13	—			13
O128	2(1) —(1)	2	+	74–100	87	0
	2	34	—	1–92	24.6	1
	—	4	—	5–50	27.5	2
O142	8	7	—	2–42	13	0
	11	1	—	22		0
	4(1) 6(3)	4	+	20–100	62	0
	6(2) 10(1)	16	—	1–22	5.8	5
	23(1) 34(6)					
	35(2) 36(2)					
	52(1) —(1)					

*A selection of LA strains of all serotypes was FAS positive. The *eae* DA O55 strains were FAS negative.

†These four strains hybridised with the *eae* and DAEC probes.

positive strains. Certain O:H combinations such as O111:H25, O119:H2, O127:H— and O127:H40 showed a high proportion of *eae* probe-positive, LA-negative strains; in particular, 37 of 47 serogroup O127 strains gave this result (Table 3).

A selection from all serogroups of the strains that showed LA was examined in the FAS test; all these strains gave positive results. However, four O55:H— strains that hybridised with the *eae* and DAEC probes only showed diffuse adhesion and were FAS-negative.

The results of the EAggEC and DAEC probe tests were also correlated with adhesion assays (Table 4). A total of 122 probe-positive strains was examined and 99 showed either the aggregative or diffuse adhesion pattern. The 23 strains that showed no adhesion to HEP-2 cells included all 11 strains of serotype O128:H35 that hybridised with the EAggEC probe.

Discussion

Over 70% of the strains from the 13 EPEC serogroups hybridised with at least one of the DNA probes for virulence factors or putative virulence factors. The *eae* property was found in >80% of strains belonging to O serogroups 26, 55, 119, 125, 127 and 142 and in >40% of strains of O serogroups 86, 111, 114, 126 and 128. The majority of these *eae* probe-positive strains gave LA and were FAS test positive. However, >90% of these *eae* probe-positive strains were unlike typical EPEC in that they did not hybridise with the EAF probe. Some of the EAF-positive strains belonged to serotypes usually associated with outbreaks of infantile gastroenteritis such as O55:H—, O114:H2, O119:H6, O126:H2, O127:H6, O128:H2 and O142:H6. The majority of the EAF-negative strains belonged to serotypes that were different from those of the EAF-positive strains. Therefore, it is unlikely that the

Table 4. Correlation of EAggEC and DAEC probes with cell tests

Serogroup	H type (n)	Number of strains	Probe result		Adhesion type
			EAggEC	DAEC	
O44	7(1) 18(11)	12	+	+	Agg
	18(1) 41(1)	2	+		Agg
O55	27	1	+	+	Agg
	12	1	+		Agg
	4(1), 10(2), -(1)	4	+		None
O86	2(2) 6(2) 11(1) 18(2)	9	+		Agg
	21(1) -(1)				
	18	1	+		None
	18	1		+	DA
O111	7(2) 12(1) 21(21)	40	+		Agg
	-(15) ?(1)				
	12(1), -(1)	2	+		None
O114	-	1	+		Agg
	9	1	+	+	None
O125	9	1	+		Agg
	9	1	+		None
O126	27(25) -(1)	26	+	+	Agg
	27	2	+	+	None
	27	1		+	Agg
	27	1		+	None
	33(1), 45(1)	2		+	DA
O127	21	1	+		Agg
O128	35	11	+		None
	10	1		+	DA

EAF-negative strains resulted from loss of the EAF plasmid from positive strains.

Although most *eae* probe-positive strains showed LA, the lack of attachment in some cases may suggest that there is no or very poor expression of the *eaeA* gene or other genes. Recent studies have shown that the EAF plasmid controls the expression of the *eaeA* gene product, a 94-kDa protein termed intimin [22]. The *perA* locus, a plasmid encoded regulator, increases expression of intimin by 100-fold and this *perA* gene has been detected in all EPEC strains that carry the EAF plasmid. In this study, all the *eae*-positive, EAF-positive strains expressed LA and were FAS test-positive. The presence of the *perA* locus or a similar regulatory gene in the *eae*-positive, EAF-negative strains is not yet known. The EAF plasmid also carries the *bfpA* gene that encodes bundle-forming pili (BFP) and it has been suggested that these pili are an adhesin of EPEC [23]. The *bfpA* and EAF probes were correlated in a recent study [24] and this demonstrated that the *bfpA* probe hybridised with 137 of 138 EAF-positive strains and only with 10 EAF-negative strains. Hybridisation of the *eae*-positive, EAF-negative strains examined in the present study with a *bfpA* probe would provide further information on the distribution of these pili in EPEC.

Many strains belonging to O serogroups 44, 86, 111 and 126 were enteroaggregative by probe and adhesion tests and in a total of nine serogroups there were EAggEC probe-positive strains. This extends the range of EPEC O serogroups that contain EAggEC [4, 11, 20, 25] and supports the suggestion that EAggEC may be a significant cause of diarrhoea in Britain. Previous studies demonstrated that the aggregative pattern of

adherence is usually plasmid determined [25] and in strain 17-2, serotype O3:H2, plasmid-encoded genes specified bundle-forming fimbriae on the surface [26]. At present it is not clear whether EAggEC strains of different serotypes specify antigenically distinct adhesins. Tests suitable for clinical diagnostic laboratories need to be developed so that the importance of EAggEC can be assessed.

There has been some controversy over the virulence of EPEC and many laboratories have discontinued testing for EPEC [4, 27]. The results of this study have demonstrated that identification of EPEC by serogrouping is an effective method of identifying strains of *E. coli* with pathogenic potential, although the organisms were diverse in their properties. In particular, the virulence of *eae*-positive, EAF-negative strains and of EAggEC strains needs further evaluation.

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