BACTERIAL PATHOGENICITY

Virulence properties of atypical EPEC strains

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Virulence properties of 31 atypical enteropathogenic Escherichia coli (EPEC) strains isolated from cases of diarrhoea were examined. All except two strains adhered to HEp-2 cells in a localised adherence-like (LAL) pattern. With the exception of two strains, all were fluorescent actin staining (FAS) positive. Gentamicin HEp-2 invasion assay studies showed that all strains were invasive. Transmission electron microscopy of infected HEp-2 cells showed the characteristic attaching and effacing lesion and invasion of the cultured cells. Of the nine strains that hybridised with a DNA probe for α -haemolysin, five were haemolytic within 3 h of incubation, while the remaining strains were haemolytic only after incubation for 24 h. Three strains produced enterohaemolysin on blood agar. None of the 31 strains of E. coli induced fluid accumulation in the rabbit intestinal loop assay or displayed cytotoxic effects in HeLa and Vero cells. All the strains belonging to serotypes O26:H11, O26:H⁻ and O119:H2 expressed intimin β , whereas all the strains from serotype O55:H7 expressed intimin γ . The strains belonging to serogroup O111 expressed a non-typable intimin. The participation of intimin in LAL was supported by adhesion inhibition experiments in which antibodies to intimin significantly reduced the level of LAL.

Introduction

Enteropathogenic Escherichia coli (EPEC) remains a leading cause of infantile diarrhoea worldwide. The exact mechanisms by which EPEC strains cause diarrhoea are unknown, but one important characteristic of the histopathology of the infection is attaching and effacing (A/E) lesion. All A/E-positive bacteria have a 35-kb chromosomal pathogenicity island, called the LEE region (for locus of enterocyte effacement), that appears to encode all the necessary functions required for A/E lesion formation [1, 2]. Proteins encoded by the LEE region can be divided into three functional groups: (1) Sep, involved in a type III secretion apparatus [3]; (2) Esps, secreted proteins involved in cell signalling [4-6]; and (3) intimin, a 94kDa outer-membrane protein associated with intimate bacterial adhesion and re-arrangement of cytoskeletal actin [7]. Intimin is encoded by the gene eae, which

has been cloned and sequenced from human EPEC [8], human enterohaemorrhagic E. coli (EHEC) [9, 10], and Hafnia alvei (C-terminus only) [11], as well as from Citrobacter freundii [11, 12]. In their amino acid sequence, intimins show >90% homology over the first 659 amino acid residues, but less homology over the remaining 280 residues at the C-terminus of the protein. Frankel et al. [11, 13] have demonstrated that the binding activity of intimin is located at the Cterminus of the protein. Polymerase chain reaction (PCR) and serological tests for intimin classification have been developed, based on the eae gene sequences of EPEC O127:H6, O114:H2 and O86:H34 and EHEC O157:H7. Recently these tests showed that intimin polypeptides can be divided into at least five serologically distinct types, designated intimin α , β , δ and γ , respectively and a non-typable group [14].

Gómez and Kaper [15] described a cluster of genes (*perA*, B, C) that regulate expression of *eae* and are located in a 60-MDa plasmid referred to as the EPEC adherence factor (EAF) plasmid. The EAF plasmid also harbours the bundle-forming pilus (*bfp*) gene cluster [16] and is widely distributed among EPEC

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strains, as demonstrated by hybridisation with a DNA probe derived from EAF plasmid (EAF probe). The EAF plasmid is not essential for the A/E phenotype, as strain E2348/69 cured of EAF plasmid can still cause A/E lesions, albeit with less efficiency [17].

A new class of diarrhoeagenic E. coli was recently described and regarded as atypical EPEC strains, because they are positive for eae but do not have the EAF plasmid which is detected by the EAF probe [18]. Atypical EPEC strains are found in several EPEC O serogroups and correspond to serotypes or electrophoretic types (ETs) different from those of typical EPEC strains [19-22]. These bacteria are frequently isolated from individuals with diarrhoea in which no other pathogen can be identified. In the UK, EPEC strains not hybridising with the EAF probe may be of greater importance than the EAF-positive strains [23]. Whether atypical EPEC strains possess additional virulence factors that have yet to be discovered or whether there are specific host factors that predispose to disease with these strains is unknown [18].

The aim of this study was to investigate further the virulence characteristics of some atypical EPEC strains of serogroups O26, O55 and O111. O119:H2 strains that contain the bfpA and perA genes but do

not hybridise with the EAF probe were also included in the study.

Materials and methods

Bacterial strains

The study investigated 25 atypical EPEC strains of serogroups O26, O55 and O111 and also studied six strains of *E. coli* O119:H2 that contain plasmidencoded *bfpA* and *perA* genes, but do not react with the EAF probe and do not produce the bundle-forming pilus (BFP). All the strains included in this study were isolated from patients with diarrhoea and previously characterised as regards serotype, electrophoretic types (ETs) and virulence genes [19–22]. After isolation, the strains were kept on nutrient agar protected from light. Strain number, serotype, ET, virulence genes, isolation period and origin are shown in Table 1. Bacterial strains used as controls and for other purposes in this study are listed in Table 2 [8, 15, 24–26].

Antisera

Polyvalent antisera raised against the whole 94-kDa intimin purified from JPN15 harbouring pCVD450 which carries the entire *per* genes and overexpresses

Table 1. Serotype, electrophoretic types, virulence genes, period of isolation and origin of the atypical EPEC strains

Strain no.	Serotype	ET	Virulence genes	Period of isolation	Origin
2262-79	O26:H ⁻	13	eae, α -Hly	1979	USA
C240-52	O26:H ⁻	13	eae, α -Hly	1952	Switzerland
C814-67	O26:H11	13	eae, α-Hly	1967	Denmark
3323-61	O26:H11	13	eae, α -Hly	1961	USA
15	O26:H11	1	eae, EHEC	1985	Brazil
20	O26:H11	15	eae, EHEC	1986	Brazil
22	O26:H11	13	eae, α -Hly	1981	Brazil
47	O26:H11	15	eae, EHEC	1988	Brazil
5624-50	O55:H7	5	eae	1950	USA
660-79	O55:H7	5	eae	1979	USA
586-65	O55:H7	5	eae	1965	Sri-Lanka
148-83	O55:H7	5	eae	1983	Chile
13-81	O55:H7	5	eae	1981	Brazil
154-11	O55:H7	5	eae	1978	Brazil
340	O55:H7	5	eae	1978	Brazil
316	O55:H7	5	eae	1983	Brazil
25	O111ab:H ⁻	9	eae	1983	Chile
255	Olllab:H ⁻	9	eae, α -Hly	1993	Brazil
238	Olllab:H ⁻	9	eae, α -Hly	ND	Brazil
179	O111ab:H9	9	eae, α -Hly	1977	USA
233	O111ab:H9	9	eae, α -Hly	1993	Brazil
29	O111ab:H9	9	eae	1982	Brazil
5	O111ac:H9	9	eae	1986	Brazil
50	Olllac:H9	9	eae	1981	Brazil
263	O111ac:H9	9	eae	1993	Brazil
72	O119:H2	7	eae, bfpA*	1993	Brazil
79	O119:H2	7	eae, bfpA	1993	Brazil
80	O119:H2	7	eae, bfpA	1993	Brazil
109	O119:H2	7	eae, bfpA	ND	Mexico
112	O119:H2	8	eae, bfpA	1960	Denmark
118	O119:H2	9	eae, bfpA	1974	USA

ET, electrophoretic type; *eae*, EPEC attaching and effacing; a-*Hly*, a-haemolysin; EHEC, EHEC plasmid; *bfpA*, bundle-forming pilus. All strains were negative with probes for EAF (EPEC adherence factor), AA (aggregative adherence), DA (diffuse adherence), Inv (*E. coli* invasiveness), Stx1 and Stx2 (Shiga toxin 1 and 2), LT-I and LT-II (*E. coli* enterotoxin type 1 and 2), STh (heat-stable enterotoxin *E. coli* of human origin), STp (heat-stable enterotoxin *E. coli* of porcine origin) and CDT (cytolethal distending toxin); ND, not determined. *BFP is not expressed in these strains as verified by Western blotting with anti-BFP polyclonal serum.

Strain no.	Description	Reference
E2348/69	EPEC (O127:H6) isolated from an outbreak in Taunton, UK	[24]
JPN15	Strain E2348/69 spontaneously cured of pMAR2 plasmid	[8]
JPN15 (pCVD450)	pACYC 184 vector harbouring per genes from E2348/69	[15]
CVD206	eae (intimin) gene deletion mutant of E2348/69	[25]
ICC61	Wild-type EPEC strain (O114:H2)	[26]

intimin were used [15]. Intimin type-specific antisera were raised as described previously [14]. A polyclonal antiserum raised against the 280 amino acid C-terminal of the cell-binding domain of intimin from EPEC serotypes O127:H6 (intimin α) and O114:H2 (intimin β) was used [14].

HEp-2 cell adherence assay

The ability to adhere to HEp-2 cells was determined as described by Scaletsky *et al.* [27]. Monolayers were examined after incubation for 3 and 6 h.

Invasion assay

The invasiveness of bacteria was quantified by the method of Francis et al. [28]. Monolayers of 10⁵ HEp-2 cells in 24-well plates with minimal essential medium (MEM, Sigma) supplemented with fetal bovine serum 10% were infected with 3×10^8 bacteria. After incubation for 3 h, non-adherent bacteria were removed from the monolayers by washing with phosphate-buffered saline (PBS), pH 7.2. MEM containing gentamicin 100 mg/L was added to each well for 1 h to kill any remaining extracellular bacteria. The monolayers were then washed with PBS, and the epithelial cells were lysed with Triton X-100 1% for 10 min. Serial dilutions of the resulting suspension were plated on MacConkey agar and the relative number of intracellular bacteria was expressed as the number of cfu of bacteria recovered from the lysed monolayer after treatment with gentamicin for 1 h. All assays were performed in duplicate.

Fluorescent actin staining test

The fluorescent actin staining (FAS) test, which demonstrates the presence of filamentous actin beneath attached bacteria, was performed as described by Knutton *et al.* [29]. The strains were tested after incubation for 3 and 6 h.

Transmission electron microscopy

The association (adherence and invasion) of atypical EPEC with HEp-2 cells was analysed by transmission electron microscopy (TEM) as described by Pedroso et

al. [30]. Preparations were fixed for TEM after incubation of bacteria with HEp-2 cells for 6 h.

Haemolysin assay

Production of α -haemolysin (α -Hly) and enterohaemolysin (E-Hly) was investigated on blood agar as described by Beutin *et al.* [31] with a heavy streak inoculum. Strains were considered positive for α -Hly production when haloes of haemolysis were observed around the growth after incubation for 3 h and for E-Hly production only after incubation for 24 h. Hybridisation with a DNA probe for α -haemolysin was performed as described by Welch *et al.* [32].

Toxin production

All strains were tested for production of cytotoxins active against Vero and HeLa cells [33] and in ligated rabbit ileal loops [34].

Detection of intimin α , β and γ by PCR and Western blotting

PCR. Table 3 shows the specific forward primers used in the PCR to amplify a segment within the 3' end of the eae gene encoding the 280 amino acid C-terminal of the cell-binding domain of intimin from EPEC serotypes O127:H6 (intimin α), O114:H2 (intimin β) and O157:H7 (intimin γ). These primers were used together with a universal reverse primer (Int-Ru) that was synthesised according to the absolutely conserved and universal amino acid sequence WAAGANKY. Amplification was performed in a Thermal Cycler (Minicycler, MJ Research) for 30 cycles as follows: 95°C 20 s, 45°C 1 min and 74°C 1 min after denaturation at 95°C for 5 min. The reaction conditions were a 50- μ l reaction mixture of the following constitution: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), gelatin 0.01%, template DNA 100 ng, 200 μ M dNTPs, 1.5 mM MgCl₂, 25 pmol of each of the primers (Table 3) and 1.5 U Taq DNA polymerase (Gibco-BRL).

Western blotting. Bacterial whole-cell lysates were electrophoresed in SDS-PAGE 8% gels as described by Laemmli [35], transferred on to nitrocellulose membranes (BioRad) and immunoblotted according to Towbin *et al.* [36]. Membranes were blocked overnight in a solution consisting of skimmed milk 3% in PBS containing Tween 20 0.5% (PBST), washed three times with PBST and incubated with polyvalent anti-intimin (1 in 2000) or anti-intimin α (1 in 750) or anti-intimin

 Table 3. List of primers used to classify intimin subtypes

Primer	Orientation	Sequence		
Int α	Forward	5'CCTTAGGTAAGTTAAGT		
Int β	Forward	5'TAAGGATTTTGGGACCC		
Int γ	Forward	5'ACAAACTTTGGGATGTTC		
Int Ru	Reverse	5'TTTTACACAARYKGCAWAAGC		

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 β (1 in 5000) sera diluted in PBST for 1 h at room temperature. The membranes were washed three times with PBST and then incubated for a further 1 h at room temperature with goat anti-rabbit immunoglobulin G antibodies conjugated to alkaline phosphatase (Sigma). The proteins were detected with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma). As no antiserum was available against intimin γ , this intimin was detected only by PCR.

Adhesion inhibition assay

Briefly, 50 μ l of bacterial cultures grown overnight at 37°C in Tryptic Soy Broth (Difico) were pre-incubated with 1 ml of polyvalent anti-intimin antiserum (1 in 40 dilution in MEM supplemented with fetal bovine serum 2% and D-mannose 1%) for 1 h at 37°C. This mix was added to each well with a monolayer of the HEp-2 cells and the plates were incubated for 6 h at 37°C. The cells were then washed with PBS, fixed with methanol and stained for 5 min with May-Grünwald stain and for 20 min with Giemsa stain. The effect of antiserum upon EPEC adherence to HEp-2 cells was determined by calculating the percentage of cells with four or more attached bacteria in relation to the control experiment done under the same conditions without pre-incubation with antiserum. At least 300 cells were observed in each experiment.

Results

Adherence patterns

Among all strains studied, only two belonging to O55:H7 and O26:H11 serotypes adhered to HEp-2 cells after incubation for 3 h, forming large clusters of adherent bacteria, a pattern termed localised adherence (LA) and which is characteristic of typical EPEC strains (Fig. 1A) [27]. The remaining strains adhered only weakly in the usual 3-h adhesion test (Fig. 1B). However, after incubation for a further 3 h, these strains showed the LA-like (LAL) pattern described elsewhere [20] (Fig. 1C).

Invasion assay

When atypical strains were analysed for invasive properties all strains invaded HEp-2 cells, although with less efficiency than strain E2348/69. However, no significant differences were seen between the strains studied. The number of bacteria recovered (by plating) from HEp-2 cells after incubation with gentamicin ranged from 10^3 to 10^5 cfu/ml (data not shown).

FAS test

With the exception of two strains (nos. 15 and 20), all were FAS positive after incubation for 3 or 6 h.



Fig. 1. (A) E. coli O26 strain 47 showing a LA pattern after incubation for 3 h (\times 4000). (B) E. coli O111ab:H9 strain 50 showing a non-characteristic adherence pattern after incubation for 3 h (\times 3570). (C) The same strain showing a LAL pattern after incubation for 6 h (\times 3525).

Electron microscopy

Examination of HEp-2 cells by electron microscopy after incubation with bacterial cells for 6 h at 37°C showed characteristic A/E lesions, i.e., effacement of microvilli, and cup and pedestal formation at sites of bacterial attachment (Fig. 2A and B). Bacteria were frequently seen enclosed in membrane-bound vacuoles (Fig. 2B), confirming the invasive features demonstrated by the HEp-2 cell gentamicin invasion assay.



Fig. 2. Electron micrography of HEp-2 cells after incubation for 6 h at 37°C with *E. coli* O111ac:H9 strain 263. (A) Bacteria are seen closely adhering to the cell membrane, causing effacement of microvilli and pedestal formation at the sites of attachment. (B) Bacterial cells can be seen closely adhering on surface of epithelial cell (\blacksquare) and enclosed in membrane-bound vacuole (\rightarrow). Magnification: A, \times 32 600; B, \times 17 140.

Haemolytic activity

The five O26 strains that reacted with the α -Hly probe produced α -Hly within 3 h of incubation. Three O26 and four O111 strains produced haemolysis only after incubation for 24 h and the latter, but not the former, reacted with the α -Hly probe.

Toxin production

None of the 31 strains had any effect in Vero or HeLa cells or provoked fluid accumulation in the rabbit intestinal loop assay.

Detection of intimin α , β and γ

Amplification of intimin genes (α , β and γ) of different EPEC serotypes with specific primers revealed that only E2348/69 contained intimin α , whereas all strains from serotypes O26:H11, O26:H⁻, O119:H2 and O114:H2 contained intimin β . Strains belonging to O55:H7 were specifically amplified with the intimin γ primer. None of the strains from serogroup O111 gave amplification products with any of the primers specific for intimin α , β and γ genes, suggesting that these strains may contain a different intimin type.

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Regardless of the intimin type, all EPEC strains reacted with the polyvalent anti-intimin antiserum (data not shown). Thus, the production of intimin by all strains investigated was confirmed. When typespecific antisera were used, intimin α was detected only in E2348/69, whereas strains that were positive for intimin β by PCR reacted with anti-intimin β antibodies. Those strains belonging to serogroup O111 which could not be typed by PCR did not show a typable intimin (Fig. 3).

Inhibition of adherence by polyvalent anti-intimin antiserum

The participation of intimin in LAL was supported by the fact that anti-intimin polyvalent antiserum significantly reduced the level of LAL of all strains studied (Fig. 4). As this antiserum is raised against the whole intimin molecule of JPN15, a slightly higher inhibition of adherence was observed in this strain. The preimune rabbit serum did not show any inhibition of the LAL phenotype. Although the results were reported in terms of the reduced number of HEp-2 cells with attached bacteria, the cluster size was greatly reduced after serum treatment (Fig. 5).

Discussion

The results obtained show that 29 of the 31 strains exhibited the LAL adherence pattern and two exhibited an adherence pattern similar to LA. The reason for this



Fig. 3. Western blot analysis of various EPEC strains with intimin β antiserum. Anti-intimin β was used at a dilution of 1 in 5000. Mol. wt markers (kDa) are shown on the left. Strains E2348/69 (lane 1); ICC61 (2); CVD206 (3); 47 (O26:H11) (4); 20 (O26:H11) (5); 3323-61 (O26:H11) (6); 72 (O119:H2) (7); C586-65 (O55:H7) (8); 263 (O111ac:H9) (9); (\leftarrow) intimin β .



Fig. 4. The effect of the polyvalent anti-intimin serum on LAL *E. coli* adherence was determined by calculating the percentage of cells with five or more attached bacteria in relation to the control performed with PBS. Values are means and SEM from three experiments.



Fig. 5. Adherence of *E. coli* O119:H2 strain 72 to HEp-2 cells in the absence (**A**) and in the presence of polyvalent anti-intimin serum after incubation for 6 h (**B**). Magnification: A, \times 4280; B, \times 4320.

difference was not investigated, but it is probable that these LA strains have adhesins not present in the other strains. Regarding the LAL strains, it has been generally accepted that the adhesion of EPEC strains to cultured epithelial cells is mediated initially by bundle-forming pili (BFP) and later by intimin [8, 16]. There is also evidence that the aggregation of the bacterial cells to form the LA cluster on the surface of the cultured epithelial cell is mediated by BFP. As all 29 LAL strains lacked BFP, it is likely that the delayed appearance and the weaker density of this pattern are due to the lack of BFP production by these strains. On the other hand, as all the strains produced intimin and a polyvalent anti-intimin serum substantially reduced the LAL pattern, it is probable that this pattern is mediated at least partially by intimin. The absence of BFP may also explain why the strains were less invasive than the control strain (E2348/69) and why some strains showed a positive FAS test only after incubation for 6 h. Furthermore, these results, as well as the LAL pattern, may have been influenced by the fact that the O26, O55 and O111 strains did not have per genes. In

a recent study, Knutton *et al.* [37] showed that the presence of the *per* genes encoded by the EAF plasmid was responsible for the increased number of EPEC cells expressing surface intimin during the logarithmic growth phase. It is in accordance with this fact that the six O119:H2 strains that are *per* positive had a positive FAS test in 3 h.

The haemolytic pattern exhibited by the strains was interesting. The O26 strains that reacted with the α -Hly probe were haemolytic within 3 h of incubation and so clearly produced the classical *E. coli* α -Hly. In contrast, the O111 strains that were positive with the α -Hly probe were haemolytic only after 24 h, which indicates that these strains may have had secretion dysfunction of α -Hly or were producing an enterohaemolysin-like substance. In view of their probe positivity, the former explanation seems more likely. Three O26 strains, which were negative for α -Hly probe, were positive for E-Hly.

None of the 31 strains of *E. coli* displayed a cytotoxic effect in HeLa and Vero cells or provoked fluid accumulation in the rabbit intestinal loop assay.

Only *eae* positive strains were included in this study [19–22]. With an anti-intimin polyclonal antiserum, Western blotting confirmed that all the strains expressed intimin. The N-termini of cloned intimin polypeptides have been shown to be highly conserved, whereas the C-termini are considerably more variable [10, 13]. Frankel *et al.* [13] reported that the binding domain of intimin resides in the C-terminus. On the basis of the variation within the cell-binding domain Adu-Bobie *et al.* [14] have recently subtyped the intimin family into groups designated α , β , γ and δ .

In the present study, PCR and Western blotting were used to study the antigenic variation within the cellbinding domain of intimin expressed by the atypical EPEC strains. In agreement with Adu-Bobie et al. [14], with PCR primers designed on the basis of the eae sequences of EPEC strains O127:H6 (intimin α), O114:H2 (intimin β) and O157:H7 (intimin γ), only E2348/69 (O127:H6) was found to have intimin α , whereas all the strains belonging to serotypes O26:H11, O26:H⁻, O119:H2 and ICC61 (O114:H2) expressed intimin β . The eight strains of serotypes O55:H7 harboured intimin γ . The strains of serogroup O111 tested had a non-typable intimin, suggesting that there are still other intimin types yet to described. The strains that produced a specific PCR product with the intimin α or intimin β primers also were recognised by anti-intimin α or anti-intimin β in Western blotting.

The production of intimin γ by the O55:H7 strains reinforces the phylogenetic similarity between this serotype and serotype O157:H7 [38] and the failure of the atypical O111 strains to produce intimin β represents an additional difference between the 48 J. S. PELAYO ET AL.

atypical and the typical O111 strains, because the latter produce intimin β [14].

From the whole series of results, it may be concluded that most atypical EPEC and typical EPEC strains have similar virulence properties, with the exception of those coded by the EAF plasmid. The behaviour of the O119:H2 strains probably is also due to the non-production of the BFP. However, studies under way in this laboratory suggest that most atypical EPEC strains have the *astA* gene encoding EAST-1 toxin while the typical ones do not [39].

Regarding the capacity of atypical EPEC to cause diarrhoea, more studies are necessary to elucidate their virulence mechanisms. However, volunteer studies performed by Levine *et al.* [40] have shown that JPN15 (a plasmid-less EPEC strain) caused diarrhoea, but less severely than the wild-type strain. Thus, it is probable that atypical EPEC lacking plasmid-encoded determinants also have the ability to cause diarrhoea. This hypothesis is reinforced by the fact that these atypical EPEC have been isolated from individuals with diarrhoea in which no other pathogen can be identified [23].

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