Allelic variability of critical virulence genes (*eae*, *bfpA* and *perA*) in typical and atypical enteropathogenic *Escherichia coli* in Peruvian children

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Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of infantile diarrhoea in developing countries. The aim of this study was to describe the allelic diversity of critical EPEC virulence genes and their association with clinical characteristics. One hundred and twenty EPEC strains isolated from a cohort diarrhoea study in Peruvian children were characterized for the allele type of *eae* (intimin), *bfpA* (bundlin pilin protein of bundle-forming pilus) and *perA* (plasmid encoded regulator) genes by PCR-RFLP. Atypical EPEC strains (*eae*+, *bfp*–) were the most common pathotype in diarrhoea (54/74, 73 %) and control samples from children without diarrhoea (40/46, 87 %). Overall, there were 13 *eae* alleles; the most common were beta (34/120, 28 %), theta (24/120, 20 %), kappa (14/120, 12 %) and mu (8/120, 7 %). There were five *bfpA* alleles; the most common were beta1/7 (10/26), alpha3 (7/26) and beta5 (3/26). There were three *perA* alleles: beta (8/16), alpha (7/16) and gamma (1/16). The strains belonged to 36 distinct serogroups; O55 was the most frequent. The gamma-intimin allele was more frequently found in diarrhoea episodes of longer duration (>7 days) than those of shorter duration (3/26, 12 % vs 0/48, 0 %, *P*<0.05). The kappa-intimin allele had the highest clinical severity score in comparison with other alleles (*P*<0.05). In Peruvian children, the virulence genes of EPEC strains are highly variable. Further studies are needed to evaluate additional virulence markers to determine whether relationships exist between specific variants and clinical features of disease.

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Abbreviations: AE, Attaching and effacing; Bfp, bundle-forming pilus; EAF, *Escherichia coli* adherence factor; EPEC, enteropathogenic *E. coli*; LA, localized adherence; LEE, locus of enterocyte effacement; Per, plasmid-encoding regulator.

Two supplementary tables are available with the online version of this paper.

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) is an important diarrhoeal pathogen of young children living in developing countries (Scaletsky *et al.*, 2002). A hallmark phenotype of EPEC is the ability to produce attaching and effacing (AE) lesions. In the AE lesion, the bacteria attach tightly to the

host cell membrane causing a dramatic disruption of the cell surface leading to distortion of microvilli and close adherence of bacteria to the enterocyte. All of the genetic elements required for the production of the AE lesion are encoded in a 35 kb chromosomal pathogenicity island called the locus of enterocyte effacement (LEE). The intimate attachment is mediated by the outer-membrane protein called intimin (Jerse *et al.*, 1990). Intimin is highly variable in the carboxy-terminal region, known as Int-280, and several methods have been developed to recognize its alleles. Ramachandran *et al.* (2003) designed universal PCR primers to amplify the Int-280 region and identified types by RFLP. Others have used microarrays (Garrido *et al.*, 2006) or a heteroduplex mobility assay (Ito *et al.*, 2007). To date, more than 25 major allelic variants of *eae* have been described (Lacher *et al.*, 2006).

EPEC is classified into typical and atypical strains based on the presence of the plasmid *E. coli* adherence factor (EAF). There are two important operons on this plasmid, *bfp* and *per*, the first encoding the type IV bundle-forming pilus (Bfp) and the second encoding a transcriptional activator called plasmid-encoded regulator (Per) which is required for optimal activation and function of LEE-encoded genes and Bfp expression. Lacher and co-workers found 11 distinct EAF plasmid types based on combinations of *bfpA* and *perA* alleles (Lacher *et al.*, 2007). Typical EPEC strains produce the localized adherence (LA) phenotype, associated with the production of Bfp (Giron *et al.*, 1991). Bfp is responsible for the formation of microcolonies on cultured cells (Giron *et al.*, 1991) and is associated with virulence in experimental infection in human volunteers (Bieber *et al.*, 1998).

Little is known about the allelic distribution of *eae*, *bfpA* and *perA* or about whether specific alleles are related to specific clinical characteristics. Most studies have only described the allele distribution between diarrhoea and control samples (Knutton *et al.*, 2001; Vieira *et al.*, 2001; Jenkins *et al.*, 2006; Kozub-Witkowski *et al.*, 2008). In this study, we characterized a collection of strains obtained from a cohort study in Lima, Peru, using PCR-RFLP analysis to describe the allelic diversity, the relationships between alleles of *eae*, *bfpA* and *perA*, and to determine whether clinical features of illness were clearly related to specific alleles.

METHODS

Strains. The strains examined in this study were isolated from a cohort epidemiological study of diarrhoea in infants from 2 to 12 months of age. The study was conducted in peri-urban communities of Lima, Peru, from September 2006 to December 2007 (Ochoa *et al.*, 2009). In the study, 1065 stool samples were obtained from 1034 children; 936 samples were from children with diarrhoea (case patients) and 424 were from children without diarrhoea (controls). Samples were examined for enteric pathogens (*Shigella*, *Salmonella*, *Vibrio*, *Campylobacter*, *Giardia lamblia*, *Cryptosporidium* and rotavirus) by conventional methods (Murray, 2007). Five lactose-positive colonies were isolated from MacConkey plates and tested with specific

DNA primers designed to detect enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enterohaemorrhagic *E. coli*, enteroaggregative *E. coli*, diffusely adherent *E. coli* and EPEC, as described previously (Guion *et al.*, 2008). The number of positive EPEC colonies per sample varied from 1 to 5, but for the present study, only one *eae*+ colony per sample was selected for further analysis.

Clinical data. Clinical information on the diarrhoeal episodes was obtained from the cohort study. We used a modified Vesikari score (Ruuska & Vesikari, 1990) to determine the severity of the episode. The score included: duration of diarrhoea in days (0–3 points), maximum number of stools per day during the episode (1–3), presence of vomiting (0–1), maximum number of emesis per day during the episode (0–3), fever (0–1), dehydration (0–1) and treatment (0–2). The maximum possible score was 14.

Serotyping. Serotyping was performed at the *E. coli* Reference Center, Pennsylvania State University, according to standard methods for determining the O antigen (Orskov *et al.*, 1977). H typing was performed using a *flhC* PCR-RFLP method (Machado *et al.*, 2000).

Detection of virulence genes. EPEC isolates were examined for *eae*, *bfpA* and *perA* by PCR with the primers and conditions listed in Supplementary Table S1 (available in JMM Online). PCR for the three genes was performed in a 25 µl reaction mixture containing 2.5 µl 2.5 mM of each dNTP (Promega), 2 µl 25 mM MgCl₂, 0.5 µl 20 µM of each primer (Invitrogen), 2.5 µl 10× buffer II (Applied Biosystems), 1 U (for *bfpA* and *perA*) or 1.5 U (for *eae*) AmpliTaq Gold (Applied Biosystems) and 2 µl DNA template. Amplification reactions were performed in a thermo cycler (iCycler; Bio-Rad) and, for all amplification reactions, the mixture was heated at 94 °C for 10 min prior to thermocycling. The mixture was held at 72 °C for 7 min after the final cycle before cooling at –20 °C. Amplified products were analysed by using 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide. A 100 bp Plus DNA ladder (Fermentas) was used as a molecular size marker in all gels. The positive PCR control was EPEC strain E2348/69 (*eae*+ *bfpA*+) and the negative control was *E. coli* C600.

Primers for intimin (*eae*) were designed by Lacher *et al.* (2006) to amplify 1.8–2.0 kb of the 3' end of *eae* to the 3' end of *escD* for all known intimin subtypes. This region of *eae* encodes the C-terminal 280 amino acids and was used for RFLP analysis because it possesses the greatest degree of sequence variation between subtypes.

RFLP. The *in silico* RFLP tool of the EcMLST website (<http://www.shigatox.net/cgi-bin/mlst7/insilicorflp>) was used to predict the restriction patterns of the alleles (Supplementary Table S2, available in JMM Online). Two different restriction enzyme digests were used for *eae*. Digestion with *AluI* was performed in 30 µl reaction mixtures with 10 U enzyme (1 µl) (New England BioLabs), 3.0 µl 10× reaction buffer, 20.0 µl unpurified PCR product and 6.0 µl distilled water; the samples were incubated overnight at 37 °C. Digestion with *BstNI* was performed in 30 µl reaction mixtures with 10 U of enzyme, 3.0 µl 10× reaction buffer, 0.3 µl 100× BSA, 20.7 µl unpurified PCR product and 5.0 µl distilled water followed by an overnight incubation at 60 °C. The *bfpA* and *perA* digests were performed as described previously (Lacher *et al.*, 2007). Briefly, *bfpA* PCR products were digested with three separate restriction enzymes (*AluI*, *BstNI* and *BfaI*), while *perA* amplicons were digested with two separate enzymes (*DdeI* and *Sau96I*). After incubation, 15 µl of the digests was separated on ethidium bromide-stained 3.0% agarose gels and visualized by illumination with UV light. The positive controls for *eae*, *bfpA* and *perA* RFLP patterns were provided by Dr M. S. Donnenberg.

Statistical analysis. The allelic frequencies obtained in each group and the allelic distributions of each population were compared using the program GenALEX 6. The comparisons between groups were made using Chi-squared or Fisher's exact test. Student's *t*-test was used for the comparison between severity scores.

RESULTS AND DISCUSSION

Characterization of clinical isolates

Although many studies have found that EPEC infection is significantly associated with infant diarrhoea, EPEC strains were isolated with similar frequency from diarrhoeal samples (74/936, 8%) and from healthy controls (46/424, 11%) in this study, as also described elsewhere (Ochoa *et al.*, 2008). The frequency of asymptomatic colonization might relate to the age of the children and the high frequency of breastfeeding in this age group. Colonization rather than illness may result from the complex interaction of multiple factors, including host susceptibility to infection (the child's age, presence of protective maternal factors such as breastfeeding and trans-placental antibodies, and nutritional and immunological status), and environmental factors (poor hygiene and high faecal contamination). Atypical EPEC (94/120, 78%) constituted the majority of *eae*-positive isolates in this study and were common both in patients with diarrhoea (54/74, 73%) and in controls (40/46, 87%), as has been recently reported in developing and developed countries (Knutton *et al.*, 2001; Afset *et al.*, 2003, 2004; Blanco *et al.*, 2006a, b; Ochoa *et al.*,

2008). Overall, atypical strains were significantly more common in controls (40/424, 9%) than in diarrhoeal cases (54/936, 6%) ($P < 0.05$). The prevalence of *perA* among typical EPEC strains was 55% (11/20) in diarrhoeal patients and 83% (5/6) in controls. Among atypical EPEC strains, the prevalence of *perA* was 7% (4/54) in diarrhoeal patients and 3% (1/40) in controls. The low prevalence of *perA* in typical strains is uncommon and may have two possible explanations: the primers used in the PCR may have failed to recognize new alleles; alternatively, *perA* may not have an important role in infection or other regulators may compensate for its absence.

The role of atypical EPEC in childhood diarrhoea remains to be defined. The association of these strains with diarrhoea is not completely clear, since some studies have reported that atypical EPEC was significantly associated with endemic diarrhoea (Scaletsky *et al.*, 1999; Vieira *et al.*, 2001; Dulguer *et al.*, 2003) and diarrhoea outbreaks (Hedberg *et al.*, 1997; Yatsuyanagi *et al.*, 2002; Jenkins *et al.*, 2003); however, others have reported no significant association with diarrhoea (Knutton *et al.*, 2001; Afset *et al.*, 2004). In our study, atypical EPEC strains were actually significantly more common in control than in ill patients; however, many of these previous studies have demonstrated a tendency for the infants with diarrhoea to have a higher prevalence of atypical EPEC than typical EPEC. Atypical EPEC strains appear to be a diverse group expressing other virulence factors (i.e. *afa*, afimbrial adhesion; EAST1, heat-stable toxin 1; E-hly, EHEC

Table 1. Characterization of typical EPEC strains

Serotypes: R, rough, untypable; O– or H–, did not react with standard antisera; H+, positive H reaction, does not match with known reference standards; X, unclassified O types; NG, strain not recovered for serotyping; NT, non-tyable. NA, Not applicable.

Intimin type	Diarrhoea samples (n=20)				Control samples (n=6)			
	n (%)	<i>bfpA</i> (n)*	<i>perA</i> (n)*	Serotypes	n (%)	<i>bfpA</i> (n)*	<i>perA</i> (n)*	Serotypes
alpha	0 (0)	NA	NA	NA	1 (17)	alpha1	beta	O– : H5
beta	7 (35)	beta1/7 (4), alpha3 (2), NT	alpha (2), beta (2), negative (3)	O26:H11†, O111:H+, X18:H19, O– : H8, O– : H19, O– : H–	2 (33)	alpha3 (2)	beta (2)	O97:H7, OX9:H7
beta2	1 (5)	alpha3	beta	H119:O6 or O41	0	NA	NA	NA
epsilon	1 (5)	beta1/7	Negative	O123:H31	0	NA	NA	NA
gamma	1 (5)	beta1/7	Negative	NG	0	NA	NA	NA
iota	1 (5)	NT	alpha	O55:H21 or H36	0	NA	NA	NA
kappa 1	2 (10)	alpha1, beta1/7	alpha (2)	O– : H20, O2:H+	0	NA	NA	NA
mu	1 (5)	beta5	alpha	O55:H36	1 (17)	beta5	alpha	O55:H21 or H36
theta	3 (15)	alpha3 (1), NT (2)	beta, gamma, negative	O91:H27, O– : H27, O6:H16	0	NA	NA	NA
xi	2 (10)	beta1/7 (2)	Negative (2)	O55:H7†, O171:H19	0	NA	NA	NA
NT	1 (5)	beta1/7	Negative	OR:H+	2 (33)	beta5, alpha3	beta	O69:H27, O– : H+

*n is only given if >1.

†Previously reported as classic EPEC O:H serotypes.

haemolysin; *efa/lifA*, EHEC factor for adherence/lymphocyte inhibitory factor) that may compensate for the loss of EAF (Trabulsi *et al.*, 2002; Afset *et al.*, 2006). Atypical EPEC strains are capable of colonizing the intestinal mucosa and producing AE lesions (Knutton *et al.*, 2001).

Typical EPEC strains are more homogeneous in their virulence characteristics than atypical strains (Trabulsi *et al.*, 2002). *bfpA* is an important virulence determinant of typical EPEC; *perA* may not be (Vieira *et al.*, 2001; Dulguer *et al.*, 2003). We have not found significant differences between the clinical characteristics of *perA* + and *perA* – strains.

Distribution of intimin alleles

Seven strains (6 %) (four atypical and three typical EPEC) were non-typable for intimin based on the RFLP pattern (Supplementary Table S2). Of the typable intimins, 13 different alleles were found. Beta-intimin was the most common subtype (28 %) and was found with similar

prevalence in diarrhoeal patients (31 %) and controls (24 %). The other prevalent alleles were theta (20 %), kappa (12 %) and mu (7 %) (Tables 1 and 2). The intimin alleles beta, theta, gamma and mu are most frequently found among EPEC isolated from humans (Dulguer *et al.*, 2003; Blanco *et al.*, 2006a, b; Jenkins *et al.*, 2006; Kozub-Witkowski *et al.*, 2008). The distribution of intimin alleles found in this study is similar to that obtained in samples from other Latin American countries, although the order of frequency is not the same in all studies (Blanco *et al.*, 2006b; Dulguer *et al.*, 2003). Intimin alleles alpha2, eta, nu, rho and tau were not found in this study. There were no significant differences in the distribution of intimin alleles between diarrhoea and control samples or in their distribution between typical and atypical strains using GenAEx 6.

The strains studied belonged to 36 distinct serogroups, of which O55 was the most frequent (six from children with diarrhoea and two from the control group). Only 62/120

Table 2. Characterization of the atypical EPEC strains

Serotype abbreviations are given in the legend to Table 1. NA, Not applicable.

Intimin type	Diarrhoea samples (n=54)			Control samples (n=40)		
	n (%)	<i>perA</i> (n)*	Serotypes	n (%)	<i>perA</i> (n)*	Serotypes
alpha	0 (0)	NA	NA	1 (3)	Negative	O142:H34†
beta	16 (30)	Negative (16)	O10:H5, O15:H2, O20:H30, O25:H4, O119:H8 (2), O119:H7, O128:H2† (3), O153:H7, O–:H8, O–:H38 (2), O–:H5, O–:H–	9 (22)	Negative (9)	O35:H2, O128:H2†, O153:H7, O–:H11 (2) O–:H8, O–:H25, O–:H2, O–:H–
beta2	1 (2)	Negative	O–:H6 or H41	0 (0)	NA	NA
epsilon	2 (4)	Negative (2)	O69:H32, O–:H31	4 (10)	Negative (4)	O2:H27, O157:H–, O153:H19, O–:H27
gamma	2 (4)	Negative (2)	O126:H19, O–:H7	2 (5)	Negative (2)	O55:H7†, O153:H19
iota	1 (2)	Negative	O88:H8	1 (3)	gamma	O–:H10
kappa	8 (15)	Negative (8)	O15:H+, O157:H39, O88:H8, OR:H5, O–:H10, O–:H5, O–:H+, O–:H–	4 (10)	Negative (4)	O–:H10, O–:H39, O–:H5, NG
lambda	1 (2)	Negative	O–:H10	3 (8)	Negative (3)	O–:H10, O–:H30, OR:H34
mu	5 (9)	alpha (3), negative (2)	O55:H21 or O36 (2), O–:H21 or H36, O–:H5, NG	1 (3)	Negative	O–:H11
omicron	2 (4)	Negative (2)	O13:H11, O–:H40	1 (3)	Negative	O13:H11
theta	10 (18)	Negative (10)	O2:H27, O5:H27, O8:H8, O75:H5, O91:H27, O–:H27 (3), O–:H7, O108:H21 or H36	11 (28)	Negative (11)	O34:H9 (2), O89:H8, O82:H–, O91:H27, O134:H27, O–:H27 (2), O–:H8, O–:H9, O–:H21 or H36
xi	3 (6)	Negative (3)	O91:H2, O98:H5, O–:H5	1 (3)	Negative	OR:H5
zeta	1 (2)	Negative	O55:H7†	0 (0)	NA	NA
NT	2 (4)	alpha, negative	O85:H31, O–:H7	2 (5)	Negative (2)	O73:H18, O–:H5

*n is only given if >1.

†Previously reported as classic EPEC O:H serotypes.

(52 %) strains were O typable. Sixty-nine different serotypes were found (Tables 1 and 2). Among these, we found four serotypes (O128:H2, O55:H7, O26:H1 and O142:H34) previously described as classic EPEC strains (Trabulsi *et al.*, 2002). Serotype distribution of EPEC strains varied considerably, as in other studies, and did not correlate with specific intimin types (Vieira *et al.*, 2001; Dulguer *et al.*, 2003). In general, efforts to correlate serotype with pathogenesis have yielded disappointing results.

Distribution of *bfpA* and *perA* alleles

Of the ten *bfpA* alleles cited in the literature (Lacher *et al.*, 2007), four were found in these strains: beta1/7 38 % (10/26), alpha3 27 % (7/26), beta5 12 % (3/26) and alpha1 8 % (2/26). Strains with the beta1/7-*bfpA* were found only in children with diarrhoea [50 % (10/20) vs 0 % (0/6), $P < 0.05$]. For *perA*, three alleles were found: beta 31 % (8/26), alpha 27 % (7/26) and gamma 4 % (1/26). The majority of strains (10/26, 38 %) were negative for *perA* by PCR, whereas beta-*perA* tended to be more frequent in EPEC from controls samples [20 % (4/20) vs 67 % (4/6), $P < 0.05$]. In general, the diversity in the *eae*, *bfpA* and *perA* alleles found was similar to that reported by Lacher *et al.* (2007). In this study, we found 12 EAF types, including seven new combinations of *bfpA* and *perA* alleles, relative to the ones described by Lacher *et al.* (2007) (Table 3). We

also found that the alpha1-*bfpA* allele was associated with two different *perA* alleles (beta and alpha). In contrast, Lacher *et al.* (2007) found that each *bfpA* allele was associated with only one *perA* allele class.

Association with clinical characteristics

There were no clear age-related differences in intimin alleles (data not shown). For both typical and atypical EPEC, most diarrhoeal episodes were acute (≤ 7 days) 65 % (48/74), 27 % (20/74) lasted 8–13 days and 8 % (6/74) were persistent (≥ 14 days). Among acute episodes, *eae* alleles beta (25 %; 12/48), theta (23 %; 11/48) and kappa (10 %; 5/48) were the most prevalent in both typical and atypical cases. Among the episodes of longer duration, beta-intimin was also the most prevalent (42 %; 11/26) followed by kappa (19 %; 5/26). Among isolates from the six persistent diarrhoea episodes, there were three beta and one each of theta, kappa and gamma. Gamma-intimin was more frequently found in strains from diarrhoea episodes of longer duration (> 7 days) than shorter episodes, with frequencies of 12 % (3/26) vs 0 % (0/48), respectively, $P < 0.05$.

To determine whether clinical characteristics of the diarrhoea episodes might be related to specific intimin alleles, only infections due to a single pathogen were evaluated. Overall, severity, as suggested by the modified Vesikari score, tended to be worse in children infected with

Table 3. Distribution of *eae*, *bfpA* and *perA* alleles, and EAF types among typical and atypical strains in children with diarrhoea and control children

NA, Not applicable; NT, non-typable.

EAF type	<i>bfpA</i>	<i>perA</i> *	Diarrhoea	Intimin type (n)	Control	Intimin type (n)
1†	alpha1	alpha	1	kappa (1)	0	NA
3†	alpha3	beta	4	beta (2), beta2 (1), theta (1)	3	beta (2) and NT (1)
4†	beta1/7‡	alpha	2	beta (1), kappa (1)	0	NA
8†	beta5	alpha	1	mu (1)	1	mu (1)
11†	Negative	<u>gamma</u>	0	NA	1	iota (1)
12	alpha1	beta	0	NA	1	alpha (1)
13	beta5	Negative	0	NA	1	NT (1)
14	beta1/7‡	Negative	8	beta (3), xi (2), epsilon (1), gamma (1), NT (1)	0	NA
15	NT	alpha	2	beta (1), iota (1)	0	NA
16	NT	<u>gamma</u>	1	theta (1)	0	NA
17	NT	Negative	1	theta (1)	0	NA
18	Negative	<u>alpha</u>	4	mu (3), NT (1)	0	NA
19	Negative	<u>Negative</u>	52	beta (17), beta 2 (1), epsilon (2), gamma (2), iota (1), kappa (9), lambda (1), mu (2), omicron (2), theta (10), xi (3), zeta (1), NT (1)	39	alpha (1), beta (9), epsilon (4), gamma (2), kappa (4), lambda (3), mu (1), omicron (1), theta (11), xi (1), NT (2)

*Underlined alleles indicate *perA* alleles that correspond to atypical EPEC strains.

†Previously reported EAF type.

‡These two alleles (beta1 and beta7) could not be distinguished under standard electrophoretic conditions.

Table 4. Clinical characteristics of EPEC diarrhoea episodes by intimin type in patients with no other pathogen isolated

Mean $\pm$ SD values are given for age and severity score; median (range) values are given for duration, vomiting and stool.

Allele	<i>n</i>	Age (months)	Blood in stools (<i>n</i> ; %)	Fever (<i>n</i> ; %)	Duration (days)	Vomiting (<i>n</i> ; %)	Vomiting*	Stool†	ORS (<i>n</i> ; %) [‡]	Severity score§
beta	11	6.7 $\pm$ 2.0	1 (9)	5 (46)	8 (1–24)	9 (82)	1 (0–9)	5 (4–8)	6 (55)	8.8 $\pm$ 1.8
theta	10	9.8 $\pm$ 2.8	0 (0)	5 (50)	2.5 (1–22)	4 (40)	0 (0–9)	6 (4–12)	6 (60)	7.0 $\pm$ 3.1
mu	5	6.8 $\pm$ 3.0	0 (0)	0 (0)	7 (1–10)	1 (20)	0 (0–3)	5 (4–7)	3 (60)	6.6 $\pm$ 3.2
kappa	4	9.2 $\pm$ 1.5	1 (25)	2 (50)	7 (3–10)	4 (100)	5.5 (1–7)	5.5 (5–11)	2 (50)	10.5 $\pm$ 1.9
xi	3	6.7 $\pm$ 0.9	0 (0)	1 (33)	5 (1–8)	1 (33)	0 (0–5)	6 (6–8)	1 (33.3)	7.3 $\pm$ 4.9
Others¶	14	9.1 $\pm$ 2.6	0 (0)	6 (43)	3.5 (1–15)	4 (29)	0 (0–4)	4 (3–11)	5 (36)	5.7 $\pm$ 2.6

*Maximum number of emesis per day during the episode.

†Maximum number of stools per day during the episode.

‡ORS, oral rehydration solutions given.

§Modified Vesikari score.

||*P* < 0.05 for the comparison between kappa and other alleles.

¶Includes beta2, epsilon, gamma, iota, lambda, omicron, zeta and two non-typable strains.

EPEC strains carrying beta- and kappa-intimin compared with other *eae* alleles (Table 4). The correlation between intimin type and diarrhoea severity could be relevant to pathogenesis via either the intensity or the specific targeting of bacterial adherence. It has been suggested that tissue tropism is influenced by intimin type (Fitzhenry *et al.*, 2002, 2003); this could explain the association of beta-intimin with more prolonged diarrhoea episodes and perhaps could relate to the severity of the episodes observed in beta- and kappa-intimin. However, other virulence factors are likely to play an important role in the expression of disease. Vieira *et al.* (2001) analysed the virulence potential of atypical EPEC and concluded that a lack of expression of the LEE genes related to virulence. Interpreting the possible relationships between intimin or other virulence genes and clinical illness could be complicated by many factors including protective factors in breast milk, previous infection with related intimin types, age of infection, genes associated or not associated with intimin in a given strain, and genetic predisposition of the host.

In summary, in the cohort of Peruvian infants, the virulence genes of the EPEC strains isolated were highly variable. Some intimin alleles were associated with diarrhoeal episodes of longer duration and higher clinical severity scores. Further studies are needed to evaluate additional virulence markers to determine which factors are crucial for disease.

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