Typing of intimin (*eae*) genes from enteropathogenic *Escherichia coli* (EPEC) isolated from children with diarrhoea in Montevideo, Uruguay: identification of two novel intimin variants (μ B and ξ R/ β 2B)

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A total of 71 enteropathogenic *Escherichia coli* (EPEC) strains isolated from children with diarrhoea in Montevideo, Uruguay, were characterized in this study. PCR showed that 57 isolates carried *eae* and *bfp* genes (typical EPEC strains), and 14 possessed only the *eae* gene (atypical EPEC strains). These EPEC strains belonged to 21 O:H serotypes, including eight novel serotypes not previously reported among human EPEC in other studies. However, 72 % belonged to only four serotypes: O55:H– (six strains), O111:H2 (13 strains), O111:H– (14 strains) and O119:H6 (18 strains). Nine intimin types, namely, α 1 (two O142 strains), β 1 (29 strains, including 13 O111:H2 and 14 O111:H–), γ 1 (three O55:H– strains), θ (five strains, including three strains with H40 antigen), κ (two strains), ε 1 (one strain), λ (one strain), μ B (six strains of serotypes O55:H51 and O55:H–) and ξ R/ β 2B (22 strains, including 18 O119:H6) were detected among the 71 EPEC strains. The authors have identified two novel intimin genes (μ B and ξ R/ β 2B) in typical EPEC strains of serotypes O55:H51/H– and O119:H6/H–. The complete nucleotide sequences of the novel μ B and ξ R/ β 2 variant genes were determined. PFGE typing after *Xba*I DNA digestion was performed on 44 representative EPEC strains. Genomic DNA fingerprinting revealed 44 distinct restriction patterns and the strains were clustered in 12 groups. Only 15 strains clustered in six groups of closely related (similarity > 85 %) PFGE patterns, suggesting the prevailing clonal diversity among EPEC strains isolated from children with diarrhoea in Montevideo.

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INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) was first recognized as a cause of infantile diarrhoea in the 1940s, and was associated with outbreaks in hospitals and nurseries in the UK. During one such outbreak, Bray (1945) prepared antiserum to a strain of *E. coli* isolated from a patient with

diarrhoea and used this antiserum to show that the epidemic strains belonged to the same serogroup, later recognized as *E. coli* O111. In 1987, the World Health Organization (1987) recognized EPEC serotypes of 12 different O serogroups (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158). Although large outbreaks of infant diarrhoea due to EPEC have largely disappeared from industrialized countries, EPEC remains an important cause of potentially fatal infant diarrhoea in developing countries (Trabulsi *et al.*, 2002). In Brazil, for example, EPEC strains are recovered from up to 30 % of cases of diarrhoea in infants of low socioeconomic level (Gomes *et al.*, 1996). In Uruguay, diarrhoeagenic *E. coli* (DEC) are the most frequently identified bacterial agents associated with gastroenteritis of children in low-income population

Abbreviations: AEEC, attaching and effacing *E. coli*; A/E, attaching and effacing; DEC, diarrhoeagenic *E. coli*; EPEC, enteropathogenic *E. coli*; STEC, Shiga-toxin-producing *E. coli*; UPGMA, unweighted pair group method with arithmetic means.

The GenBank/EMBL/DDBJ accession numbers for the complete nucleotide sequences of the novel μ B and ξ R/ β 2B variant intimin genes are AJ705049 and AJ715407, respectively.

groups. Although rotavirus is increasingly recognized as a frequent cause of these infections, EPEC still accounts for a vast proportion of diarrhoea cases. *Shigella flexneri* and *Campylobacter jejuni* are also frequently isolated, especially from episodes of bloody diarrhoea (Torres *et al.*, 2001).

For decades, the mechanisms by which EPEC caused diarrhoea were unknown, and this diarrhoeagenic pathotype could only be identified on the basis of O:H serotyping. However, since 1979, numerous advances in our understanding of the pathogenesis of EPEC diarrhoea have been made through the application of tissue culture and molecular genetic methods (Frankel *et al.*, 1998; Trabulsi *et al.*, 2002; Kaper *et al.*, 2004). One of the central mechanisms of EPEC pathogenesis is the formation of attaching and effacing (A/E) lesions, which is characterized by the intimate attachment of the bacteria to the enterocyte membrane and by the effacement of the microvilli of the enterocyte (Jerse *et al.*, 1990; Kaper *et al.*, 1998). The ability to produce A/E lesions has also been detected in strains of Shiga-toxin-producing *E. coli* (STEC) and in strains of other bacterial species. STEC and EPEC that cause characteristic A/E lesions in the intestinal mucosa are also classified as attaching and effacing *E. coli* (AEEC). The genetic determinants for the production of A/E lesions are located on a large chromosomal pathogenicity island, the locus of enterocyte effacement (LEE; Kaper *et al.*, 1998). The central portion of LEE encodes intimin (Eae, a 94–97 kDa outer-membrane protein) and Tir, the intimin receptor, which is translocated into the host cell membrane by a type III secretion system. Differentiation of intimin alleles represents an important tool for EPEC and STEC typing in routine diagnostics as well as in pathogenesis, epidemiological, clonal and immunological studies. The C-terminal end of intimin is responsible for receptor binding, and it has been suggested that different intimins may be responsible for different host tissue cell tropisms (Torres *et al.*, 2005). The 5' regions of *eae* genes are conserved, whereas the 3' regions are heterogeneous. This observation has led to the construction of universal PCR primers and allele-specific PCR primers, which have made it possible to differentiate at present 21 variants of the *eae* gene encoding 21 different intimin types and subtypes: $\alpha 1$, $\alpha 2$, $\beta 1$, $\xi R/\beta 2B$, $\delta/\beta 2O$, κ , $\gamma 1$, $\gamma 2$, θ , $\epsilon 1$, $\nu R/\epsilon 2$, ζ , $\eta 1$, $\eta 2$, $\iota 1$, $\mu R/\iota 2$, λ , μB , νB , ξB and o) (Gannon *et al.*, 1993; Adu-Bobie *et al.*, 1998; Oswald *et al.*, 2000; Tarr & Whittam, 2002; Zhang *et al.*, 2002; Blanco *et al.*, 2004a, b, d, 2005, 2006; Garrido *et al.*, 2006).

In 1996, EPEC strains were defined as intimin-containing DEC isolates that possess the ability to form A/E lesions on intestinal cells and do not possess Shiga toxin (*stx*) genes (Kaper, 1996). However, EPEC strains can be further classified as typical or atypical. Typical EPEC strains possess a virulence plasmid (EAF plasmid) that includes genes encoding the bundle-forming pilus (Bfp), which is required for localized adherence on cultured epithelial cells; atypical EPEC strains do not possess the EAF plasmid with the *bfpA* gene (Trabulsi *et al.*, 2002). In industrialized countries,

atypical EPEC (*eae*⁺ *bfpA*[−] *stx*[−]) are more frequently isolated from diarrhoeal cases, whereas typical EPEC (*eae*⁺ *bfpA*⁺ *stx*[−]) dominate in developing countries (Trabulsi *et al.*, 2002).

The aim of this study was to establish the serotypes, intimin types and genetic diversity of typical and atypical EPEC strains isolated from children with diarrhoea in Montevideo, Uruguay.

METHODS

***E. coli* isolates and control strains.** A total of 71 EPEC isolates from children with diarrhoea (sporadic cases) studied in Montevideo between 1990 and 1999 were characterized in this study (Torres *et al.* 2001; F. Schelotto and others, unpublished data). Cultures of faeces yielding EPEC strains were performed as a part of medical and microbiological surveys aimed at the determination of the aetiology of acute and persistent diarrhoea, the antibiotic resistance of identified pathogens, and the adequacy of procedures for feeding and rehydrating affected children. Most infections were acquired in the community, but were studied when the children attended Montevideo Children's Hospital.

E. coli strains used as controls were: EPEC-E2348/69 (human, O127:H6, *bfpA*, *eae-α1*), AEEC-IH2498a (human, O125:H6, *eae-α2*), EPEC-337 (human, O111:H2, *bfpA*, *eae-β1*), EPEC-359 (human, O119:H6, *bfpA*, *eae-ξR/β2B*), EPEC-BL152.1 (human, O86:H34, *bfpA*, *eae-δ/β2O*), AEEC-6044/95 (human, O118:H5, *eae-κ*), STEC-EDL933 (human, O157:H7, *stx1*, *stx2*, *eae-γ1*), STEC-TW07926 (human, O111:H8, *stx1*, *stx2*, *eae-θ*), STEC-VTB-286 (bovine, O103:H2, *stx1*, *eae-ε1*), AEEC-IH3205a (human, O123:H19, *eae-νR/ε2*), STEC-VTO-50 (ovine, O156:H−, *stx1*, *eae-ζ*), AEEC-CF11201 (human, O125:H−, *eae-η1*), H03/53199a (human, O125:H45, *eae-η2*), AEEC-7476/96 (human, O145:H4, *eae-ι1*), AEEC-217-2 (human, O101:H−, *eae-μR/ι2*), AEEC-68-4 (human, O34:H−, *eae-λ*), EPEC-373 (human, *bfpA*, O55:H51, *eae-μB*), AEEC-IH1229a (human, O10:H−, *eae-νB*), STEC-B49 (bovine, O80:H−, *stx1*, *eae-ξB*), IH2997f (human, O129:H−, *eae-o*) and K12-185 (negative for *stx1*, *stx2*, *bfpA* and *eae* genes). Strains were stored at room temperature in nutrient broth with 0.75% agar.

Serotyping. The determination of O and H antigens was carried out by the method of Guinée *et al.* (1981), employing all available O (O1–O185) and H (H1–H56) antisera. All antisera were obtained and absorbed with the corresponding cross-reacting antigens to remove non-specific agglutinins. The O antisera were produced in the Laboratorio de Referencia de *E. coli* (LREC), Lugo, Spain (<http://www.lugo.usc.es/ecoli>), and the H antisera were obtained from the Statens Serum Institut, Copenhagen, Denmark. *E. coli* strains representing the novel O groups O182–O185 were kindly provided by Flemming Scheutz, Statens Serum Institut.

Detection of DEC genes. Detection of virulence genes of EPEC and other DEC was performed by PCR using specific primers for amplification of eight virulence genes of distinct DEC groups: STEC (*stx1* and *stx2*) (Blanco *et al.*, 2003), EPEC/STEC (*eae*) (Blanco *et al.*, 2003), EPEC (*bfpA*) (Gunzburg *et al.*, 1995), enteroinvasive *E. coli* (EIEC) (*ipaH*) (Tornieporth *et al.*, 1995), enteroaggregative *E. coli* (EAEC) (pCDV432) (Schmidt *et al.*, 1995) and enterotoxigenic *E. coli* (ETEC) (*eltA* and *est*) (Schultsz *et al.*, 1994; Blanco *et al.*, 2006).

Typing of intimin (*eae*) genes. Typing of intimin genes into *eae* $\alpha 1$, $\alpha 2$, $\beta 1$, $\xi R/\beta 2B$, $\delta/\beta 2O$, κ , $\gamma 1$, $\gamma 2$, θ , $\epsilon 1$, $\nu R/\epsilon 2$, ζ , $\eta 1$, $\eta 2$, $\iota 1$, $\mu R/\iota 2$, λ , μB , νB , ξB and o was performed by PCR as previously described (Blanco *et al.*, 2003, 2004b, 2005, 2006). Base sequences and

predicted sizes of amplified products for the specific oligonucleotide primers used in this study are shown in Table 1. The oligonucleotide primers were designed by us according to the nucleotide sequences of the virulence genes. Isolates positive for the *eae* gene with EAE-1 and EAE-2 primers were further analysed with all different variant primers. Due to the high sequence similarity, specific primers could not be designed to distinguish between *eae*- γ 2 and *eae*- θ , *eae*- δ and *eae*- κ , or *eae*- η 1 and *eae*- η 2 genes. In these cases it was necessary to establish the nucleotide sequence of a fragment from the 3' variable region of the *eae* gene.

Sequencing of the intimin (*eae*) genes. The nucleotide sequence of the amplification products purified with a QIAquick DNA purification kit (Qiagen) was determined by the dideoxynucleotide triphosphate chain-termination method of Sanger, with the BigDye Terminator v3.1 Cycle Sequencing kit and an ABI 3100 Genetic Analyser (Applied Bio-Systems).

Nucleotide sequence accession numbers. The *eae* sequences of strains analysed were deposited in the European Bioinformatics Institute (EMBL Nucleotide Sequence Database), and the accession numbers assigned are indicated in Table 2 and in Results.

Phylogenetic analyses. The percentage identities of nucleotides of the 21 *eae* variants were calculated with the CLUSTAL W program (Thompson *et al.*, 1994) included in the EMBL software (<http://www.ebi.ac.uk/clustalw/>). An unweighted pair group method with arithmetic means (UPGMA) tree for amino acid sequences was constructed from a matrix of uncorrected p distances by using MEGA 3.1 (Kumar *et al.*, 2004). Robustness of the tree was tested with bootstrapping (1000 replicates). The tree was rooted using the midpoint rooting option (<http://www.megasoftware.net/mega.html>).

Macrorestriction fragment analysis by PFGE. PFGE was performed in a CHEF Mapper system (Bio-Rad) at 14 °C in 0.5 × Tris/borate/EDTA by the Enternet-proposed standard-protocol for PFGE (http://www.foodborne-net.de/content/e25/e70/e580/index_ges.html). Cleavage of the agarose-embedded DNA was achieved with 0.2–0.8 U μ l⁻¹ *Xba*I (Roche) according to the manufacturer's instructions. Run times and pulse times were 2.20–54.0 s for 22 h with linear ramping. To perform the comparison of the PFGE pulsotypes, TIFF files were analysed with BioNumerics software (Applied Maths). Cluster analysis of the Dice similarity indices based on UPGMA was done to generate a dendrogram describing the relationship among EPEC pulsotypes. A difference of at least one restriction fragment in the patterns was considered the criterion for discriminating between clones.

RESULTS

Virulence genes

A total of 71 EPEC isolates were characterized in this study. PCR showed that 57 isolates carried *eae* and *bfpA* genes (typical EPEC strains), and 14 possessed only the *eae* gene (atypical EPEC strains). All 71 EPEC strains were negative for the other six DEC virulence genes (*stx*₁, *stx*₂, *ipaH*, pCDV432, *eltA* and *sta*) investigated.

Serotypes

EPEC strains belonged to 11 O serogroups, 10 H flagellar antigen types and 21 O:H serotypes, including eight novel serotypes not previously reported among human EPEC in other studies (Table 2). Of the strains, 83 % were of three O serogroups (O55, O111 and O119), 45 % expressed two H

flagellar antigens (H2 and H6) and 72 % belonged to only four serotypes: O55:H– (six strains), O111:H2 (13 strains), O111:H– (14 strains) and O119:H6 (18 strains).

Typing of *eae* (intimin) genes

Nine intimin types, namely, α 1 (two O142 strains), β 1 (29 strains, including 13 O111:H2 and 14 O111:H–), ξ R/ β 2B (22 strains, including 18 O119:H6), γ 1 (three O55:H– strains), θ (five strains, including three strains with H40 antigen), κ (two strains), ϵ 1 (one strain), λ (one strain) and μ B (six strains of serotypes O55:H51 and O55:H–) were detected among the 71 EPEC strains, and none of the strains was positive for intimin types α 2, γ 2, δ / β 2O, ζ , η 1, η 2, ι 1, μ R/ ι 2, ν B, ν R/ ϵ 2 or ξ B (Table 2).

Identification of two novel intimin variant genes: sequence comparison and evolutionary analysis of *E. coli* intimin genes

A fragment of the 3' variable region of the *eae* gene from the 24 representative EPEC strains was sequenced. We identified in typical EPEC strains of serotypes O55:H51/H– and O119:H6/H– two novel intimin genes *eae*- μ B and *eae*- ξ R/ β 2B that show less than 95 % nucleotide sequence identity with existing intimin genes. The complete nucleotide sequences of the novel μ B (AJ705049) and ξ R/ β 2B (AJ715407) variant genes were determined (Table 2). As the complete sequence of *eae* genes encoding intimins δ / β 2O (AJ875027), λ (AJ715409) and ξ B (AJ705051) was not available in public databases, they were also sequenced.

We determined the genetic relationship of the 21 *eae* variants: α 1 (M58154), α 2 (AF530555), β 1 (AF200363), ξ R/ β 2B (AJ715407), δ / β 2O (AJ875027), κ (AJ308552), γ 1 (AF071034), γ 2 (AF025311), θ (AF449418), ϵ 1 (AF116899), ν R/ ϵ 2 (AF530554), ζ (AJ271407), η 1 (AJ308550), η 2 (AJ876652), ι 1 (AJ308551), μ R/ ι 2 (AF530553), λ (AJ715409), μ B (AJ705049), ν B (AJ705050), ξ B (AJ705051) and o (AJ876648) (Table 3). Since the nucleotide sequences analysed were of different lengths, we used CLUSTAL W (Thompson *et al.*, 1994) for optimal sequence alignment. Identities of 93, 91 and 91 % were calculated between the novel *eae*- μ B variant and *eae*- γ 1, *eae*- γ 2 and *eae*- θ genes, respectively. The percentage identities between the novel *eae*- ξ R/ β 2B gene and the *eae*- β 1, *eae*- δ / β 2O and κ genes were 90, 93 and 94 %, respectively.

The phylogenetic tree for amino acid sequences of the intimin variants constructed by the UPGMA method (uncorrected p distances) of MEGA 3.1 revealed six groups of closely related intimins: (i) α 1, α 2, ζ , ν B and o ; (ii) λ ; (iii) β 1, ξ R/ β 2B, δ / β 2O and κ ; (iv) ϵ 1, ξ B, ν R/ ϵ 2, η 1 and η 2; (v) γ 1, μ B, γ 2 and θ ; and (vi) ι 1 and μ R/ ι 2 (Fig. 1).

Genetic diversity of EPEC: PFGE patterns

Forty-four EPEC strains were selected to be analysed by PFGE, including the most prevalent serotypes. These included 16 O111 strains (eight O111:H2, seven

Table 1. PCR primers used for amplifying and typing of intimin *eae* genes

Gene	Primer	Oligonucleotide sequence (5'–3')	Fragment size (bp)	Annealing temperature	Primer coordinates*	Accession number
<i>eae</i> †	EAE-1	GGAACGGCAGAGGTAACTCTGCAG	775	55 °C	1441–1460	AF022236
	EAE-2	GGCGCTCATCATAGTCTTTC			2193–2215	
<i>eae-α1</i>	EAE-FB	AAAACCGCGGAGATGACTTC	820	60 °C	1909–1928	AF022236
	EAE-A	CACTCTTCGCATCTTGAGCT			2709–2728	
<i>eae-α2</i>	IH2498aF	AGACCTTAGGTACATTAAGTAAGC	517	60 °C	2099–2122	AF530555
	IH2498aR	TCCTGAGAAGAGGGTAATC			2597–2615	
<i>eae-β1</i>	B1F	CACAATTAATGCACCGGGT	241	55 °C	2499–2517	AF453441
	B1R	GCTTGATACACCTGATGACT			2720–2739	
<i>eae-ξR/β2B‡§</i>	B2F	TGAAGGGGGGAACCCCTGTG	564	62 °C	2054–2073	AF530556
	B2R	TTTCTTTTGAAGTGTCTAAAGC			2596–2617	
<i>eae-δ/κ/β2O </i>	EAE-FB	AAAACCGCGGAGATGACTTC	830	60 °C	1909–1928	U66102
	EAE-D	CTTGATACACCCGATGGTAAC			2718–2738	
<i>eae-γ1</i>	EAE-FB	AAAACCGCGGAGATGACTTC	804	60 °C	1909–1928	AF071034
	EAE-C1	AGAACGCTGCTCACTAGATGTC			2691–2712	
<i>eae-θ/γ2¶</i>	EAE-C2F	AGAACGTTACTGGTGACTTA	414	58 °C	2303–2322	AF025311
	EAE-C2R	CTGATATTTTATCAGCTTCA			2697–2716	
<i>eae-ε1</i>	EAE-FB	AAAACCGCGGAGATGACTTC	722	66 °C	1909–1928	AF116899
	LP5	AGCTCACTCGTAGATGACGGCAAGCG			2605–2630	
<i>eae-νR/ε2§</i>	EAE-E2F	AATACAGAAGTTAAGGCAT	378	58 °C	2230–2248	AF530554
	EAE-E2R	ACGACCACTATTCATTTTC			2590–2607	
<i>eae-ζ</i>	Z1	GGTAAGCCGTTATCTGCC	206	62 °C	2062–2079	AF449417
	Z2	ATAGCAAGTGGGGTGAAG			2250–2267	
<i>eae-η1/η2</i>	EAE-FB	AAAACCGCGGAGATGACTTC	702	60 °C	1899–1918	AJ308550
	ETA-B	TAAGCGACCACTATTTCGTG			2582–2600	
<i>eae-η1</i>	ETA-FN	CGCTTTGTTTAAATGCCGATAAA	410	62 °C	1074–1095	AJ308550
	ETA-RN	GACTGCGTAATGCACTG			1467–1483	
<i>eae-ι1</i>	EAE-FB	AAAACCGCGGAGATGACTTC	651	55 °C	1909–1928	AJ308551
	IOTA-B	GTCATATTTAACTTTTACACTA			2538–2559	
<i>eae-μR/ι2§</i>	Iota2-F	CTGGTAAAGCGATAGTCAAAC	936	58 °C	1850–1870	AF530553
	Iota2-R	GCGTTTTTGAAGAAACATTTTGC			2763–2785	
<i>eae-λ</i>	68-4F	CGGTCAGCCTGTGAAGGGC	370	64 °C	2061–2079	AJ715409
	68-4R	AATACCGGAAGAGGCATCTAT			2410–2430	
<i>eae-μB#</i>	EAE-FB	AAAACCGCGGAGATGACTTC	655	60 °C	1909–1928	AJ705049
	FV373R	ACTCATCATAATAAGCTTTTTTG			2541–2563	
<i>eae-νB#</i>	IH1229aF	CACAGCTTACAATTGATAACA	311	60 °C	2255–2275	AJ705050
	IH1229aR	CTCACTATAAGTCATACGACT			2545–2565	
<i>eae-ξB#</i>	EAE-FB	AAAACCGCGGAGATGACTTC	468	66 °C	1909–1928	AJ705051
	B49R	ACCACCTTTAGCAGTCAATTTG			2355–2376	
<i>eae-ο</i>	H2997fF	AGCGTTAGCAATGCCGCAGTTGAT	271	60 °C	2203–2226	AJ876647
	IH2997fR	CAACGGTAATTGTTGTTTCC			2454–2473	

*Location within *eae* gene. The oligonucleotide primers were designed by us according to the nucleotide sequences of the virulence genes.

†Universal oligonucleotide primer pair EAE-1 and EAE-2 with homology to the 5' conserved region of the *eae* gene (detects all types of *eae* variant). Isolates positive for *eae* with EAE-1 and EAE-2 primers were further analysed with all variant primers.

‡The intimin β2 of Blanco *et al.* (2003) and this study is identical to the intimin ξR described by Ramachandran *et al.* (2003). In this study, our β2 is referred to as ξR/β2B.

§Intimins μ, ν and ξ described by Ramachandran *et al.* (2003) in ruminant *E. coli* strains. When Ramachandran *et al.* (2003) submitted the nucleotide sequences of μ, ν and ξ genes, these intimin types were termed ι2, ε2 and β2, respectively.

||The intimin δ of Adu-Bobie *et al.* (1998) is very similar to intimin κ of Zhang *et al.* (2002). The intimin δ of Adu-Bobie *et al.* (1998) was also termed β2 by Oswald *et al.* (2000). Thus, in the present study, this intimin is referred to as δ/β2O. Due to the high level of sequence identity, specific primers could not be designed to differentiate *eae-δ* and *eae-κ* genes.

¶The intimin θ of Tarr & Whittam (2002) is very similar to intimin γ2 of Oswald *et al.* (2000). Due to the high level of sequence identity, specific primers could not be designed to distinguish *eae-γ2* and *eae-θ* genes.

#Intimins μ, ν and ξ described by Blanco *et al.* (2003, 2004b, 2006) and this study.

Table 2. Intimin types and serotypes of typical and atypical EPEC strains isolated from children with diarrhoea in Montevideo

Intimin type	Serotype	Number of strains	<i>stx1, stx2</i>	<i>eae</i>	<i>bfp</i>	Accession no. of sequence (strain)
Typical EPEC strains (<i>n</i> = 57)						
$\alpha 1$	O142:H21*†	1	—	+	+	AJ716138 (strain FV335)‡
$\alpha 1$	O142:HNT*†	1	—	+	+	AJ875028 (strain FV361)‡
$\beta 1$	O111:H2*§	13	—	+	+	AJ716139 (strain FV337)‡
$\beta 1$	O111:H—*§	14	—	+	+	AJ716140 (strain FV335)‡
$\xi R/\beta 2$	O119:H6*§	18	—	+	+	AJ715407 (strain FV359)
$\xi R/\beta 2$	O119:H—*§	1	—	+	+	AJ744877 (strain FV386)‡
$\xi R/\beta 2$	ONT:H51†	2	—	+	+	AJ716141 (strain FG190)‡
$\gamma 1$	O55:H—*§	1	—	+	+	AJ875029 (strain FV365)‡
θ	O127:H40*§	1	—	+	+	AJ716144 (strain FV381)‡
μB	O55:H51*	2	—	+	+	AJ705049 (strain FV373)
μB	O55:H—*§	3	—	+	+	AJ716147 (strain FG275)‡
Atypical EPEC strains (<i>n</i> = 14)						
$\beta 1$	O119:H—*§	1	—	+	—	AJ744877 (strain FV551)‡
$\beta 1$	O128:H12*†	1	—	+	—	AJ875030 (strain FV400)‡
$\xi R/\beta 2$	O33:H6	1	—	+	—	AJ875033 (strain FV632)‡
$\gamma 1$	O55:H—*§	2	—	+	—	AJ579369 (strain FV376)‡
θ	O34:H—†	1	—	+	—	AJ744878 (strain FG513)‡
θ	O51:H40†	1	—	+	—	AJ744879 (strain FG547)‡
θ	O55:H40*†	1	—	+	—	AJ716142 (strain FG27)‡
θ	O111:H25*§	1	—	+	—	AJ716143 (strain FG175)‡
κ	O49:H29†	1	—	+	—	AJ744880 (strain FG305)‡
κ	ONT:H10	1	—	+	—	AJ875031 (strain FV245)‡
$\varepsilon 1$	O111:H21*	1	—	+	—	AJ716145 (strain FG157)‡
λ	O101:H33	1	—	+	—	AJ716146 (strain FG274)‡
μB	O55:H51*	1	—	+	—	AJ875032 (strain FV276)‡

*Classical EPEC O serogroups.

†Novel serotypes not previously reported in human typical and atypical EPEC strains.

‡Sequence of the 3' variable region of the *eae* gene.

§Classical EPEC O:H serotypes.

||Complete sequence of the *eae* gene.

O111:H—, one O111:H21); 13 O119:H6 strains; eight O55 strains (four O55:H—, three O55:H51, one O55:H40), and other serotypes (O127:H40, O33:H6, O127:H—, O142:HNT, O142:H21, O153:H—, O153:H51) with one intimin each. Genomic DNA fingerprinting of these 44 EPEC strains by PFGE revealed 44 distinct *Xba*I restriction patterns, considering a difference of at least one restriction fragment in the patterns as the criterion for discriminating between them. None of the strains presented an identical PFGE pattern to another. In the dendrogram produced by the UPGMA algorithm, the isolates were clustered in 12 groups (1–12 strains per group) of 60 % similar strains according to the Dice similarity index (Fig. 2). Only 15 strains clustered in six groups of closely related (similarity > 85 %) PFGE patterns: (i) FV336 and FV347; (ii) FV354, FV337, FV367, FV344 and FV345; (iii) FG187 and FV362; (iv) FG240 and FV376; (v) FV359 and FV360; and (vi) FV361 and FV385.

Analysing each of the 12 groups represented in the dendrogram, all groups but two (groups I and XI) corresponded to a particular serogroup. Thus, 14 strains of serogroup O111 with intimin $\beta 1$ were clustered in two clearly differentiated groups (III and IV), both including O111 strains with H2 or H— antigen, suggesting that perhaps those H— by serotyping actually carried genes for H2. Group IV included PFGE patterns more closely related (five strains with similarity > 85 %). Most of the 13 strains of serotype O119:H6 with intimin type $\xi R/\beta 2B$ were clustered in group IX, with only two strains (FG187 and FV362; similarity > 90 %) clearly differentiated in another group (V). A high heterogeneity was observed in group IX, in which genetic relatedness ranged from 65 to 92 %. Strains of serogroup O55 clustered in three different groups (II, VII and VIII). Group II included a single strain of serotype O55:H40 with intimin θ . Group VIII included two closely related strains (FG240 and FV376; similarity 93 %) of

Fig. 1. Phylogenetic tree for amino acid sequences of the intimin variants constructed by the UPGMA method (uncorrected p distances) of MEGA 3.1. Numbers at nodes are the percentage of bootstrap replications in which a particular node was supported. The genetic distance is shown on the scale bar. Phylogenetic analysis revealed six groups of closely related intimins: (i) $\alpha 1$, $\alpha 2$, ζ , νB and o ; (ii) λ ; (iii) $\beta 1$, $\xi R/\beta 2B$, $\delta/\beta 2O$ and κ ; (iv) $\epsilon 1$, ξB , $\nu R/\epsilon 2$, $\eta 1$ and $\eta 2$; (v) $\gamma 1$, μB , $\gamma 2$ and θ ; and (vi) $i 1$ and $\mu R/i 2$.

serotype O55:H– with intimin $\gamma 1$, and group VII five strains of serotypes O55:H–/H51 with intimin μB , of which the genetic relatedness ranged from 65 to 80 %.

DISCUSSION

Diarrhoeal illness is a major public health problem worldwide, with over 2 million deaths occurring each year, particularly among infants younger than 5 years (http://www.who.int/vaccine_research/diseases/e_coli/en/). One of the most common causes of infantile diarrhoea is EPEC. Typical EPEC strains belonging to classical EPEC serotypes were associated historically with outbreaks of infantile diarrhoea in industrialized countries, particularly during the 1940s and 1950s, but at present they are very rare (Trabulsi *et al.*, 2002; Jenkins *et al.*, 2003; Blanco *et al.*, 2006). Recently, however, Gerner-Smidt *et al.* (2003) have reported that classical EPEC is the most common bacterial cause of diarrhoea in children less than 2 years old in Denmark. In

industrialized countries, atypical EPEC are more frequently isolated from diarrhoeal cases than typical EPEC. Furthermore, Nguyen *et al.* (2006) have recently shown that, in contrast to patients infected with other pathogens, patients infected with atypical EPEC are far more likely to experience diarrhoea past 14 days, the point long recognized as a clinical watershed that heralds an increased risk of illness and death. Interestingly, atypical EPEC strains have also been isolated frequently from healthy infants (Afset *et al.*, 2003; Beutin *et al.*, 2003).

The situation in developing countries is not well defined, but several studies in Brazil, Chile, Uruguay and Bangladesh (Albert *et al.*, 1995; Gomes *et al.*, 1996; Levine *et al.*, 1996; Nunes *et al.*, 2003; Vidal *et al.*, 2004) have shown a high frequency of typical EPEC serotypes in stools from children with diarrhoea. However, some recent studies performed in Brazil, Thailand and Vietnam have shown a very low frequency of typical EPEC and a relatively high frequency of atypical EPEC (Pelayo *et al.*, 1999; Vieira *et al.*, 2001; Ratchtrachenchai *et al.*, 2004; Rodrigues *et al.*, 2004; Vu Nguyen *et al.*, 2005). Our results confirm that typical EPEC strains belonging to classical EPEC serotypes O55:H–, O111:H2, O111:H– and O119:H6 are important pathogens associated with diarrhoea of children in Uruguay (Torres *et al.*, 2001). These serotypes are the most frequent EPEC serotypes implicated in infantile diarrhoea in Brazil, and have also been frequently isolated in other countries (Trabulsi *et al.*, 2002; Nunes *et al.*, 2003; Ratchtrachenchai *et al.*, 2004).

Specific intimin subtypes may be involved in mediating both tissue tropism and host specificity, and may provide information on the association of EPEC and STEC with severe disease and on the nature of the bacterium–host relationship. In addition, host immunity to the surface-exposed proteins produced by one *E. coli* strain may not provide protection against intestinal colonization by *E. coli* strains which bear distinct intimin types (Adu-Bobie *et al.*, 1998; Torres *et al.*, 2005). We have identified six novel intimin variant genes that we originally designated $\beta 2$, $\eta 2$, μ , ν , ξ and o when the sequences were submitted to the EMBL Nucleotide Sequence Database (Blanco *et al.*, 2003, 2004b, 2005, 2006), and before knowing the results obtained by Ramachandran *et al.* (2003). The intimin $\beta 2$ that we have found in all 18 typical EPEC strains of classical EPEC serotype O119:H6 (Blanco *et al.*, 2003; this study) is identical to intimin ξ described by Ramachandran *et al.* (2003) in one bovine strain of serotype ONT:HNT. Thus, in this study, our $\beta 2$ intimin is referred to as $\xi R/\beta 2B$. We have found the $\xi R/\beta 2B$ intimin in another four human strains of serotypes O119:H–, ONT:H51 and O33:H6 in the present study, in six human strains of serotypes O56:H6 (*bfpA* negative), O110:H6 (*bfpA* negative), O113:H6 (*bfpA* negative), O137:H6 (*bfpA* negative) and O167:H6 (*bfpA* positive) isolated in Spain (Blanco *et al.*, 2006), and in two *E. coli* strains of serotypes O139:H14 (*bfpA* negative) and O167:H6 (*bfpA* positive) isolated from neotropical

Table 3. Pairwise alignments calculated with CLUSTAL W

Percentage identities are shown. Values in bold type represent the maximum percentage identities between different genes.

<i>eae</i> gene	$\alpha 1$	$\alpha 2$	$\beta 1$	$\xi R/\beta 2B$	$\delta/\beta 2O$	κ	$\gamma 1$	$\gamma 2$	θ	$\varepsilon 1$	$\nu R/\varepsilon 2$	ζ	$\eta 1$	$\eta 2$	$\iota 1$	$\mu R/\iota 2$	λ	μB	νB	ξB	<i>o</i>
$\alpha 1$	100	95	83	88	88	88	87	85	86	84	87	91	86	87	88	82	86	88	89	84	87
$\alpha 2$		100	83	88	87	87	87	85	85	83	87	91	85	87	87	82	87	87	90	83	87
$\beta 1$			100	90	88	88	84	85	85	88	84	83	83	84	84	82	83	83	82	88	82
$\xi R/\beta 2B$				100	93	94	86	85	85	83	87	86	86	88	87	82	86	87	83	83	83
$\delta/\beta 2O$					100	99	87	85	85	82	87	86	85	87	87	82	87	89	84	84	83
κ						100	87	85	85	82	87	86	85	87	87	82	87	89	84	84	83
$\gamma 1$							100	91	91	84	88	86	85	86	86	82	89	93	83	85	83
$\gamma 2$								100	99	84	86	84	83	84	87	83	86	91	83	84	83
θ									100	84	86	84	83	84	87	83	86	91	83	86	83
$\varepsilon 1$										100	91	83	92	92	85	82	85	82	81	97	81
$\nu R/\varepsilon 2$											100	86	93	94	89	83	88	87	83	91	80
ζ												100	85	86	86	81	86	86	89	82	86
$\eta 1$													100	98	86	83	85	85	84	91	83
$\eta 2$														100	88	83	86	87	82	91	82
$\iota 1$															100	89	88	88	83	85	83
$\mu R/\iota 2$																100	83	83	86	81	85
λ																	100	87	84	85	85
μB																		100	84	84	83
νB																			100	82	91
ξB																				100	82
<i>o</i>																					100

non-human primates with diarrhoea in Brazil (Blanco *et al.*, 2004c). The other five intimins described by us ($\eta 2$, μ , ν , ξ and *o*) are different to the existing intimin types and are referred to as $\eta 2$, μB , νB , ξB and *o*, respectively. Interestingly, all six EPEC strains positive for the novel μB intimin belonged to classical EPEC serogroup O55 (serotypes O55:H51 and O55:H-). However, we also detected the $\gamma 1$ (O55:H-) and θ (O55:H40) intimin variant genes in human EPEC strains belonging to serogroup O55 characterized in this study. The novel intimin $\eta 2$ was identified in one human atypical EPEC strain of serotype ONT:H45 isolated in Spain and in three bovine typical EPEC strains of serotype ONT:H45 isolated in Switzerland (Blanco *et al.*, 2005). The novel intimins νR and *o* were detected in human atypical EPEC strains of serotypes O10:H-, O84:H- and O129:H- isolated in Spain (Blanco *et al.*, 2006), whereas the intimin ξB was observed in two Spanish bovine STEC strains of serotype O80:H- (Blanco *et al.*, 2004b).

Our phylogenetic analysis revealed six groups of closely related intimin genes: (i) $\alpha 1$, $\alpha 2$, ζ , νB and *o*; (ii) λ ; (iii) $\beta 1$, $\xi R/\beta 2B$, $\delta/\beta 2O$ and κ ; (iv) $\varepsilon 1$, ξB , $\nu R/\varepsilon 2$, $\eta 1$ and $\eta 2$; (v) $\gamma 1$, μB , $\gamma 2$ and θ ; and (vi) $\iota 1$ and $\mu R/\iota 2$. This analysis is in accordance with the finding of Zhang *et al.* (2002). However, the intimin alleles $\alpha 2$, $\xi R/\beta 2$, $\eta 2$, μB , $\mu R/\iota 2$, νB , $\nu R/\varepsilon 2$, ξB and *o* were not analysed in the study of Zhang *et al.* (2002) because no nucleotide sequences were available.

Ramachandran *et al.* (2003) classified the 14 intimin genes they analysed into nine distinct phylogenetic families ($\alpha 1$ - $\alpha 2$, $\beta 1$ - $\xi R/\beta 2B$, $\gamma 1$, κ , ε - η - $\nu R/\varepsilon 2$, ι - $\mu R/\iota 2$, λ , θ and ζ).

In recent years, DNA macrorestriction analysis by PFGE has increasingly been used for the molecular subtyping of a wide range of bacterial pathogens, and is now considered the 'gold standard' for the molecular subtyping of many pathogenic organisms (Mora *et al.*, 2004). However, to our knowledge, very few studies are available concerning PFGE typing of EPEC strains. In our study we have analysed 44 EPEC strains by PFGE, including the most prevalent serotypes. None of the strains presented an identical PFGE pattern to another. In the dendrogram produced by the UPGMA algorithm, the isolates were clustered in 12 groups (1–12 strains per group) of 60 % similar strains according to the Dice similarity index. Only 15 strains were clustered in six groups of closely related (similarity > 85 %) PFGE patterns, indicating the prevailing clonal diversity among EPEC strains isolated from children with diarrhoea in Montevideo.

In conclusion, we have identified two novel intimin types in typical EPEC of serotypes O55:H51/H- (*eae*- μB) and O119:H6/H- (*eae*- $\xi R/\beta 2B$) that could become novel targets for vaccine development. Our results indicate that typical EPEC strains of serotypes O55:H51/H- (*eae*- μB), O111:H2/H- (*eae*- $\beta 1$) and O119:H6 (*eae*- $\xi R/\beta 2$) are important pathogens associated with diarrhoea in children in Uruguay.

Fig. 2. Dendrogram generated by Bionumeric software, showing distance calculated by the Dice similarity index of PFGE *Xba*I patterns among 44 EPEC strains. The degree of similarity (%) is shown on the scale at the top left of the figure. The strains were clustered in 12 groups generated by the UPGMA algorithm of 60% similarity according to the Dice index.

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REFERENCES

- Adu-Bobie, J., Frankel, G., Bain, C., Goncalves, A. G., Trabulsi, L. R., Douce, G., Knutton, S. & Dougan, G. (1998). Detection of intimins α , β , γ , and δ , four intimin derivatives expressed by attaching and effacing microbial pathogens. *J Clin Microbiol* **36**, 662–668.
- Afset, J. E., Bergh, K. & Bevanger, L. (2003). High prevalence of atypical enteropathogenic *Escherichia coli* (EPEC) in Norwegian children with diarrhoea. *J Med Microbiol* **52**, 1015–1019.
- Albert, M. J., Faruque, S. M., Faruque, A. S. G., Neogi, P. K. B., Ansaruzzaman, M., Bhuiyan, K., Alam, N. A. & Akbar, M. S. (1995). Controlled study of *Escherichia coli* diarrheal infections in Bangladeshi children. *J Clin Microbiol* **33**, 973–977.
- Beutin, L., Marchés, O., Bettelheim, K. A., Gleier, K., Zimmermann, S., Schmidt, H. & Oswald, E. (2003). Hep-2 cell adherence, actin aggregation, and intimin types of attaching and effacing *Escherichia coli* strains isolated from healthy infants in Germany and Australia. *Infect Immun* **71**, 3995–4002.
- Blanco, M., Blanco, J. E., Mora, A. & 9 other authors (2003). Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from healthy sheep in Spain. *J Clin Microbiol* **41**, 1351–1365.
- Blanco, J. E., Blanco, M., Alonso, M. P., Mora, A., Dahbi, G., Coira, M. A. & Blanco, J. (2004a). Serotypes, virulence genes and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from human patients: prevalence in Lugo (Spain) from 1992 through 1999. *J Clin Microbiol* **42**, 311–319.
- Blanco, M., Blanco, J. E., Mora, A., Dahbi, G., Alonso, M. P., González, E. A., Bernárdez, M. I. & Blanco, J. (2004b). Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from cattle in Spain and identification of a new intimin variant gene (*eae-ξ*). *J Clin Microbiol* **42**, 645–651.
- Blanco, M., Blanco, J. E., Carvalho, V. M., Lopes Onuma, D. & Pestana de Castro, A. F. (2004c). Typing of intimin (*eae*) genes in attaching and effacing *Escherichia coli* strains from monkeys. *J Clin Microbiol* **42**, 1382–1383.
- Blanco, M., Podola, N. L., Krüger, A. & 11 other authors (2004d). Virulence genes and intimin types of Shiga-toxin-producing *Escherichia coli* isolated from cattle and beef products in Argentina. *Int Microbiol* **7**, 269–276.
- Blanco, M., Schumacher, S., Tasara, T., Zweifel, C., Blanco, J. E., Dahbi, G., Blanco, J. & Stephan, R. (2005). Serotypes, intimin variants and other virulence factors of *eae* positive *Escherichia coli* strains isolated from healthy cattle in Switzerland. Identification of a new intimin variant gene (*eae-η2*). *BMC Microbiol* **5**, 23.
- Blanco, M., Blanco, J. E., Dahbi, G. & 8 other authors (2006). Identification of two new intimin types in atypical enteropathogenic *Escherichia coli*. *Int Microbiol* **9**, 103–110.
- Bray, J. (1945). Isolation of antigenically homogenous strains of *Bact. Coli neapolitanum* from summer diarrhoea in infants. *J Pathol Bacteriol* **57**, 239–247.
- Frankel, G., Phillips, A. D., Rosenshine, I., Dougan, G., Kaper, J. B. & Knutton, S. (1998). Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Mol Microbiol* **30**, 911–921.
- Gannon, V. P. J., Rashed, M., King, R. K. & Golsteyn Thomas, E. J. (1993). Detection and characterization of the *eae* gene of Shiga-like toxin-producing *Escherichia coli* using polymerase chain reaction. *J Clin Microbiol* **31**, 1268–1274.
- Garrido, P., Blanco, M., Moreno-Paz, M., Briones, C., Dahbi, G., Blanco, J. E., Blanco, J. & Parro, V. (2006). STEC-EPEC oligonucleotide microarray: a new tool for typing genetic variants of the LEE pathogenicity island of human and animal Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC). *Clin Chem* **52**, 192–201.
- Gerner-Smidt, P., Jensen, C., Olsen, K. E. P., Scheutz, F., Molbak, K. & Olesen, B. (2003). Diarrheagenic potential of *Escherichia coli* in children in a developed country. *J Clin Microbiol* **41**, 5836–5837.
- Gomes, T. A., Griffin, P. M., Ivey, C., Trabulsi, L. R. & Ramos, S. R. (1996). EPEC infections in Sao Paulo. *Rev Microbiol* **27**, 25–33.
- Guinée, P. A. M., Jansen, W. H., Wadström, T. & Sellwood, R. (1981). *Escherichia coli* associated with neonatal diarrhoea in piglets and calves. In *Laboratory Diagnosis in Neonatal Calf and Pig Diarrhoea: Current Topics in Veterinary and Animal Science*, vol 13, pp 126–162. Edited by P. W. Leeww & P. A. M. Guinée. The Hague: Martinus-Nijhoff.
- Gunzburg, S. T., Tornieporth, N. G. & Riley, L. W. (1995). Identification of enteropathogenic *Escherichia coli* by PCR-based detection of the bundle-forming pilus gene. *J Clin Microbiol* **33**, 1375–1377.
- Jenkins, C., Lawson, A. J., Cheasty, T., Willshaw, G. A., Wright, P., Dougan, G., Frankel, G. & Smith, H. R. (2003). Subtyping intimin genes from enteropathogenic *Escherichia coli* associated with outbreaks and sporadic cases in the United Kingdom and Eire. *Mol Cell Probes* **17**, 149–156.
- Jerse, A. E., Yu, J., Tall, B. D. & Kaper, J. B. (1990). A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc Natl Acad Sci U S A* **87**, 7839–7843.
- Kaper, J. B. (1996). Defining EPEC. *Rev Microbiol* **27**, 130–133.
- Kaper, J. B., Elliott, S., Sperandio, V., Perna, N. T., Mayhew, G. F. & Blattner, F. R. (1998). Attaching and effacing intestinal histopathology and the locus of enterocyte effacement. In *Escherichia coli O157:H7 and other Shiga toxin-producing E. coli Strains*, pp. 163–182. Edited by J. B. Kaper & A. D. O'Brien. Washington, DC: American Society for Microbiology.
- Kaper, J. B., Nataro, J. P. & Mobley, H. L. T. (2004). Pathogenic *Escherichia coli*. *Nature Rev Microbiol* **2**, 123–140.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Levine, M. M., Prado, V., Lior, H. & Lagos, R. (1996). Epidemiologic studies of diarrhea associated with enteropathogenic *Escherichia coli* (EPEC) in Santiago, Chile. *Rev Microbiol* **27**, 40–44.
- Mora, A., Blanco, M., Blanco, J. E. & 7 other authors (2004). Phage types and genotypes of human and animal Shiga

toxin-producing *Escherichia coli* O157:H7 in Spain. Identification of two predominating phage types (PT2 and PT8). *J Clin Microbiol* **42**, 4007–4015.

Nguyen, R. N., Taylor, L. S., Tauschek, M. & Robins-Browne, R. M. (2006). Atypical enteropathogenic *Escherichia coli* infection and prolonged diarrhea in children. *Emerg Infect Dis* **12**, 597–603.

Nunes, E. B., Saridakis, H. O., Irino, K. & Pelayo, J. S. (2003). Genotypic and phenotypic characterization of attaching and effacing *Escherichia coli* (AEEC) isolated from children with and without diarrhoea in Londrina, Brazil. *J Med Microbiol* **52**, 499–504.

Oswald, E., Schmidt, H., Morabito, S., Karch, H., Marchès, O. & Caprioli, A. (2000). Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: characterization of a new intimin variant. *Infect Immun* **68**, 64–71.

Pelayo, J. S., Scaletsky, I. C., Pedroso, M. Z., Sperandio, V., Giron, J. A., Frankel, G. & Trabulsi, L. R. (1999). Virulence properties of atypical EPEC strains. *J Med Microbiol* **48**, 41–49.

Ramachandran, V., Brett, K., Hornitzky, M. A., Dowton, M., Bettelheim, K. A., Walker, M. J. & Djordjevic, S. P. (2003). Distribution of intimin subtypes among *Escherichia coli* isolates from ruminant and human sources. *J Clin Microbiol* **41**, 5022–5032.

Ratchtrachenchai, O. A., Subpasu, S., Hayashi, H. & Ba-Thein, W. (2004). Prevalence of childhood diarrhoea-associated *Escherichia coli* in Thailand. *J Med Microbiol* **53**, 237–243.

Rodrigues, J., Thomazini, C. M., Morelli, A. & de Batista, G. C. M. (2004). Reduced etiological role for enteropathogenic *Escherichia coli* in cases of diarrhea in Brazilian infants. *J Clin Microbiol* **42**, 398–400.

Schmidt, H., Knop, C., Franke, S., Aleksic, S., Heesemann, J. & Karch, H. (1995). Development of PCR for screening of entero-aggregative *Escherichia coli*. *J Clin Microbiol* **33**, 701–705.

Schultsz, C., Pool, G. J., Van Ketel, R., De Wever, B., Speelman, P. & Dankert, J. (1994). Detection of enterotoxigenic *Escherichia coli* in stool samples by using nonradioactively labeled oligonucleotide DNA probes and PCR. *J Clin Microbiol* **32**, 2393–2397.

Tarr, C. L. & Whittam, S. (2002). Molecular evolution of the intimin gene in O111 clones of pathogenic *Escherichia coli*. *J Bacteriol* **184**, 479–487.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Tornieporth, N. G., John, J., Salgado, K., De Jesus, P., Latham, E., Melo, M. C. N., Gunzburg, S. T. & Riley, L. W. (1995). Differentiation of pathogenic *Escherichia coli* strains in Brazilian children by PCR. *J Clin Microbiol* **33**, 1371–1374.

Torres, M. E. M. C., Pérez, F., Schelotto, F. & 13 other authors (2001). Etiology of children's diarrhea in Montevideo, Uruguay: associated pathogens and unusual isolates. *J Clin Microbiol* **39**, 2134–2139.

Torres, A. G., Zhou, X. & Kaper, J. B. (2005). Adherence of diarrheagenic *Escherichia coli* strains to epithelial cells. *Infect Immun* **73**, 18–29.

Trabulsi, L. R., Keller, R. & Gomes, T. A. T. (2002). Typical and atypical enteropathogenic *Escherichia coli*. *Emerg Infect Dis* **8**, 508–513.

Vidal, R., Vidal, M., Lagos, R., Levine, M. & Prado, V. (2004). Multiplex PCR for diagnosis of enteric infections associated with diarrheagenic *Escherichia coli*. *J Clin Microbiol* **42**, 1787–1789.

Vieira, M. A. M., Andrade, J. R. C., Trabulsi, L. R., Rosa, A. C. P., Dias, A. M. G., Ramos, S. R. T. S., Frankel, G. & Gomes, T. A. T. (2001). Phenotypic and genotypic characteristics of *Escherichia coli* strains of non-enteropathogenic *E. coli* (EPEC) serogroups that carry *eae* and lack the EPEC adherence factor and Shiga toxin DNA probe sequences. *J Infect Dis* **183**, 762–772.

Vu Nguyen, T., Van, P. L., Huy, C. L., Gia, K. N. & Weintraub, A. (2005). Detection and characterization of diarrheagenic *Escherichia coli* from young children in Hanoi, Vietnam. *J Clin Microbiol* **43**, 755–760.

World Health Organization (1987). *Programme for Control of Diarrhoeal Diseases. Manual for laboratory investigation of acute enteric infections*. Geneva: World Health Organization.

Zhang, W. L., Köhler, B., Oswald, E., Beutin, L., Karch, H., Morabito, S., Caprioli, A., Suerbaum, S. & Schmidt, H. (2002). Genetic diversity of intimin genes of attaching and effacing *Escherichia coli* strains. *J Clin Microbiol* **40**, 4486–4492.