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Identification of two new intimin types in atypical enteropathogenic *Escherichia coli*

Summary. Stool specimens of patients with diarrhea or other gastrointestinal alterations who were admitted to Xeral-Calde Hospital (Lugo, Spain) were analyzed for the prevalence of typical and atypical enteropathogenic *Escherichia coli* (EPEC). Atypical EPEC strains (*eae*$^+$ *bfp*$^-$) were detected in 105 (5.2%) of 2015 patients, whereas typical EPEC strains (*eae*$^+$ *bfp*$^+$) were identified in only five (0.2%) patients. Atypical EPEC strains were (after *Salmonella*) the second most frequently recovered enteropathogenic bacteria. In this study, 110 EPEC strains were characterized. The strains belonged to 43 O serogroups and 69 O:H serotypes, including 44 new serotypes not previously reported among human EPEC. However, 29% were of one of three serogroups (O26, O51, and O145) and 33% belonged to eight serotypes (O10:H$^-$, O26:H11, O26:H$^-$, O51:H49, O123:H19, O128:H2, O145:H28, and O145:H$^-$). Only 14 (13%) could be assigned to classical EPEC serotypes. Fifteen intimin types, namely, α1 (6 strains), α2 (4 strains), β1 (34 strains), ξR/β2 (6 strains), γ1 (13 strains), γ2/θ (16 strains), δ/k (5 strains), ε1 (9 strains), νR/ε2 (5 strains), ζ (6 strains), ι1 (1 strain), μR/ι2 (1 strain), νB (1 strain), ξB (1 strain), and ο (2 strains), were detected among the 110 EPEC strains, but none of the strains was positive for intimin types μ1, μ2, λ, or μB. In addition, in atypical EPEC strains of serotypes O10:H$^-$, O84:H$^-$, and O129:H$^-$, two new intimin genes (*eae*-νB and *eae*-ο) were identified. These genes showed less than 95% nucleotide sequence identity with existing intimin types. Phylogenetic analysis revealed six groups of closely related intimin genes: (i) α1, α2, ζ, νB, and ο; (ii) ι1 and μR/ι2; (iii) β1, ξR/β2B, δ/β2O, and κ; (iv) ε1, ξB, η1,η2, and νR/ε2; (v) γ1, μB, γ2, and θ; and (vi) λ. These results indicate that atypical EPEC strains belonging to large number of serotypes and with different intimin types might be frequently isolated from human clinical stool samples in Spain. [**Int Microbiol** 2006; 9(2):103-110]

Key words: attaching and effacing *E. coli* · enteropathogenic *E. coli* · *eae* gene · intimin · locus of enterocyte effacement

Introduction

Five classes of diarrheagenic *Escherichia coli* (DEC) have been described: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), Shiga-toxin-producing (STEC), and enteroaggregative (EAEC). Diffuse adhering *E. coli* (DAEC) may represent a sixth category, but this has not been clearly established [24]. EPEC was the first pathotype of *E. coli* to be described. In 1955, the term enteropathogenic *E. coli* (EPEC) was proposed by Neter et al. [25] to designate certain serotypes of *E. coli* that were associated with outbreaks of infantile diarrhea. In 1987, the World Health Organization [41] recognized EPEC serotypes of 12 different O serogroups (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158). For decades, the mechanisms by

which EPEC caused diarrhea were unknown and this diarrheagenic pathotype could only be identified on the basis of O:H serotyping. In the past 20 years, however, the tools for identifying EPEC have been refined as the molecular basis of EPEC pathogenesis has begun to be elucidated and specific virulence genes have been discovered [10,12,38].

The central mechanism of EPEC pathogenesis is a lesion called attaching and effacing (A/E), which is characterized by microvilli destruction, intimate adherence of bacteria to the intestinal epithelium, pedestal formation, and aggregation of polarized actin and other elements of the cytoskeleton at sites of bacterial attachment. The ability to induce A/E lesions is encoded by genes located on a 35-kb pathogenicity island called the locus of enterocyte effacement (LEE), which contains the genes encoding intimin, a type III secretion system, secreted proteins (Eps), and the translocated intimin receptor (Tir). Homologues of LEE are also found in STEC and in animal A/E *E. coli* strains [4,6,13,20,22,29,40,44].

Intimin, a 94-kDa outer-membrane protein encoded by the gene *eae*, is responsible for the intimate adherence of bacteria to enterocyte membranes [20]. The intimin protein is highly variable between different EPEC and STEC serotypes and at least five distinct antigenic variants have been identified [1]. Differentiation of intimin alleles represents an important tool for STEC and EPEC 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 tropism. The 5´ regions of *eae* genes are conserved, whereas the 3´ regions are heterogeneous. This observation led to the construction of universal PCR primers and allele-specific PCR primers, which has made it possible to differentiate between 21 variants of the *eae* gene that encode 21 different intimin types and subtypes (α1, α2, β1, ξR/β2B, δ/β2O, κ, γ1, γ2, θ, ε1, νR/ε2, ζ, η1, η2, ι1, μR/ι2, λ, μB, νB, ξB, and ο) [1,6,8,9, 13,18,27,30,34,43].

In 1995, EPEC strains were defined as intimin-containing diarrheagenic *E. coli* isolates that possess the ability to form A/E lesions on intestinal cells and that do not possess Shiga-toxin genes [21]. However, EPEC strains can be further classified as typical or atypical. Typical EPEC strains have a virulence plasmid (EAF plasmid) that encodes genes for the bundle-forming pilus (Bfp), which is required for localized adherence on cultured epithelial cells; atypical EPEC strains do not possess the EAF plasmid containing the *bfpA* gene [10,12,38]. The EAF plasmid is not essential for the formation of A/E lesions, although its presence enhances the efficiency with which they occur, probably because of the influence of a cluster of regulatory genes (*perA*, *perB*, and *perC*). However, BFP remains a potentially important virulence factor, as shown by volunteer challenge studies in which a mutant in one of the *bfp* genes (*bfpF*) failed to disperse from microcolonies on infected Hep-2 cells and severely impaired the ability to cause diarrhea in volunteers [10,12,38]. In industrialized countries, atypical EPEC (*eae*$^+$, *bfpA*$^-$) are more frequently isolated from patients with diarrhea, although typical EPEC (*eae*$^+$, *bfpA*$^+$) dominate in developing countries [2,3, 11,14,18,19,28,37–39]. However, few studies have compared the prevalence of typical and atypical EPEC strains in patients with diarrhea in different geographic regions.

The objectives of this study were to determine the prevalence of typical and atypical EPEC in the stool samples submitted for routine pathogen identification to the clinical microbiology laboratory of the Hospital Xeral-Calde of Lugo, Spain, and to establish the serotypes, virulence genes, and intimin types of EPEC isolated between 1996 and 1999. Here, we report two new *eae* variant genes (νB and ο) in atypical EPEC strains and elaborate a PCR scheme for amplification and typing of the 21 *E. coli* intimin variant genes currently known. This is the first study on typical and atypical EPEC in Spain. Our findings suggest that atypical EPEC strains belonging to a large number of serotypes and with different intimin types might be frequently isolated from human clinical stool samples in our country.

Materials and methods

Clinical specimens, culture, EPEC screening and serotyping. Over more than two years, from October 1996 to August 1999, unduplicated fecal samples (only one per patient) obtained from inpatients and outpatients of all ages and submitted to the clinical microbiology laboratory for routine pathogen identification were screened for EPEC and other types of diarrheagenic *E. coli* (DEC). Fecal samples were plated onto MacConkey agar (MAC) and cefixime tellurite sorbitol MacConkey (CTSMAC) medium. EPEC were detected by PCR using specific primers for amplification of eight virulence genes of distinct DEC groups (Table 1S, ONLINE) [5,16,31,32,36]). For PCR, a loopful of bacterial growth taken from the first streaking area of the fecal culture plates was suspended in 0.5 ml of sterile distilled water and boiled for 5 min to release the DNA. From each PCR-positive fecal culture, ten *E. coli*-like colonies obtained from MAC and/or CTSMAC plates were analyzed by PCR in order to obtain DEC isolates for further characterization. If no positive single colony was found among the first ten, at least 40 more colonies were tested. All EPEC isolates were subsequently characterized biochemically with the API 20E system (bioMérieux, France) and serotyped by the method of Guinée et al. [15] employing all available O (O1–O185) and H (H1–H56) antisera. All stool specimens were cultured to identify bacterial enteric pathogens, including *Salmonella*, *Shigella*, *Yersinia*, *Campylobacter*, and *Aeromonas*, by standard methods.

Typing of intimin (*eae*) genes. Intimin genes were typed into *eae* α1, α2, β1, ξR/β2B, δ/β2O, κ, γ1, γ2, θ, ε1, νR/ε2, ζ, η1, η2, ι1, μR/ι2, λ, μB, νB, ξB, and ο by PCR as previouly described [5,6,9,13]. The base sequences and predicted sizes of amplified products for the specific oligonucleotide primers used in this study are shown in Table 2S, ONLINE. We designed oligonucleotide primers according to the nucleotide sequences of the virulence genes. Isolates positive for *eae* with EAE-1 and EAE-2 primers were further analyzed with all the different variant primers.

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 Analyzer (Applied Bio-Systems).

Nucleotide sequence accession numbers. The *eae* sequences of the strains analyzed were deposited in the European Bioinformatics Institute (EMBL Nucleotide Sequence Database). The accession numbers assigned are indicated in Table 3S, ONLINE.

Phylogenetic analyses. Genetic distances and phylogenetic trees of *eae* sequences were calculated and constructed with the CLUSTAL W program [35] included in the EMBL software [http://www.ebi.ac.uk/clustalw/].

Results

Prevalence of EPEC in stool samples. Stool specimens of patients with diarrhea or other gastrointestinal alterations who were admitted to Xeral-Calde Hospital (Lugo City, Spain) were analyzed for the prevalence of typical and atypical enteropathogenic *Escherichia coli* (EPEC). Of 2015 patients investigated, atypical EPEC strains (*eae*$^+$ *bfp*$^-$) were detected in 105 (5.2%). In contrast, typical EPEC (*eae*$^+$ *bfp*$^+$) were identified in only five (0.2%) patients (Table 1). *Salmonella* and atypical EPEC corresponded to the groups of enteropatogenic bacteria that were recovered with higher frequencies.

Virulence genes. In this study, 110 EPEC isolates were characterized. PCR showed that five isolates carried *eae* and *bfp* genes (typical EPEC strains) and 105 possessed only the *eae* gene (atypical EPEC strains). All 110 EPEC strains were negative for the other six DEC virulence genes (*stx*$_1$, *stx*$_2$, *ipaH*, pCDV432, *eltA*, *est*) investigated.

Serotypes. EPEC strains could be classified according to 43 O serogroups, 22 H flagellar antigens, and 69 O:H serotypes, including 44 new serotypes not previously reported among human EPEC. However, 29% of the strains were of three serogroups (O26, O51, and O145), 46% expressed eight flagellar antigens (H2, H6, H11, H16, H19, H28, H40, and H49) and 33% belonged to eight serotypes (O10:H$^-$, O26:H11, O26:H$^-$, O51:H49, O123:H19, O128:H2, O145:H28, and O145:H$^-$). Only 14 (13%) possessed classical EPEC serotypes (O26:H11, O26:H$^-$, O111:H25, O125:H6, O128:H2) (Table 2).

Typing of *eae* (intimin) genes. Fifteen intimin types, namely, α1 (6 strains), α2 (4 strains), β1 (34 strains), ξR/β2 (6 strains), γ1 (13 strains), γ2/θ (16 strains), δ/κ (5 strains), ε1 (9 strains), νR/ε2 (5 strains), ζ (6 strains), ι1 (1 strain), μR/ι2 (1 strain), νB (1 strain), ξB (1 strain), and ο (2 strains), were detected among the 110 EPEC strains. None of the strains was positive for intimin types η1, η2, λ, or μB. H6 flagellar antigen was expressed by all six strains with intimin ξR/β2, whereas H19 was found in all five strains positive for intimin type νR/ε2. Of the 15 strains with intimin γ2/θ, seven possessed the H40 flagellar type (Table 2).

Identification of two new 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 51 representative EPEC strains was sequenced (see Table 3S, ONLINE). A nearly total correlation was observed between the results obtained with the type-specific (TS)-PCR approach and those determined by sequencing of the intimin *eae* gene. In addition, in atypical EPEC strains of serotypes O10:H$^-$, O84:H$^-$, and O129:H$^-$, two new intimin genes *eae*-νB and *eae*-ο were identified that showed less than 95% nucleotide sequence identity with existing intimin genes. The complete nucleotide sequences of the new νB (AJ705050) and ο (AJ876647 and AJ876648) variant genes were determined. We also determined the complete nucleotide sequence of the gene *eae*-ξR/β2 of the atypical EPEC strain IH2664b (O113:H6)

Table 1. Prevalence of diarrheagenic *E. coli* (DEC) and other bacterial enteropathogens in human clinical stool samples (Lugo, Spain, 1996–1999)

Bacterial enteropathogen	Total number analyzed	Number of positive samples
Typical EPEC *eae*$^+$ *bfp*$^+$	2015	5 (0.2%)
Atypical EPEC *eae*$^+$ *bfp*$^-$	2015	105 (5.2%)
STEC (or VTEC)	3212	90 (2.8%)
ETEC	2467	17 (0.7%)
EIEC	2467	6 (0.2%)
EAEC	2467	51 (2.1%)
Salmonella spp.	3794	282 (7.4%)
Campylobacter spp.	3794	159 (4.2%)
Yersinia spp.	3794	10 (0.3%)
Shigella spp.	3794	2 (0.05%)
Aeromonas spp.	3794	9 (0.2%)

Table 2. Intimin types and serotypes of typical and atypical EPEC strains

Intimin type	Number of strains	Serotypes (no. of strains)
Typical EPEC strains (*eae*$^+$ *bfp*$^+$ *stx*$^-$) (n = 5)		
α1	3	O131:H46[a,b] (1), O157:H45 (2)
ξR/β2	1	O167:H6[b] (1)
ι1	1	O153:H8[b] (1)
Atypical EPEC strains (*eae*$^+$ *bfp*$^-$ *stx*$^-$) (n = 105)		
α1	3	O51:H49 (3)
α2	4	O63:H33[a,b] (1), O125:H6[c] (1), O132:H1[a,b] (1), O132:H34 (1)
β1	34	O4:H16[a,b] (2), O6:H1[a,b] (1), O11:H16 (1), O15:H2 (1), O26:H$^-$[c] (5), O26:H11[c] (3), O49:H2[a,b] (1), O51:H$^-$[a] (1), O51:H19[a,b] (1), O51:H49[a] (2), O80:H26[a,b] (1), O103:H$^-$ (2), O115:H8[a,b] (2), O128:H2[c] (3), O145:H10[a,b] (2), O153:H7 (1), O167:H9 (1), O177:H$^-$[b] (1), O177:H11[b] (2), ONT:H2[a] (1)
ξR/β2	5	O56:H6[a] (1), O110:H6[a,b] (1), O113:H6[a,b] (2), O137:H6[a,b] (1)
δ/κ	5	O49:H$^-$[b] (1), O71:H49[a,b] (1), O88:H$^-$ (2), ONT:H$^-$[a] (1)
γ1	13	O55:H11[a,b] (1), O145:H28 (4), O145:H$^-$ (8)
γ2/θ	16	O2:H40[a,b] (1), O5:H11[b] (1), O10:H$^-$[a,b] (2), O11:H25[a,b] (1), O51:H40[a,b] (2), O71:H40[a,b] (2), O76:H7[a,b] (1), O103:H40[a,b] (1), O111:H25[a,c] (1), O117:H40[a,b] (1), O153:H$^-$[a,b] (1), O159:H4[a,b] (1), ONT:H$^-$ (1)
ε1	9	O26:H$^-$[a,c] (1), O45:H40[a,b] (1), O80:H26[a,b] (1), O103:H2 (1), O140:H31[a,b] (1), O152:H38[a,b] (2), O157:H16[a,b] (1), ONT:H16[a,b] (1)
R/ε2	5	O6:H19[a,b] (1), O123:H19[a,b] (4)
ζ	6	O85:H31[a,b] (1), O85:H$^-$ (1), O85:HNT[a,b] (1), O156:H25 (1), O156:H$^-$[b] (1), O179:HNT[a,b] (1)
μR/ι2	1	ONT:H45[a] (1)
νB	1	O10:H$^-$[a,b] (1)
ξB	1	O80:H2[a,b] (1)
o	2	O84:H$^-$[a,b] (1), O129:H$^-$[a,b] (1)

[a]Serotypes not previously reported to possess these intimin types.
[b]New serotypes (n = 44) not previously reported in human typical and atypical EPEC strains.
[c]Classical EPEC serotypes.

(AJ715408). The sequence was identical to the *eae*-ξR/β2 allele sequenced by Ramachandran et al. [30] from the bovine strain KB411 (ONT:HNT) (AF530556) and to the allele *eae*-ξR/β2 sequenced by Blanco et al. [unpublished data] from the human typical EPEC strain FV359 (O119:H6) (AJ715407). Furthermore, we determined the complete nucleotide sequence of the gene *eae*-ξB of the atypical EPEC strain IH2475B (O80:H2) (AM180621). That sequence was identical to the *eae*-ξB allele sequenced by Blanco et al. [unpublished data] from the bovine STEC strain B49 (O80:H$^-$) (AJ705051).

In another approach, the genetic relationship of 21 *eae* variants (α1, α2, β1, ξR/β2B, δ/β2O, κ, γ1, γ2, θ, ε1, νR/ε2, ζ, η1, η2, ι1, μR/ι2, λ, μB, νB, ξB and o) was established (Table 3). Since the nucleotide sequences analyzed differed in length, CLUSTAL W was used for optimal sequence alignment. Identities of 89, 90, 89, and 91% were calculated between the new *eae*-ν*B* variant and the genes *eae*-α1, *eae*-α2, *ea*-ζ and *eae*-o, respectively. The genetic distances between the new *eae*-o gene and *eae*-α1, *eae*-α2, *eae*-ζ and *eae*-ν*B* were 87, 87, 86, and 91%, respectively. Phylogenetic analysis revealed six groups of closely related intimin genes (Fig. 1).

Discussion

Typical EPEC strains are diarrheagenic *E. coli* historically associated with outbreaks of infantile diarrhea, particularly during the 1940s and 1950s. Although large outbreaks of infant diarrhea due to typical EPEC have largely disappeared from industrialized countries, typical EPEC strains remain an important cause of potentially fatal infant diarrhea in developing countries [38]. In Brazil and Uruguay, for example, EPEC strains were recovered in 30% or more of the samples obtained from infants of low socioeconomic level who were treated for diarrhea [14,37].

Our findings suggest that atypical EPEC strains could be a significative cause of human infections in Spain and confirm that, in developed countries, atypical EPEC are more frequently isolated from patients with diarrhea (5.2%) than typical EPEC (0.2%). It is also remarkable that, with respect to enteropathogenic bacteria, atypical EPEC strains were the second most frequently isolated group. However, as atypical EPEC strains have been frequently isolated from healthy

Table 3. Designations, sequence characteristics, and origins of the intimin alleles analyzed in this study. Genetic relationship of the new intimin genes (νB and ο) detected in atypical human EPEC strains and the remaining *eae* variants: Pairwise alignments calculated with CLUSTAL W

Designation of intimin	ORF length (bp)	Reference strain	Serotype	Origin	Accession number	Genetic relationship identities with intimin νB	Genetic relationship identities with intimin ο	Reference
α1	2820	E2348/69	O127:H6	Human	M58154	89%	87%	[27]
α2	2820	*E. coli*	O125:H6	Human	AF530555	90%	87%	[30]
β1	2820	RDEC1	O15:H$^-$	Rabbit	AF200363	82%	82%	[44]
ξR/β2B	2820	KB411	ONT:HNT	Bovine	AF530556	83%	83%	[30]
ξR/β2B	2820	FV359	O119:H6	Human	AJ715407	83%	83%	Blanco et al. (unpublished)
δ/β2O	2820	BL152.1	O86:H34	Human	AJ875027	84%	83%	Blanco et al. (unpublished)
κ	2820	6044/95	O118 :H5	Human	AJ308552	84%	83%	[43]
γ1	2805	EDL933	O157:H7	Human	AF071034	83%	83%	[29]
γ2	2808	95NR1	O111:H$^-$	Human	AF025311	83%	83%	[40]
θ	2808	CL-37	O111:H8	Human	AF449418	83%	83%	[34]
ε1	2847	PMK5	O103:H2	Human	AF116899	81%	81%	[27]
νR/ε2	2847	VR64/4	O2related:H19	Ovine	AF530554	83%	80%	[30]
ζ	2817	4795/95	O84:H4	Human	AJ271407	89%	86%	[43]
η1	2847	CF11201	O125:H$^-$	Human	AJ308550	84%	83%	[43]
η2	2847	H03/53199a	ONT:H45	human	AJ876652	82%	82%	[9]
ι1	2814	7476/96	O145:H4	Human	AJ308551	83%	83%	[43]
μR/ι2	2814	VR45	OR:H$^-$	Ovine	AF530553	86%	85%	[30]
λ	2817	EPEC-68.4	O34:H$^-$	Human	AJ715409	84%	85%	Blanco et al. (unpublished)
μB	2808	EPEC-373	O55:H51	Human	AJ705049	84%	83%	Blanco et al. (unpublished)
νB	2823	IH1229a	O10:H$^-$	Human	AJ705050	100%	91%	This study
ξB	2847	STEC-B49	O80:H$^-$	Bovine	AJ705051	82%	82%	Blanco et al. (unpublished)
ο	2820	IH2997f	O129.H$^-$	Human	AJ876648	91%	100%	This study

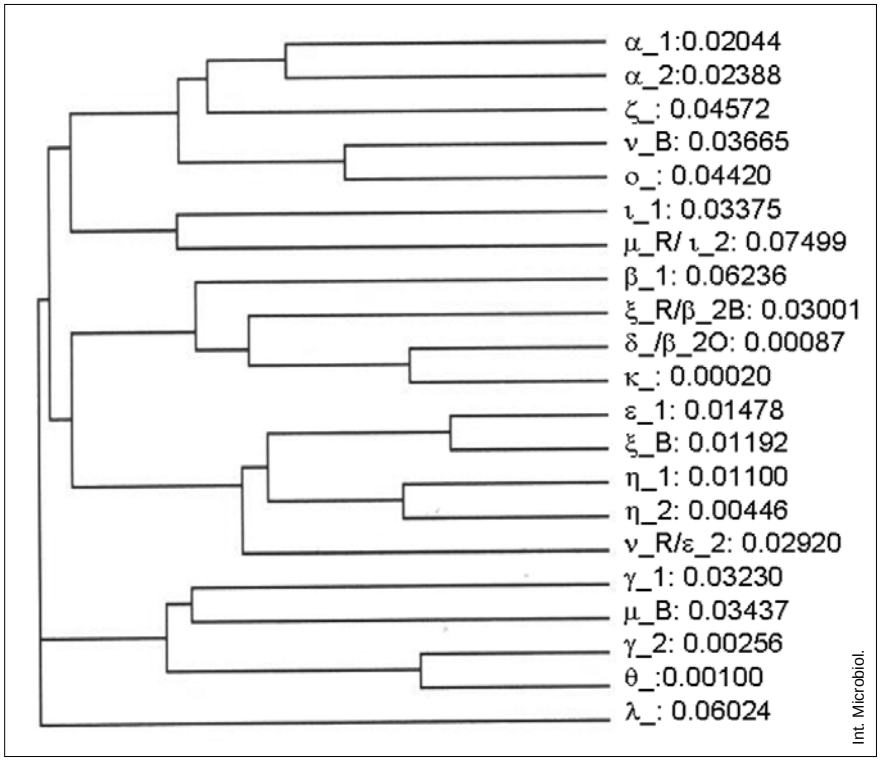

Fig. 1. Phylogenetic tree of the intimin variant genes. The tree was constructed using the CLUSTAL W program. Numbers on the branches denote genetic distances. Phylogenetic analysis revealed six groups of closely related intimin genes: (i) α1, α2, ζ, νB, and ο; (ii) ι1 and μR/ι2; (iii) β1, ξR/β2B, δ/β2O, and κ; (iv) ε1, ξB, η1, η2, and νR/ε2; (v) γ1, μB, γ2, and θ; and (vi) λ.

infants [3,19,28,39], further studies are required to establish the clinical significance of this finding. In Switzerland, Pabst et al. [28] detected EPEC (*eae*$^+$ *stx*$^-$) in the specimens of 30 (16%) of 187 children with diarrhea and in those of 15 (11%) of 137 controls, with similar frequencies in all age groups. Of the 1010 fecal specimens obtained from children in Brazil, Vieira et al. [39] found that 32 (6.3%) of 505 diarrhea patients and 27 (5.3%) of 505 control patients carried atypical EPEC strains bearing only the *eae* gene. Twenty-five atypical EPEC strains were isolated from 12 healthy children (6.5%) in Australia and from 12 healthy children (5.9%) in Berlin by Beutin et al. [3]. Atypical EPEC strains were isolated with a similar frequency from healthy infants in France (6.5%) [11]. Although it is not clear whether, taken as a whole group, atypical EPEC strains can be considered as human enteropathogens, some atypical EPEC strains of serotypes O39:H$^-$, O88:H5, O91:H7, O111:H9, and ONT:H10 have caused large outbreaks of diarrheal disease involving both children and adults in the UK and the USA [17,18,42]. An O39:H$^-$ atypical EPEC strain (*eae*$^+$ EAF$^-$) was responsible for a foodborne diarrheal outbreak in 1991, involving 100 adults in Minnesota [17]. Furthermore, Nguyen et al. [26] recently showed that, in contrast to patients infected with other pathogens, those infected with atypical EPEC are far more likely to experience diarrhea for more than 14 days, the time-point long recognized as a clinical watershed that heralds increased risk for illness and death.

In order to determine the pathogenic potential of atypical EPEC strains isolated from healthy children, Beutin et al. [3] investigated the strains for their AE phenotype using the FAS assay. They found that most of atypical EPEC from healthy infants either do not or only weakly express an AE phenotype, which could explain why these strains did not cause diarrheal disease. However, most atypical EPEC from healthy infants were not affected with respect to the function of LEE-associated genes because the strains became clearly AE positive after the uptake of EAF plasmid. Beutin et al. [3] suggested that atypical EPEC strains play a dual role, as strains that naturally immunize against intimin in humans and as reservoirs for newly emerging human pathogenic EPEC strains. Further studies are required to compare the serotypes and virulence factors of atypical EPEC strains isolated from children with and without diarrhea in prospective case-control studies. It is possible that only some atypical EPEC strains belonging to determined serotypes and with specific virulence factors can cause diarrhea in humans.

In agreement with previous reports [3,26,43], we have found that humans can be infected with a large spectrum of serologically different atypical EPEC strains. In the present study, atypical EPEC strains belonged to 65 O:H serotypes, including 43 new serotypes not previously described among human EPEC. Interestingly, only 14 (13%) of 105 atypical EPEC strains possessed classical EPEC serotypes (O26:H11, O26:H$^-$, O111:H25, O125:H6, O128:H2). It is also remarkable that, among the five typical EPEC strains isolated in this study, three new serotypes, O131:H46, O153:H8, and O167:H6, were found. The two remaining typical EPEC strains belonged to the non-classical EPEC serotype O157:H45. Makino et al. [23] reported the first large outbreak with EPEC O157:H45 in Japan in 1998. Recently, Stephan et al. [33] isolated typical EPEC O157:H45 strains from cattle in Switzerland.

The differentiation of intimin alleles is an important tool for EPEC and STEC typing in routine diagnostics as well as in pathogenesis, epidemiological, clonal, and immunological studies [1,4,6,9,18,27,30,34,43]. We recently identified six new intimin variant genes [6,9, Blanco et al. unpublished data] that we originally designated as β2, η2, μ, ν, and ξ when the sequences were submitted to the EMBL Nucleotide Sequence Database, and before becoming aware of the results obtained by Ramachandran et al [30]. The intimin β2 (AJ715407) [Blanco et al. unpublished data], which we found in typical EPEC strains of classical EPEC serotype O119:H6, is identical to the intimin ξ described by Ramachandran et al. [30] in a bovine strain of serotype ONT:HNT. Thus, in this study, the β2 intimin described by our group is referred to as ξR/β2B. In the present report, ξR/β2B intimin was found in six strains of serotypes O56:H6 (*bfpA* negative), O110:H6 (*bfpA* negative), O113:H6 (*bfpA* negative), O137:H6 (*bfpA* negative), and O167:H6 (*bfpA* positive). Additionally, this intimin was also identified in two *E. coli* strains of serotypes O139:H14 (*bfpA* negative) and O167:H6 (*bfpA* positive) isolated in Brazil from neotropical nonhuman primates with diarrhea [7]. The other five intimins that we discovered (η2, μ, ν, ξ, and ο) differ from the previously known intimin types and are referred to as η2, μB, νB, ξB, and ο, respectively. The new intimin η2 was identified in one human atypical EPEC strain of serotype ONT:H45 isolated in Spain in 2003 and in three bovine typical EPEC strains of serotype ONT:H45 isolated in Switzerland [9]. Interestingly, all six EPEC strains positive for the new μB intimin belonged to classical EPEC serogroup O55 (serotypes O55:H51 and O55:H$^-$) [Blanco et al. unpublished data]. The new intimin νR was detected in the present study in one human atypical EPEC strain of serotype O10:H$^-$ isolated in Spain, whereas intimin ξB was observed in two Spanish bovine STEC strains of serotype O80:H$^-$ [6] and in an atypical human EPEC strain of serotype O80:H2 in this study. In the present study, atypical EPEC strains of serotypes O84:H$^-$ and O129:H$^-$ were found to contain the new intimin gen *eae*-ο, which showed less than 95% nucleotide sequence identity with existing intimin genes.

Our phylogenetic analysis revealed six groups of closely related intimin genes: (i) α1, α2, ζ, νB, and ο; (ii) ι1 and μR/ι2; (iii) β1, ξR/β2B, δ/β2O, and κ; (iv) ε1, ξB, η1, η2, and

νR/ε2; (v) γ1, μB, γ2, and θ; and (vi) λ. This analysis is in accordance with the report of Zhang et al. [43]. However, the intimin alleles α2, ξR/β2, η2, μB, μR/ι2, νB, νR/ε2, ξB, and ο were not analyzed in that study because no nucleotide sequences were available.

In conclusion, our findings indicate that atypical EPEC strains belonging to a large number of serotypes and with different intimin types could be frequently isolated from human clinical stools samples in Spain.

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Identificación de dos nuevos tipos de intiminas en *Escherichia coli* enteropatógenas atípicas

Resumen. En este estudio hemos analizado la prevalencia de *Escherichia coli* enteropatógenas (ECEP) típicas y atípicas en las muestras fecales de pacientes con diarrea y otras alteraciones gastrointestinales del Complexo Hospitalario Xeral-Calde de Lugo (España). Las ECEP atípicas (*eae*$^+$ *bfp*$^-$) se detectaron en 105 (5.2%) de los 2015 casos investigados, mientras que las ECEP típicas (*eae*$^+$ *bfp*$^+$) se identificaron en solamente cinco (0.2%) pacientes. Las ECEP atípicas fueron los segundos enteropatógenos más frecuentemente aislados después de *Salmonella*. Un total de 110 cepas de ECEP fueron caracterizadas en este estudio. Las cepas de ECEP pertenecían a 43 serogrupos O y 69 serotipos O:H, entre los cuales había 44 nuevos serotipos no encontrados previamente entre ECEP humanas. No obstante, el 29% de las cepas se pudieron englobar en tres serogrupos (O26, O51 y O145) y el 33% pertenecían a 8 serotipos (O10:H$^-$, O26:H11, O26:H$^-$, O51:H49, O123:H19, O128:H2, O145:H28 y O145:H$^-$). Únicamente 14 (13%) cepas presentaron serotipos de ECEP clásicos. Se detectaron 15 tipos de intiminas entre las 110 cepas de ECEP examinadas: α1 (6 cepas), α2 (4 cepas), β1 (34 cepas), ξR/β2 (6 cepas), γ1 (13 cepas), γ2/θ (16 cepas), δ/κ (5 cepas), ε1 (9 cepas), νR/ε2 (5 cepas), ζ (6 cepas), ι1 (1 cepa), μR/ι2 (1 cepa), νB (1 cepa), ξB (1 cepa) y ο (2 cepas). No se encontró ninguna cepa con las intiminas η1, η2, λ, o μB. Además, en cepas de ECEP atípicas de los serotipos O10:H$^-$, O84:H$^-$ y O129:H$^-$ se identificaron dos nuevos tipos de genes que codifican intiminas (*eae*-νB y *eae*-ο) y que mostraron menos de un 95% de identidad en su secuencia nucleótidica con las de otros tipos de intiminas. El análisis filogenético reveló que los genes que codifican los diferentes tipos de intiminas se pueden englobar en seis grupos: (i) α1, α2, ζ, νB, ο; (ii) ι1, μR/ι2; (iii) β1, ξR/β2B, δ/β2O, κ; (iv) ε1, ξB, η1, η2, νR/ε2; (v) γ1, μB, γ2, θ; (vi) λ. En conclusión, nuestros resultados indican que en muestras clínicas humanas en España podrían aislarse con frecuencia ECEP atípicas pertenecientes a un amplio margen de serotipos y con diferentes tipos de intiminas. [**Int Microbiol** 2006; 9(2):103-110]

Palabras clave: *E. coli* de adhesión y borrado · *E. coli* enteropatógenas · gen *eae* · intimina · isla de patogenicidad LEE

Identificação de dois novos tipos de intiminas em *Escherichia coli* enteropatógenas atípicas

Resumo. Neste estudo analisámos a prevalência de *Escherichia coli* enteropatógenas (ECEP) típicas e atípicas nas mostras fecais de pacientes com diarreia e outras alterações gastrointestinais do Complexo Hospitalar Xeral-Calde de Lugo (Espanha). As ECEP atípicas (*eae*$^+$ *bfp*$^-$) detectaram-se em 105 (5,2%) dos 2015 casos investigados, enquanto as ECEP típicas (*eae*$^+$ *bfp*$^+$) foram identificadas em soamente cinco (0,2%) pacientes. As ECEP atípicas foram os segundos enteropatógenos mais freqüentemente isolados depois da *Salmonella*. Um total de 110 cepas de ECEP foram caracterizadas neste estudo. As cepas de ECEP pertenciam a 43 serogrupos O e 69 serotipos O:H, incluídos 44 novos serotipos não encontrados previamente entre ECEP humanos em outros estudos. Não obstante, 29% das cepas puderam englobar-se em três serogrupos (O26, O51 e O145) e 33% pertenciam a 8 serotipos (O10:H$^-$, O26:H11, O26:H$^-$, O51:H49, O123:H19, O128:H2, O145:H28 e O145:H$^-$). Apenas 14 (13%) cepas apresentaram serotipos de ECEP clássicos. Detectaram-se 15 tipos de intiminas entre as 110 cepas de ECEP examinadas: α1 (6 cepas), α2 (4 cepas), β1 (34 cepas), ξR/β2 (6 cepas), γ1 (13 cepas), γ2/θ (16 cepas), δ/κ (5 cepas), ε1 (9 cepas), νR/ε2 (5 cepas), ζ (6 cepas), ι1 (1 cepa), μR/ι2 (1 cepa), νB (1 cepa), ξB (1 cepa) y ο (2 cepas). Não se encontrou nenhuma cepa com as intiminas η1, η2, λ, o μB. Além disso, identificámos em cepas de ECEP atípicos dos serotipos O10:H$^-$, O84:H$^-$ e O129:H$^-$, dois novos tipos de genes que codificam intiminas (*eae*-νB e *eae*-ο). Estes genes mostraram menos de 95% de identidade na sua seqüência nucleótidica relativamente aos de outros tipos de intiminas. A análise filogenética revelou que os genes que codificam os diferentes tipos de intiminas se podem englobar em seis grupos: (i) α1, α2, ζ, νB, ο; (ii) ι1, μR/ι2; (iii) β1, ξR/β2B, δ/β2O, κ; (iv) ε1, ξB, η1, η2, νR/ε2; (v) γ1, μB, γ2, θ; (vi) λ. Em conclusão, os nossos resultados indicam que em amostras clínicas humanas em Espanha se poderiam freqüentemente isolar ECEP atípicas pertencentes a um amplo abanico de serotipos e com diferentes tipos de intiminas. [**Int Microbiol** 2006; 9(2):103-110]

Palavras chave: *E. coli* de adesão e apagado · *E. coli* enteropatógenas · gene *eae* · intimina · ilha de patogenicidad LEE