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Comparison of ruminant and human attaching and effacing *Escherichia coli* (AEEC) strains

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ABSTRACT

The presence of 12 genes associated with virulence in human attaching and effacing *Escherichia coli* (AEEC) was studied within a collection of 20 enterohemorrhagic *E. coli* (EHEC) and 206 atypical enteropathogenic *E. coli* (EPEC) isolated from ruminants. In addition, virulence genes and the clonal relationship of 49 atypical EPEC O26 strains isolated from humans and ruminants were compared to clarify whether ruminants serve as a reservoir of atypical EPEC for humans. A great diversity in the content of virulence gene was found. Thus, the *espH*, *espG* and *map* genes were detected in more than 85% of ruminant AEEC strains; the *tccP2*, *espI*, *efa1*/*lifA*, *ehxA* and *paa* genes were present in 50–70% of strains; and other genes such as *tccP*, *espP*, *katP* and *toxB* were detected in <25% of strains. EHEC strains contained more virulence genes than atypical EPEC strains. Our results suggest for the first time that the *efa1/lifA* gene is associated with diarrhea in newborn ruminants and that the AEEC strains with the H11 flagellar antigen are potentially more virulent than the non-H11 AEEC strains. Importantly, we identified a new intimin variant gene, *eae*ρ, in three ruminant atypical EPEC strains. The comparison of ruminant and human EPEC O26 strains showed that some ruminant strains possess virulence gene profiles and pulse-field gel electrophoresis pulsotypes similar to those of human strains. In conclusion, our data suggest that atypical EPEC is a heterogeneous group with different pathogenic potential and that ruminants could serve as a reservoir of atypical EPEC for humans.

1. Introduction

Attaching and effacing *Escherichia coli* (AEEC) are a cause of diarrhea in humans and animals. These bacteria cause a characteristic attaching and effacing (AE) lesion in the gut mucosa (Nataro and Kaper, 1998). The formation of AE lesions is governed by the locus of enterocyte effacement, which contains the *eae* gene that encodes an outer membrane protein called intimin, which is necessary for intimate attachment to epithelial cells (Nataro and Kaper, 1998). Human enterohemorrhagic *E. coli* (EHEC) and human enteropathogenic *E. coli* (EPEC) cause AE lesions in the intestinal mucosa. In contrast to EPEC, EHEC strains produce verotoxins (VTs) (Nataro and Kaper, 1998). Domestic ruminants, mainly cattle, have been implicated as the principal reservoir of EHEC strains for humans (Nataro and Kaper, 1998). EPEC have been classified as typical or atypical. Typical EPEC harbor EAF (EPEC

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adherence plasmid) that encodes bundle forming pilus (Bfp) and are rarely found in animals, whereas atypical EPEC lack of the EAF plasmid and can be isolated from humans and animals (Blanco et al., 2006a,b; Hernandes et al., 2009; Nataro and Kaper, 1998). In contrast to EHEC, the role of ruminants as reservoirs of atypical EPEC that are pathogenic for humans is not well established (Hernandes et al., 2009).

Virulence genes of AEEC have been mainly studied in human strains. Molecular characterization of human atypical EPEC strains has shown great diversity of virulence factors among different strains (Beutin et al., 2005; Scaletsky et al., 2009). It is therefore possible that different strains within the group of atypical EPEC have different pathogenic potentials, and that only some of these strains can cause disease in humans. In contrast to human AEEC strains, only a limited number of studies have been carried out on the properties and virulence genes of atypical EPEC strains isolated from ruminants.

The present study investigated the presence of 12 genes associated with virulence in a large collection of AEEC strains with different serotypes and intimin variants isolated from ruminants, and in atypical EPEC O26 strains isolated from humans. It also compared the virulence gene profiles and clonal relationship between atypical EPEC O26 strains isolated from humans and ruminants, to clarify whether ruminants serve as a reservoir of atypical EPEC for humans, in addition to EHEC.

2. Materials and methods

2.1. Bacterial strains

A total of 226 AEEC strains isolated between 1993 and 2005 in Spain from calves (24 strains), lambs (17 strains) and goat kids (7 strains) with diarrhea and from healthy cattle (39 strains), sheep (62 strains) and goats (77 strains) were used in this study. Twenty of these strains were EHEC (*eae*$^+$, VT$^+$) and 206 atypical EPEC (*eae*$^+$, VT$^-$, *bfpA*$^-$). These strains have previously been tested for their VT types, *eae* variants and serogrouped (Cid et al., 2001; Orden et al., 2003, 2010; Cortés et al., 2005). Twelve of the 20 EHEC strains produced VT1, 4 VT2, and 4 VT1 + VT2. The variants of the *eae* gene of the strains were: β1 (100 strains), β2 (1), γ1 (18), γ2/θ (56), δ/κ (2), ε (22), ζ (10), ι (11), xi (3), and ρ (3). The *eaeρ* gene is a new allele identified in the present study in three ruminant EPEC strains that carried unknown intimin types (Orden et al., 2003). From a representative group of 90 strains that were previously tested in the rabbit ileal loop assay, 84 (93.3%) were able to induce AE lesions (De la Fuente et al., 2004). Only one strain with different characteristics (serotype and production of VT) per animal was included in the study. Twenty-three atypical EPEC strains isolated from humans, which belonged to serogroup O26 were also included for comparison with atypical EPEC O26 strains isolated from ruminants. These human isolates were obtained between 1996 and 2007 from patients with diarrhea or other gastrointestinal alterations at the Xeral–Calde Hospital, Lugo (Spain) (Blanco et al., 2006a; Arbeloa et al., 2009).

2.2. Serotyping

In most of the strains tested, the H antigen had not been previously analyzed, therefore, this antigen was determined by agglutination for the present study in the Laboratorio de Referencia de *E. coli* (Lugo, Spain) using all available H (H1–H56) antisera. Strains that did not react with H antisera were classified as non-typeable, and those that were non-motile were denoted HNM.

2.3. Detection of virulence genes by PCR

The presence of the virulence genes *espG*, *map*, *espH*, *tccP*, *tccP2*, *espI*, *efa1*/*lifA*, *paa*, *ehxA*, *espP*, *katP* and *toxB* was determined by PCR. In addition, subtypes of *espG* (α and β) and *espP* (α, β, γ and δ) were studied. Previously described primers were used to detect the majority of these genes (Schmidt et al., 1995; Tarr et al., 2002; Mundy et al., 2004; Beutin et al., 2005; Garmendia et al., 2005a; Geue et al., 2006; Smollett et al., 2006; Brockmeyer et al., 2007; Ogura et al., 2007). However, primers specific for the *espH* (espH-F: GTAGATGAAMTKCARYTTAAAGC and espH-R: AGTTCGAGGAAACCAGGTTG) and *map* (map-F: ATGTTTAGTCCAATGACAATG and map-R: CTTGTGA-GACTTCTATCATC) genes were designed for the present study. The *E. coli* strains used as controls were as follows: EDL933 (*espGα*, *map*, *espH*, *tccP*, *espI, paa*, *ehxA*, *espPα*, *katP*, *toxB*), PMK5 (*espGβ*, *tccP2*, *efa1*/*lifA*, *ehxA*, *katP*), 36/03 (*espPβ*), 100/04 (*espPγ*), 4795/97 (*espPδ*), and HS (negative control).

2.4. Sequencing of the tccP2 gene and the new intimin variant gene eaeρ

Not all *tccP2*-positive AEEC strains harbor an intact functional *tccP2* gene (Ogura et al., 2007). Thus, typical EHEC O157:H7 strains contain a *tccP2* gene with a deletion of a single (T/A) base pair at codon 28, which introduces a translation frame shift and a premature stop codon. Six *tccP2*-positive AEEC strains that were representative of the most frequent serotype–intimin combinations of ruminant AEEC (O3:HNM *eaeβ*1 EPEC, O26:H11 *eaeβ1* EHEC, O26:HNM *eaeβ1* EPEC, O103:H2 *eaeε* EPEC, O153:HNM *eaeβ1* EPEC, and O177:H11 *eaeβ1* EPEC), one of the three O157:H7 *eaeγ1* EHEC strains included in this study, and three of the non-O157 AEEC strains that were positive for *tccP* and *tccP2* (O103:H25 *eaeγ2* EPEC, O156:H25 *eaeζ* EPEC, and ONT:HNM *eaeζ* EPEC) were used to sequence the *tccP2* gene to establish if this gene was intact. PCR was performed as described by Ooka et al. (2007). The PCR product was purified by using SpinPrep™ PCR Clean-Up Kit (Novagen®) and DNA was sequenced at the Sequencing Unit of CIB-CSIC (Madrid, Spain) and SECUGEN S.L. (Madrid, Spain). The CLUSTAL X program was used to align the sequences.

To identify the intimin type of the three ruminant EPEC strains that carried unknown intimin types, the complete nucleotide sequence of the *eae* gene was determined by PCR and sequencing as previously described (Blanco et al., 2006b; Garrido et al., 2006). We determined the genetic relationship of the new intimin variant gene *eaeρ* and the