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)

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Abstract

Background: Enteropathogenic Escherichia coli (EPEC) and Shigatoxin-producing Escherichia coli (STEC) share the ability to introduce attaching-and-effacing (A/E) lesions on intestinal cells. The genetic determinants for the production of A/E lesions are located on the locus of enterocyte effacement (LEE), a pathogenicity island that also contains the genes encoding intimin (eae). This study reports information on the occurrence of eae positive E. coli carried by healthy cattle at the point of slaughter, and on serotypes, intimin variants, and further virulence factors of isolated EPEC and STEC strains.

Results: Of 51 eae positive bovine E. coli strains, 59% were classified as EPEC and 41% as STEC. EPEC strains belonged to 18 O:H serotypes, six strains to typical EPEC serogroups. EPEC strains harbored a variety of intimin variants with eae-β1 being most frequently found. Moreover, nine EPEC strains harbored astA (EAST1), seven bfpA (bundlin), and only one strain was positive for the EAF plasmid. We have identified a new intimin gene (η2) in three bovine bfpA and astA-positive EPEC strains of serotype ONT:H45. STEC strains belonged to seven O:H serotypes with one serotype (O103:H2) accounting for 48% of the strains. The majority of bovine STEC strains (90%) belonged to five serotypes previously reported in association with hemolytic uremic syndrome (HUS), including one O157:H7 STEC strain. STEC strains harbored four intimin variants with eae-ε1 and eae-γ1 being most frequently found. Moreover, the majority of STEC strains carried only stx1 genes (13 strains), and was positive for ehxA (18 strains) encoding for Enterohemolysin. Four STEC strains showed a virulence pattern characteristic of highly virulent human strains (stx2 and eae positive).

Conclusion: Our data confirm that ruminants are an important source of serologically and genetically diverse intimin-harboring E. coli strains. Moreover, cattle have not only to be considered as important asymptomatic carriers of O157 STEC but can also be a reservoir of EPEC and eae positive non-O157 STEC, which are described in association with human diseases.
Background
Enteropathogenic *Escherichia coli* (EPEC) and Shiga toxin-producing *Escherichia coli* (STEC) represent two of the at least six different categories of diarrheagenic *E. coli* recognized at present [1]. Unlike other diarrheagenic *E. coli*, EPEC and STEC share the ability to introduce attaching-and-effacing (A/E) lesions on intestinal epithelial cells. A/E lesions are characterized by destruction of the microvillus brush border through restructuring of the underlying cytoskeleton by signal transduction between bacterial and host cells, intimate adherence of strains to the intestinal epithelium, pedestal formation and aggregation of polymerized actin at the sites of bacterial attachment [1,2]. The genetic determinants for the production of A/E lesions are encoded in a pathogenicity island called the locus of enteroocyte effacement (LEE) [3]. The LEE encodes the outer membrane protein intimin, which is encoded by the *eae* gene (for *E. coli*attachment effacement) localized in the central region of LEE, a third type secretion system, a number of secreted proteins (ESP), and Tir (for translocated intimin receptor), a protein encoded upstream of the *eae* gene, which is translocated into host cells [2]. Characterization of *eae* genes revealed the existence of different variants. At present, 17 genetic variants (α1, α2, β1, δ/R/β2B, δ/R/β2O, γ1, θ/γ2, ε1, VR/e2, η, ι1, μ/R/12, λ, μB, vB, ζB) have been identified (Table 4) [4-9]. The heterogeneous C-terminal (3′) end of intimin is responsible for receptor binding, and it is discussed that different intimin variants may be responsible for different host and tissue cell tropism [6,10-12]. Adu-Bobie et al. [4] found that antigenic variation exists within the cell-binding domain of intimins expressed by different clinical human EPEC and STEC isolates and defined four intimin types (α, β, γ, δ) based on type-specific PCR assays that used oligonucleotide primers complementary to the 3′end of specific *eae* genes. Oswald et al. [5] described a type-specific PCR assay which identifies a fifth intimin variant (intimin ε; referred in the present study as ε1). They divided the intimin alleles α, β, and γ based on restriction fragment length polymorphism (RFLP) analysis of PCR products into α1, α2, β1, β2, γ1 and γ2 subtypes. Furthermore, Oswald et al. [5] reclassified the δ type of Adu-Bobie et al. [4] as a β2 subtype (referred in the present study as d/β2O). Tarr and Whitam [13] presented a paper on the molecular evolution of intimin genes in human STEC and EPEC O111 clones and found two new types (ζ and θ). The new ζ intimin type also was identified by Jores et al. [11] in bovine STEC and Blanco et al. [7] in ovine STEC. Zhang et al. [6] observed that the sequences of eae-γ2 and eae-θ were almost identical (99%), and they believe that these two sequences should be considered one *eae* variant (γ2/θ), as is referred in the present study. Recently, Zhang et al. [6] determined the sequences of three new intimin variant genes (κ, η, and ι; intimin t referred in the present study as t1) found in human STEC strains. The sequence of the 3′variable region of *eae* gene of a new intimin (λ) has been submitted to GenBank by China (unpublished data) (Accession no. AF439538) and the whole intimin λ gene was sequenced by Blanco et al. (unpublished data) from a human strain (Accession no. AJ715409) and Ramachandran et al. [14] (Accession no. AF530557) from a bovine strain. Ramachandran et al. [14] identified three new intimin genes in ruminant *E. coli* strains belonging to serotypes ONT:H- (intimin μ, referred in the present study as μR/12), O2-related:H19 (intimin v, referred in the present study as vR/e2) and ONT:HNT (intimin ξ, referred in the present study as ξR/β2B) and they have also developed an intimin typing PCR-RFLP scheme that reliably differentiates 14 intimin variants. When Ramachandran et al. [14] submitted to GenBank the nucleotide sequences of μ, v, and ξ genes these intimin types were designated t2, e2 and β2, respectively. Blanco et al. [8] have identified four new intimin variant genes that they originally designated as β2, μ, v, and ξ when the sequences were submitted to EMBL Nucleotide Sequence Database, and before knowing the results obtained by Ramachandran et al. [14]. The intimin β2 found by Blanco et al. (unpublished data) in human typical EPEC strains of classical EPEC serotype O119:H6 is identical to intimin ξ described by Ramachandran et al. [14] in one bovine strain of serotype ONT:HNT. Thus, in this study our β2 intimin is referred as ξR/β2B. The other three intimins (μ, v, and ξ) discovered by Blanco et al. [8], unpublished data] are different to the existing intimin types and are referred to as μB, vB, and ζB in this study, respectively.

EPEC strains are defined as *eae*-haboring diarrheagenic *E. coli* that possess the ability to form A/E lesions on intestinal cells and that do not possess Shiga toxin genes [15]. Most EPEC strains belong to a series of O antigenic groups known as EPEC serogroups: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158. EPEC are further classified as typical, when possessing the EAF (for EPEC adherence factor) plasmid that encodes localized adherence (LA) on cultured epithelial cells mediated by the Bundle Forming Pilus (BFP); whereas atypical EPEC strains do not possess the EAF plasmid [15,16]. Typical EPEC, a major cause of infant diarrhea in developing countries, are rare in industrialized countries, where atypical EPEC seem to be a more important cause of diarrhea [1,16]. Typical and atypical EPEC strains usually belong to certain serotype clusters, differ in their adherence patterns on cultured epithelial cells (typical: LA; atypical: diffuse adherence (DA); aggregative adherence (AA); localized adherence-like adherence (LAL)), are typically found in different hosts (typical EPEC strains have only been recovered from humans), and display differences in their intimin variants [16]. Atypical EPEC appear to be more closely related to STEC and as such are considered emerging pathogens [16,17].
Table 4: Genetic relationship of the new intimin gene (ξ)2 (AJ879898, AJ879899, AJ879900) detected in bovine EPEC strains of serotype ONT:H45 and the remaining eae variants: Pairwise alignments calculated with CLUSTAL W

| Designation of intimins in this study | Designation of intimins in previous studies (References) | ORF length (bp) | Reference strain | Serotype | Origin | Accession number | Genetic relationship identities |
|--------------------------------------|-----------------------------------------------------------|----------------|-----------------|----------|--------|-----------------|-------------------------------|
| α1                                   | α (4) and α1 (5)                                          | 2820           | E2348/69        | O127:H6  | Human  | M58154          | 87%                           |
| α2                                   | α (4) and α2 (5)                                          | 2820           | E.coli          | O125:H6  | Human  | AF530555        | 87%                           |
| β1                                   | β (4) and β1 (8)                                          | 2820           | RDEC1           | O15:H-   | Rabbit | AF200363        | 84%                           |
| ξR/β2B                               | ξ (14) and β2 (8)                                         | 2820           | KB411           | ONT:HNT  | Bovine | AF530556        | 88%                           |
| ξR/β2B                               | ξ (14) and β2 (8)                                         | 2820           | FV359           | O119:H6  | Human  | AJ715407        | 87%                           |
| δ/β2O                                | δ (4) and β2 (5)                                          | 2820           | BL152.1         | O86:H34  | Human  | AJ875027        | 87%                           |
| η                                      | η (6)                                                     | 2820           | 6044/95         | O118:H5  | Human  | AJ308552        | 87%                           |
| γ1                                   | γ (4) and γ1 (5)                                          | 2805           | ELD933          | O157:H7  | Human  | AF071034        | 86%                           |
| γ2/θ                                  | γ2 (5) and θ (13)                                         | 2808           | CL-37           | O111:H8  | Human  | AF449418        | 84%                           |
| e1                                    | e (5)                                                     | 2847           | PMK5            | O103:H2  | Human  | AF116899        | 92%                           |
| ν(14)/ε2                              | ν (14) and ε2 (GenBank AF530554)                         | 2847           | VR64/4          | O2related:H19 | Ovine | AF530554        | 94%                           |
| ζ1                                    | ζ (13)                                                    | 2817           | 4795/95         | O84:H4   | Human  | AJ271407        | 86%                           |
| η1                                    | η (6)                                                     | 2847           | CF11201         | O125:H-  | Human  | AJ308550        | 98%                           |
| η2                                    | new (this study)                                          | 2847           | H03/53199a      | ONT:H45  | Human  | AJ876652        | 100%                          |
| λ                                      | λ (6)                                                     | 2814           | 7476/96         | O145:H4  | Human  | AJ308551        | 88%                           |
| µR/θ2                                | µ (14) and θ2 (GenBank AF530553)                         | 2814           | VR45            | O34:H-   | Ovine  | AF530553        | 83%                           |
| λ                                     | λ (8, 14)                                                 | 2817           | EPEC-68.4       | O34:H-   | Human  | AJ715409        | 86%                           |
| µB                                    | µ (8)                                                     | 2808           | EPEC-373        | O55:H51  | Human  | AJ705049        | 87%                           |
| νB                                    | ν (8)                                                     | 2823           | IH11229a        | O10:H-   | Human  | AJ705050        | 82%                           |
| ξB                                    | ξ (8)                                                     | 2847           | STEC-B49        | O80:H-   | Bovine | AJ705051        | 91%                           |

EPEC and STEC [16,20,32,33]. This study reports information on the occurrence of eae positive E. coli carried by healthy cattle at the point of slaughter, and on serotypes, intimin variants, and further virulence factors of isolated EPEC and STEC strains.

Results

Detection and isolation of eae positive E. coli strains

Of the 330 fecal samples collected from cattle at slaughter, 132 (40%) tested positive for eae genes. From 51 randomly selected eae PCR-positive samples, 51 eae positive E. coli strains were isolated by colony dot blot hybridization, confirmed as E. coli by biochemical properties, and further characterized by phenotypic and genotypic traits. Thirty strains carried no stx genes and were therefore classified as EPEC, whereas the remaining 21 strains possessed at least one Shiga toxin gene, and were therefore considered as STEC.

Serotypes of bovine eae positive E. coli strains

The 51 eae positive E. coli strains belonged to 18 O serogroups, 9 H types, and 23 O:H serotypes. However, 46% of strains were of three serogroups, namely O26 (6 strains), O103 (12 strains), and O145 (5 strains), and 43% belonged to two H types, namely H2 (17 strains), and H45 (5 strains). Thirty-two percent of strains were of three serogroups, namely O26 (6 strains), O103 (12 strains), and O145 (5 strains). EPEC strains belonged
to 18 O:H serotypes and eight strains were nontypeable (Table 1). STEC strains comprised seven O:H serotypes with one serotype (O103:H2) accounting for 48% of STEC strains (Table 2). Furthermore, all serotypes found in this study have been previously reported in human STEC. Five (O5:H-, O26:H-, O103:H2, O145:H-, and O157:H7) of the STEC serotypes comprising 19 (90%) strains have previously been associated with human STEC causing HUS. Serotypes O26:H- and O103:H2 were found in both EPEC and STEC strains.

**Typing of intimin (eae) genes**

Overall, the 51 isolated eae positive bovine *E. coli* strains comprised a variety of 10 intimin variants, namely α1 (1 strain), β1 (17 strains), ξR/β2B (2 strains), γ1 (6 strains), γ2/θ (2 strains), ε1 (13 strains), ζ (3 strains), η2 (3

| Serotype | No. of strains | eae | astA | bfpA |
|----------|----------------|-----|------|------|
| O2:H45   | 1              | k   | -    | -    |
| O8:H19   | 1              | β1  | +    | -    |
| O10:H-   | 1              | γ2/θ| -    | -    |
| O15:H2   | 3              | β1  | -    | -    |
| O26:H-   | 1              | β1  | +    | -    |
| O26:H+   | 1              | β1  | -    | -    |
| O26:H11  | 2              | β1  | -    | -    |
| 035:H2   | 1              | β1  | -    | -    |
| O64:H25  | 1              | ε1  | -    | -    |
| O77:H19  | 1              | β1  | -    | -    |
| O103:H2  | 1              | ε1  | -    | -    |
| O103:H8  | 1              | ε1  | +    | +    |
| O113:H6  | 2              | ξR/β2B | -  | -    |
| O119:H8  | 1              | ε1  | -    | +    |
| O128:H2  | 1              | β1  | -    | -    |
| O156:H25 | 1              | ζ   | -    | -    |
| O157:H45 | 1              | α1  | +    | +    |
| O177:H11 | 1              | β1  | +    | +    |
| ONTH-H   | 1              | ε1  | -    | -    |
| ONTH-H   | 1              | κ   | -    | -    |
| ONTH:H19 | 1              | ε1  | +    | -    |
| ONTH:H25 | 2              | ζ   | -    | -    |
| ONTH:H45 | 3              | η2  | +    | +    |

ONT: O antigen nontypeable with O1 to O185 antisera.

**Table 1: Serotypes and virulence factors of EPEC strains isolated from cattle in Switzerland (n = 30)**

| Serotype | No. of strains | eae astA bfpA |
|----------|----------------|---------------|
| O5:H1 b  | 1              | stx1 β1       |
| O5:H1 b  | 1              | stx1 β1       |
| O26:H- b | 1              | stx2 β1       |
| O26:H+ b | 1              | stx2 β1       |
| O103:H2 b| 9              | stx1 ε1       |
| O103:H2 b| 1              | stx1 ε1       |
| O111:H2 b| 1              | stx1 γ2/θ     |
| O145:H- b| 4              | stx1, stx2 γ1|
| O145:H+ b| 1              | stx2 γ1       |
| O157:H7 b| 1              | stx2 γ1       |

| Serotype | No. of strains | stx | eae | ehxA |
|----------|----------------|-----|-----|------|
| O5:H1 b  | 1              | stx1 | β1  | +    |
| O5:H1 b  | 1              | stx1 | β1  | -    |
| O26:H- b | 1              | stx2 | β1  | +    |
| O26:H+ b | 1              | stx2 | β1  | -    |
| O103:H2 b| 1              | stx1 | ε1  | +    |
| O103:H2 b| 1              | stx1 | ε1  | -    |
| O145:H- b| 1              | stx1, stx2 γ1|
| O145:H+ b| 1              | stx2 γ1 | +    |
| O157:H7 b| 1              | stx2 γ1 | +    |

a Serotypes previously found as human STEC strains
b Serotypes previously associated with human STEC strains that caused HUS
c Serotypes not yet reported within bovine STEC strains

Serotypes that caused human outbreaks are in **bold**
strains), t1 (2 strains), and κ (2 strains). Intimins α2, δ/β2O, γ1, θ/γ2, ν R/ε2, η1, μR/ι2, λ, μB, and ζB were neither detected in bovine EPEC nor in bovine STEC. EPEC strains harbored a variety of nine intimin variants (α1, β1, ε1R/β2B, κ, θ/γ2, ε1, ε2, ζ2, η2, t1). In 15 strains (52%) eae-β (twelve times B1, twice β2), was associated with nine serotypes, in three strains eae-ε1, in three strains eae-ζ2, in two strains eae-κ, and in two strains eae-η1, and in one strain eae-γ2/θ were detected (Table 1). In contrast, STEC strains harbored only four intimin variants (β1, γ1, γ2/θ, ε1) with eae-ε1 (10 strains, all of serotype O103:H2) and eae-γ1 (5 strains of serotype O145:H-), and one strain of serotype O157:H7 being most frequently found (Table 2). Intimins β1, ε1 and γ2/θ were present in both EPEC and STEC strains.

Identification of a new intimin variant gene (eae-η2).

Sequence comparison
The complete nucleotide sequence of the new η2 variant gene in three bovine bfpA and astA-positive EPEC strains of serotype ONT:H45 was determined. Furthermore, a fragment (566 bp strain FVS125 to 1728 bp strain FVS125) of the 3' variable region of the eae gene of seven representative strains was amplified and sequenced. The eae sequences were deposited in the European Bioinformatics Institute (EMBL Nucleotide Sequence Database) and the accession numbers assigned are indicated in Table 3.

By using CLUSTAL W for optimal sequence alignment, we determined the genetic relationship of the new intimin gene (η2) and the remaining eae variants (Table 4). Thus, the eae-η2 sequence is very similar to eae-η1 (identity of 98%). Phylogenetic analysis revealed six groups of the closely related intimin genes: (i) α1, α2, ζ2, and vB; (ii) β1, δ/β2O, κ, ε1R/β2B; (iii) ε1, ε2, ζ1, η2 and νR/ε2; (iv) γ1, μB, and γ2/θ; (v) λ; and (vi) t1 and μR/ι2.

Further characterization of EPEC and STEC strains
Nine EPEC strains harbored the astA gene encoding for EAST1 (Table 1). Additionally, three astA positive strains of serotype ONT:H45 harbored the bfpA gene encoding bundlein, whereas only one strain was positive for the EAF plasmid. Overall, 23 different associations of serotypes with virulence factors were identified in EPEC strains.

Of the 21 eae positive E. coli harboring genes encoding for Shiga toxins, the majority (13 strains) carried only stx1 genes, 4 strains only stx2 genes, and 4 strains both toxin genes (Table 2). The only stx1 positive strains belonged to serotypes O5:H- (2 strains), O103:H2 (10 strains) and O111:H21 (1 strain), the only stx2 positive strains to serotypes O26:H-, O26:H2, O145:H-, and O157:H7, and all the strains positive for both toxin genes to serotype O145:H-. Moreover, the majority of STEC strains (86%) tested positive for the ehxA gene encoding for Enterohe-molysin. Overall, ten different associations of serotypes with virulence factors were identified.

Discussion
Of 51 eae positive E. coli strains, 59% were classified as EPEC and 41% as STEC. The serogroups O26, O103, and O145 and serotypes O26:H-, O103:H2, O145:H- were most frequently found. Comparable distributions of EPEC (57%) and STEC (43%) strains and serogroups (44% of strains serotyped as O26, O103, O145, or O156) were reported in strains isolated from healthy cattle in Japan [34]. Of the typical EPEC serogroups (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O156), only six strains were found in the present study: four O26 strains, one O119 strain, and one

Table 3: Accession numbers of sequenced eae genes of some EPEC strains

| Strain       | Origin           | Serotype | Intimin | EMBL nucleotide sequence | bfpA | EAF plasmid | stx1 | stx2 |
|--------------|------------------|----------|---------|--------------------------|------|-------------|------|------|
| FVS109-4113/1| healthy cattle1  | ONT:H45  | η2      | AJ87998                  | +    | -           | -    | -    |
| FVS113-4115/2| healthy cattle1  | ONT:H45  | η2      | AJ879899                 | -    | -           | -    | -    |
| FVS114/1-3933/51| healthy cattle1| ONT:H45  | η2      | AJ879900                 | -    | -           | -    | -    |
| H03-53199a   | patient with diarrhea^2 | ONT:H45 | η2 | AJ876562 | nd | nd | -    | -    |
| FVS114/2-3933/52| healthy cattle1| O156:H25 | ζ | AJ879901 | - | - | -    | -    |
| FVS125-4125/1| healthy cattle1  | O157:H45 | α1 | AJ879902 | +    | -           | -    | -    |
| FVS123-4286/1| healthy cattle1  | O2:H45   | κ    | AJ879903 | -    | -           | -    | -    |
| FVS133-3988/1| healthy cattle1  | ONT:H-   | κ    | AJ879904 | -    | -           | -    | -    |
| FVS126-3951/1| healthy cattle1  | O119:H8  | ι1   | AJ879905 | +    | -           | -    | -    |
| FVS120-4075/1| healthy cattle1  | O103:H8  | ι1   | AJ879906 | +    | -           | -    | -    |
| FVS384-1070/1| healthy cattle3  | O157:H45 | α1 | AJ879907 | -    | -           | -    | -    |

^1 from Switzerland; ^2 from Spain; ^3 Stephan et al. [33]; nd: not done
O128 strain. In bovine STEC strains, only a restricted number of serotypes have been most commonly found [8,20]: the predominant serotype in the majority of surveys realized in Europe was O113:H21, in America and Australia O26:H11, and in Japan O45:H8/H- and O145:H-. As in the present study, authors in Argentina, Canada, France, Germany, Spain, and the United States have found that many STEC recovered from cattle belonged to serotypes previously associated with human disease [8,35-38].

Intimin mediates the intimate bacterial attachment to the host cell surface of EPEC and STEC, and is required for the formation of the characteristic A/E lesions. In EPEC and STEC strains isolated from human patients and from healthy infants from Germany and Australia, intimin variants α1, β1, γ1 and γ2/θ were most frequently detected [5,6,8,17,26]. Moreover, STEC serotypes commonly recovered from outbreaks of HUS and hemorrhagic colitis (O157:H7/H-; O111:H-; O26:H11/H-) typically possess intimin γ1, γ2/θ, and β1 [5,6,8,14].

Overall, the 51 isolated eae positive bovine E. coli strains comprised a variety of 10 intimin variants, and most of them harbored eae-β1 (33%), eae-ε1 (26%) and eae-γ1 (16%). As it is known that different intimin variants are associated with distinctive phylogenetic lineages of LEE-positive E. coli [4,39,40], the identified variety of bovine eae positive strains further substantiates the reservoir function of cattle for infections of humans with LEE positive strains.

Intimin β1 appears to be the most widespread variant [7,8,14,27,39,41,42]. In that it has been found in both EPEC and STEC strains from humans and several animal species, and its presence is associated with multiple E. coli serotypes. Strains harboring eae-β1 include important human diarrheagenic serotypes such as EPEC O26:H11, O111:H2, O114:H2, O126:H2, O128:H2 and STEC O26:H11/H-, O118:H16 and O177:H- [5,26,27], serotypes partly also detected in this study.

Intimin ε1 was described for the first time in human and bovine STEC of serogroup O8, O11, O45, O103, O121, and O165 [5]. In this study, eae-ε1, was detected in three bovine EPEC strains including one O103:H2 strain, and in all 9 bovine STEC O103:H2 strains. Furthermore, in a recent study performed on sheep in Switzerland, intimin ε1 was detected in all STEC O103:H2 [43]. STEC O103:H2 strains have frequently been associated with HUS and hemorrhagic colitis [26,27,44,45].

Intimin γ1 was restricted to STEC and associated with serotypes considered as highly pathogenic to humans: O145:H- (five strains) and, O157:H7 (one strain). In addition to these serotypes, eae-γ1 was previously described in association with STEC O145:H28, and with EPEC O55:H7/H- from which STEC O157:H7 are believed to have evolved [8,26,27,46].

Interestingly, one bovine EPEC O157:H45 strain harboring eae-α1 was detected. Moreover, eae-α1 was also recently detected in bovine EPEC O157:H45 [33], in two EPEC O142:H6 isolated from monkeys [47] and in a human STEC O177:H7 strain [25]. These findings disagree with the suggested restriction of intimin α1 to human EPEC strains of serotypes O55:H6, O127:H6, H34/H-, O142:H6 [4,5].

We have identified a new intimin gene (η2) in three bovine bfpA and astA-positive EPEC strains of serotype ONT:H45. The complete nucleotide sequences of the new eae-η2 (AJ879898, AJ879899, AJ879900) variant genes of the three strains were determined. The new intimin η2 also was found in one EPEC ONT:H45 strain isolated from a patient with diarrhea in Spain by Blanco et al. (unpublished data, EMBL nucleotide sequence AJ876652). Recently, a food-borne outbreak of diarrhea involving 41 students (ages 12 to 15) was reported in Japan by Yatsuyanagi et al. [48]. The implicated organism was a EPEC ONT:H45, which hybridized with the probes for eaeA, astA and bfpA genes.

In our study, about one third of EPEC strains harbored astA as further putative virulence factor; whereas only seven EPEC strains harbored bfpA encoding bundling, the structural subunit of the bundle-forming pilus in typical EPEC strains, and only one EPEC of serotype O157:H45 was positive for the EAF plasmid. Overall, bfpA seems to be isolated very rarely from bovine EPEC strains, since there are scarcely any reports of E. coli strains with bfpA isolated from cattle. However, in a recent study, we characterized 11 O157:H45 EPEC strains isolated from cattle in Switzerland and found 10 bfpA positive strains among them [33].

To assess the pathogenicity of STEC, further evaluation of virulence factors in addition to serotype is necessary. O157 and non-O157 STEC strains isolated from patients with severe symptoms such as bloody diarrhea, HC, and HUS frequently show a typical virulence spectrum, with such strains tending to be stx2 and eae positive [49-51]. Moreover, it was previously shown that bovine and human strains harboring the eae gene were statistically more likely to be positive for the ehxA gene encoding for Enterohemolysin [7,26,52]. However, the impact of Enterohemolysin, which was found in the majority of STEC strains, is controversially discussed. Additionally, STEC commonly recovered from outbreaks of HUS and HC typically possessed eae-β1, eae-γ1 and γ2/θ [5-7,39]. In
consequence, apart of the stx2 gene, which was previously reported to correlate with severe disease in humans, intimin subtyping may facilitate further understanding of associations among serotype, eae and stx subtype. In consequence, seven STEC strains (14% of all associations among serotype, intimin subtyping may facilitate further understanding of reported to correlate with severe disease in humans, E. coli biochemically properties (acid production from mannitol, the o-nitrophenyl-β-D-galactopyranoside (ONPG) test, H₂S and indole production, and proof of urease and lysine decarboxylase activities) and by PCR to be eae positive [7]. One randomly chosen colony per sample was used for further strain characterization.

Conclusion

Our data confirm that ruminants are an important source of serologically and genetically diverse intimin-harboring E. coli strains. Moreover, cattle have not only to be considered as important asymptomatic carriers of O157 STEC but can also be a reservoir of EPEC and eae positive non-O157 STEC, which are described in association with human diseases. The fecal carriage of foodborne pathogens among livestock animals at slaughter is strongly correlated with the hazard of carcasses contamination. In order to reduce the risk represented by STEC and EPEC, the maintenance of slaughter hygiene is consequently of central importance in meat production.

Methods

E. coli strains

From 330 fecal samples, collected from Swiss cattle at slaughter, 10 g were each enriched in 100 ml brilliant green bile broth (BBL, Cockeysville, Md.) at 37°C for 24 h. The enriched samples were streaked onto sheep blood agar (Difco Laboratories, Detroit, Mich.; 5% sheep blood Oxoid, Hampshire, UK), and after incubation at 37°C for another 24 h, the colonies were washed off with 2 ml of 0.85% saline solution. Two µl of each plate eluate were then evaluated by PCR with primers EAE-1 and EAE-2 (Table 5) targeting sequences at the 5’ eae conserved region detecting all types of eae described at the moment. From the 132 eae PCR-positive samples, 51 eae PCR-positive samples were then randomly selected for strain isolation with an eae DNA probe and colony dot-blot hybridization.

The eae probes were prepared by labeling eae-PCR amplicons from E. coli O157:H7 strain 857/03 with DIG High Prime kit (Roche, Mannheim, Germany). Briefly, for colony hybridization, the 51 eae positive samples were plated onto sheep blood agar and incubated overnight at 37°C. Colonies were transferred to a nylon membrane (Roche), and lysed following standard methods. After washing, crosslinking, and prehybridization in DIG-Easy-Hyb buffer (Roche) at 37°C for about 30 min, hybridization of membranes with eae DNA probes was performed overnight at 42°C. After washing in pre-heated primary and secondary wash buffers, the presence of labeled probe was detected with an alkaline phosphatase-conjugated antibody detection kit and NBT/BCIP stock solution according to the instructions of the manufacturer (Roche). Positive colonies were picked from the original sheep blood agar and confirmed as E. coli by biochemical properties (acid production from mannitol, the o-nitrophenyl-β-D-galactopyranoside (ONPG) test, H₂S and indole production, and proof of urease and lysine decarboxylase activities) and by PCR to be eae positive [7]. One randomly chosen colony per sample was used for further strain characterization.

Further strain characterization

Determination of O and H antigens was performed by the method described by Guinée et al. [53] with all available O (O1 to O185) and H (H1 to H36) antisera. E. coli isolates that are nonmotile are described as having an H flagellum type. Antisera were obtained and absorbed with corresponding cross-reaction antigens to remove nonspecific agglutinins. O antisera were produced in the Laboratorio de Referencia E. coli (LREC) http://www.lugol.usc.es/ecoli, and H antisera were obtained from the Statens Serum Institut (Copenhagen, Denmark). E. coli strains representing the new O groups O182 to O185 [unpublished data] were kindly provided by Flemming Scheutz (International Escherichia Centre, Statens Serum Institut, Copenhagen, Denmark).

All PCR assays applied in this study for characterization of eae positive E. coli strains were performed in a T3 thermocycler (Biometra, Göttingen, Germany). PCR reagents were purchased from PROMEGA (Madison, Wis.), and primers were synthesized by MICROSYNTH (Balgach, Switzerland). The 50-µl PCR mixtures normally consisted of 2 µl of bacterial suspension boiled at 100°C for 5 min in 42 µl of double-distilled water, 5 µl of 10-fold-concentrated polymerase synthesis buffer containing 2.0 mM MgCl₂, 200 µM (each) deoxynucleosid triphosphate (dNTP), 30 pmol of each primer, and 2.5 U of Taq DNA polymerase. The PCR primers, target sequences, product sizes and references are listed in Tables 5 and 6. Differences in the normal PCR mixture and cycling conditions for each PCR were previously described in the cited literature. To identify eae variants, intimin type specific PCR assays using primers complementary to the heterogeneous 3’ end of intimin genes were performed [8,9]. For detection of further putative virulence genes all strains were examined for the presence of stx genes with primers VT1 and VT2 [54]. Stx positive strains were examined for the presence of stx1 and stx2 genes [55,56]. Stx negative strains were further examined for the presence of the EAF plasmid [57], the bfpA gene on the EAF plasmid [58], and the astA gene encoding EAST1 [59].
Table 5: Sequences of oligonucleotide primers used for typing of eae intimin gene

| Gene | Primer | Oligonucleotide sequence (5’-3’)<sup>a</sup> | Fragment size (bp) | Annealing temperature | Primer coordinates | Accession number | Reference |
|------|--------|------------------------------------------|-------------------|----------------------|--------------------|------------------|------------|
| eae<sup>b</sup> | EAE-I | GGAACGGCAGGATGAAGTACGAG | 775 | 55°C | 1441–1460 | AF022236 | 7 |
| eae<sup>b</sup> | EAE-2 | GGGCCTGCAATAGCTTCTC | | | 2193–2215 |
| eae-α<sub>1</sub> | EAE-FB | AAAAAACGGGAGGATGACCTTT | 820 | 60°C | 1909–1928 | AF022236 | 8 |
| eae-α<sub>2</sub> | EAE-A | CACTCTCTGCACTTCTGATT | | | 2709–2728 |
| eae-β<sub>1</sub> | IH2498aF | AGACCTTCTGACATATTGAATG | 517 | 60°C | 2099–2122 | AF530555 | 8 |
| eae-β<sub>1</sub> | IH2498aR | TCTGAGAGAAGGTATAC | | | 2597–2615 |
| eae-β<sub>1</sub> | B1A | ACTTGCACACTAATAATGGCAG | 730 | 66°C | 1924–1944 | AF453441 | 9 |
| eae-β<sub>1</sub> | B1B | TTGACGACCCCATGTGGAAT | | | 2633–2653 |
| eae-β<sub>2</sub>b<sup>2</sup> | B2A | AAGGGGGAACCTCCGTCGCTA | 604 | 66°C | 2056–2076 | AF530556 | 9 |
| eae-β<sub>2</sub>b<sup>2</sup> | B2B | ATTTATGCGAGCCCCACGGCA | | | 2639–2659 | AJ715407 |
| eae-β<sub>2</sub>b<sup>2</sup> | EAE-FB | AAAAAACGGGAGGATGACCTTCT | 833 | 60°C | 1909–1928 | U66102 | 8 |
| eae-β<sub>2</sub>b<sup>2</sup> | EAE-D | CTCGTATACCCCGGATGTTAAC | | | 2721–2741 |
| eae-γ<sub>1</sub> | EAE-FB | AAAAAACGGGAGGATGACCTTCT | 804 | 60°C | 1909–1928 | AF071034 | 8 |
| eae-γ<sub>1</sub> | EAE-C1 | AGAGACGCTGCTACAGTATGTC | | | 2691–2712 |
| eae-γ<sub>2</sub>b<sup>2</sup> | EAE-FB | AAAAAACGGGAGGATGACCTTCT | 808 | 58°C | 1909–1928 | AF025311 | 8 |
| eae-γ<sub>2</sub>b<sup>2</sup> | EAE-C2 | CTGATTTTATCTACGTTTCA | | | 2697–2716 |
| eae-γ<sub>2</sub>b<sup>2</sup> | LPS | AGCTCAGCTGTAAGATGCGGCAACGC | 722 | 66°C | 1909–1928 | AF116899 | 8 |
| eae-γ<sub>2</sub>b<sup>2</sup> | EAE-FB | AAAAAACGGGAGGATGACCTTCT | 808 | 58°C | 1909–1928 | AF025311 | 8 |
| eae-γ<sub>2</sub>b<sup>2</sup> | LPS | AGCTCAGCTGTAAGATGCGGCAACGC | 722 | 66°C | 1909–1928 | AF116899 | 8 |

<sup>a</sup>Universal oligonucleotide primer pair EAE1 and EAE2 with homology to the 5’ conserved region of eae gene (detects all types of eae variants described at the moment). Primers used to detect the eae gene.

<sup>b</sup>The intimin β<sub>2</sub> of Blanco et al. (8) is identical to intimin β<sub>2</sub> described by Ramachandran et al. (14). In this study our β<sub>2</sub> is referred as CR/β2B.

<sup>c</sup>The intimin δ of Adu-Bobie et al. (5) is identical to intimin δ of Zhan et al. (6). The intimin δ of Adu-Bobie et al. (4) was also termed β<sub>2</sub> by Oswald et al. (5). Thus, in the present study this intimin is referred as δ/β2O.

<sup>d</sup>The intimin δ of Oswald et al. (5). Thus, in the present study this intimin is referred as δ/β2O.

<sup>e</sup>The intimin δ of Adu-Bobie et al. (4) is identical to intimin δ of Zhan et al. (6). The intimin δ of Adu-Bobie et al. (4) was also termed δ/β2O by Oswald et al. (5). Thus, in the present study this intimin is referred as δ/β2O.

<sup>f</sup>The intimin β of Tarr and Whittam (13) is the same as intimin β<sub>2</sub> of Oswald et al. (5).

<sup>g</sup>Intimins μ, ν, and ζ described by Ramachandran et al. (14) in ruminant E. coli strains. When Ramachandran et al. (14) submitted the nucleotide sequences of μ, ν, and ζ genes these intimin types were designated t1, t2, and β2, respectively.

<sup>h</sup>Intimins μ, ν, and ζ described by Blanco et al. (8, unpublished results).

**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 triphosphatase chain termination method of Sanger, with the BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 3100 Genetic Analyzer (Applied Bio-Systems).

**Phylogenetic analyses**

Genetic distances and phylogenetic trees of eae sequences were calculated and constructed with the CLUSTAL W program [60] included in the EMBL software [http://www.ebi.ac.uk/clustalw/](http://www.ebi.ac.uk/clustalw/).

**Nucleotide sequence accession numbers**

The eae sequences of strains analyzed were deposited in the European Bioinformatics Institute (EMBL Nucleotide...
Table 6: Sequences of oligonucleotide primers used for detection other virulence genes

| Target | Primer | Oligonucleotide sequence (5'-3') Sequence | Product size | Reference |
|--------|--------|------------------------------------------|--------------|-----------|
| stx    | VT1    | ATTGAGCAAAAAATATTTATATG                 | 523 bp       | 54        |
|        | VT2    | TGATGATGGCAAATTCGAT                | 520 bp       |           |
| stxI   | KS7    | CCGGACTCTCATGAAACACATTATTAATGC        | 285 bp       | 55        |
|        | KS8    | CCGGATCTAGCATTTCCTGAGTCAACG         |              |           |
| stx2   | VT2-e  | AATACATTATGGGAAAAATGAATA             | 348 bp       | 56        |
|        | VT2-f  | TAAACTGCACTTCGAAAT                   |              |           |
| esaA   | HlyA1  | GGGGCAGCAGAAAAAGTTGAG                | 311 bp       | 31        |
|        | HlyA2  | TCTCGCTGATAGTTTTTGGTA                |              |           |
| astA   | EAST11a| CCAATCAACAGCATATACTCGA              | 111 bp       | 59        |
|        | EAST11b| GGTGCGGAGTGGCAGCTTTTGT              |              |           |
| EAF    | EAF1   | CAGGTTAAAAGAAGAGTTATGAA             | 397 bp       | 57        |
|        | EAF25  | TATGGGACCATATTTATACCA               |              |           |
| bfpA   | EP1    | AATGGGACCTGCGTCCGTCGTA              | 326 bp       | 58        |
|        | EP2    | GCCGGCTTTATCCAACGTCGTA              |              |           |

Sequence Database) and the accession numbers assigned are indicated in Table 3.

Authors' contributions
RS and JB designed the study and drafted the manuscript. SS and TT isolated the strains, JEB has done serotyping of intimins, and CZ was responsible for further strain characterization. All authors read, commented on and approved of the final manuscript.

Acknowledgements
This work was partly supported by grant from the Fondo de Investigación Sanitaria (FIS G03-025-COLURED-O157), and the Xunta de Galicia (grants PGIDIT02BTF26101PR and PGIDIT04ARAG261014PR).

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